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MISIÓN

La *Revista Colombiana de Ciencias Químico-Farmacéuticas* es un órgano de difusión en el cual se publican investigaciones científicas, comunicaciones técnicas y revisiones temáticas originales en las áreas de las ciencias farmacéuticas (véanse Normas para publicación). La revista está destinada principalmente a químicos farmacéuticos, químicos, ingenieros químicos, médicos cirujanos, médicos veterinarios, y a otros profesionales de las ciencias físicas y naturales, de la ingeniería y de las profesiones sanitarias relacionadas con el uso de medicamentos.

VISIÓN

En pro de la difusión de las investigaciones, los contenidos de la *Revista Colombiana de Ciencias Químico-Farmacéuticas* son de acceso libre, con ello se espera llegar a un número mayor de lectores, propiciando la consolidación de comunidades académicas. Además, se proyecta que los contenidos publicados contribuyan al desarrollo e innovación de las ciencias farmacéuticas.

ÉTICA

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Contenido

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Compatibility study of clonazepam and excipients in solid pharmaceutical formulations

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Summary

This study has had as main objectives to apply the analysis via fourier-transform infrared spectroscopy (FTIR), thermogravimetry (TG), differential scanning calorimetry (DSC) to verify the degradation and alteration of the functional groups of clonazepam, due to their importance as a characterization tools, and to use a validated method for the quantification of clonazepam, with a new chromatographic analysis with no use of buffer in the mobile phase to be applied in the studies of stability of pharmaceutic formulations and in the analysis of degradation products. The results obtained from the thermal analysis have showed that the studied excipients did not reveal changes in the thermal behavior of the binary mixtures, clonazepam excipient, with no indication of incompatibility. The analytic method developed by high performance liquid chromatography (HPLC) has been shown adequate for quantification of clonazepam and its impurities. The implementation of thermal analysis and the application of new analytic methods have been considered important strategies in the diverse areas of the pharmaceutical industry, providing information that define the technological quality parameters of the products, aiming the development of new formulations.

Keywords: Clonazepam, Fourier-transform infrared spectroscopy (FTIR), Differential scanning calorimetry (DSC), thermogravimetry (TG), High performance liquid chromatography (HPLC), Compatibility Study.

Resumen

Estudio de compatibilidad del clonazepam y excipientes en formulaciones farmacéuticas sólidas

Este estudio ha tenido como principales objetivos aplicar el análisis mediante espectroscopía infrarroja por transformada de Fourier (FTIR), termogravimetría (TG), calorimetría diferencial de barrido (DSC) para verificar la degradación y alteración de los grupos funcionales del clonazepam, debido a su importancia como herramientas de caracterización, y utilizar un método validado para la cuantificación de clonazepam, con un nuevo análisis cromatográfico sin uso de tampón en la fase móvil para ser aplicado en los estudios de estabilidad de formulaciones farmacéuticas y en el análisis de productos de degradación . Los resultados obtenidos del análisis térmico han mostrado que los excipientes estudiados no revelaron cambios en el comportamiento térmico de las mezclas binarias, excipiente clonazepam, sin indicios de incompatibilidad. El método analítico desarrollado por cromatografía líquida de alta resolución (HPLC) se ha mostrado adecuado para la cuantificación de clonazepam y sus impurezas. La implementación del análisis térmico y la aplicación de nuevos métodos analíticos han sido consideradas estrategias importantes en las diversas áreas de la industria farmacéutica, proporcionando información que define los parámetros tecnológicos de calidad de los productos, visando el desarrollo de nuevas formulaciones.

Palabras clave: Clonazepam, espectroscopia infrarroja por transformada de Fourier (FTIR), calorimetría diferencial de barrido (DSC), termogravimetría (TG), cromatografía líquida de alta resolución (HPLC), estudio de compatibilidad.

Resumo

Estudo de compatibilidade de clonazepam e excipientes em formulações farmacêuticas sólidas

Este estudo teve como principais objetivos aplicar a análise via espectroscopia de infravermelho por transformada de Fourier (FTIR), termogravimetria (TG),

calorimetria exploratória diferencial (DSC) para verificar a degradação e alteração dos grupos funcionais do clonazepam, devido à sua importância como ferramenta de caracterização, e utilizar um método validado para quantificação do clonazepam, com uma nova análise cromatográfica sem uso de tampão na fase móvel para ser aplicada nos estudos de estabilidade de formulações farmacêuticas e na análise de produtos de degradação. Os resultados obtidos na análise térmica mostraram que os excipientes estudados não revelaram alterações no comportamento térmico das misturas binárias, excipiente clonazepam, sem indícios de incompatibilidade. O método analítico desenvolvido por cromatografia líquida de alta eficiência (CLAE) mostrou-se adequado para quantificação do clonazepam e suas impurezas. A implementação de análises térmicas e a aplicação de novos métodos analíticos têm sido consideradas estratégias importantes nas diversas áreas da indústria farmacêutica, fornecendo informações que definem os parâmetros tecnológicos de qualidade dos produtos, visando o desenvolvimento de novas formulações.

Palavras-chave: Clonazepam, Espectroscopia de infravermelho com transformada de Fourier (FTIR), Calorimetria diferencial de varredura (DSC), Termogravimetria (TG), Cromatografia líquida de alta eficiência (HPLC), Estudo de compatibilidade.

Introduction

It is a benzodiazepine derivative widely administered as an anxiolytic, anticonvulsant, muscle relaxant and sedative anesthetic [1-3]. In order for quality control to guarantee the efficacy and safety of the use of a drug, the use of sensitive analytical methods is essential [4-6].

The choice of method to be used in the analysis depends on several factors, such as the nature of the drug, purity and sample amount [7]. In addition, the laboratory conditions and the costs involved in the analysis must be taken into account.

Among the methods for this purpose, spectroscopic, chromatographic and thermal methods stand out [8-10]. In this context, thermal analysis constitutes a group of techniques of great interest in the pharmaceutical area, since it allows obtaining relevant data regarding the thermal behavior of drugs and pharmaceutical ingredients, in a relatively short time, fundamental for the evaluation of possible incompatibilities and for the development of new products [11,12].

The main thermoanalytical techniques applied in this area are differential scanning calorimetry (DSC), differential thermal analysis (DTA) [13, 14]. The application of

thermal analysis for this purpose is recent and has grown significantly in the last ten years [15, 16]. This fact motivates the evaluation of the thermal stability of this drug and drug/excipient compatibility.

The evaluations of drug-excipient interaction, through the compatibility study, were complemented through the technique of high-performance liquid chromatography (HPLC), which can be performed by analyzing the degradation product of a drug [17].

Due to the therapeutic relevance of clonazepam and its wide commercialization, the development of the present work becomes important in order to contribute to the evaluation of stability through the analysis of assay and formation of clonazepam degradation products complementing the study of interactions between clonazepam and excipients through the techniques of differential scanning calorimetry, differential thermal analysis, fourier-transform infrared spectroscopy.

Material and methods

Drug + excipient interaction study

According to Table 1, in the preparation of the binary mixtures (weight ratio of 1:1) the individual mass of each component was weighed, added separately to a porcelain capsule and ground with the drug for 2 minutes.

Table 1. Binary mixtures.

Mixtures	Components
1	Clonazepam + Lactose
2	Clonazepam + Microcrystalline Cellulose
3	Clonazepam + Starch
4	Clonazepam + Magnesium Stearate

Fourier-transform infrared spectroscopy (FTIR)

The sample was analyzed in an infrared equipment, brand Agilent Cary 6300 FT-IR, using a diffuse reflectance module to obtain the spectrum.

The absorption spectra were generated in the region of 4000 to 400 cm^{-1}, in total there were 32 spectra.

Thermogravimetry (TG)

The sample was analyzed in a TG equipment, brand Mettler Toledo, with Software STARe SW, version 10.0.

For this test, the excipients were analyzed alone and in combination with the drug. Before each thermogravimetric test, a blank was performed with the sample pan empty for the evaluated condition.

Conditions for TG: temperature range: 25 to 300 °C; gas: N_2 at 100 mL/min; heating ratio: 10 °C·min^{-1}; pan: platinum

Differential scanning calorimetry (DSC)

Sufficient amount of clonazepam was weighed in an alumina pan, then covered in a DSC equipment, brand Shimadzu, model DSC-60. In addition to clonazepam, the other raw materials and binary mixtures were tested.

Conditions for DSC: temperature range: 25 to 300 °C; gas: N_2 at 100 mL/min; heating ratio: 10 °C·min^{-1}; pan: alumina

High performance liquid chromatography (HPLC)

The HPLC experiments were initiated based on the American Pharmacopoeia, whose mobile phase is constituted by a mixture of phosphate/methanol/tetrahydrofuran buffer in the proportion of 600:520:130 v/v/v.

Due to the high amount of buffer, a new mobile phase was developed, consisting of acetonitrile/water in the proportion 600:400 v/v.

The proportions between the mobile phase constituents, injection volume, mobile phase flow rate and column analysis temperature were evaluated.

The analysis conditions tested were: injection volumes: 5 to 50 μL; column temperatures: 25 to 40 °C; flow rates: 0.8 to 1.5 mL/min; elution modes: isocratic and gradient; wavelength: 254 nm; analytical columns: C8 – 125 × 4.6 mm – 5 μm / C8 – 150 × 4.6 mm – 5 μm / C8 – 250 × 4.6 mm – 5 μm

The column was previously stabilized with the mobile phase for 30 minutes under the respective pre-established conditions.

Before the start of each analysis, a solution containing clonazepam, CR A (related compound A), CR B (related compound B) was injected in order to observe the following parameters: N (number of theoretical plates) ≥ 2000; T (asymmetry) between 0.8 and 1.2; R (resolution) ≥ 2; and the coefficient of variation between injections ≤ 2 %, in order to verify the suitability of the system.

Optimized chromatographic conditions: injection volumes: 30 μL; column temperatures: 25 °C; flow rate: 1.0 mL/min; elution modes: isocratic; wavelength: 254 nm; analytical column: C8 – 250 × 4.6 mm.

Stability Studies

The preparation of the simulated sample of excipients was performed, as described in Table 2. The samples were stored in climatic chambers, under the following conditions of temperature and humidity: 40 ± 2 °C / 75% ± 5% RH and 30 ± 2 °C/ 75% ± 5% RH for 30, 60 and 90 days.

Table 2. Simulated sample of excipients.

Components	Amount (mg)
Clonazepam	2.00
Lactose	121.50
Microcrystalline cellulose	18.70
Starch	20.40
Magnesium stearate	1.70

The prepared solutions were evaluated as described below:

- Clonazepam standard solution: 10.00 mg of clonazepam standard was added and transferred to a 100.0 mL flask. Approximately 5.0 mL of diluent was added and it was taken to ultrasound until complete dissolution. The volume was made up with diluent (0.1 mg/mL).
- Clonazepam stock sample solution 85.00 mg of clonazepam sample was weighed and transferred to a 10.0 mL flask. Approximately 5.0 mL of diluent was added and it was taken to ultrasound until complete dissolution. Make up to volume with diluent (0.1 mg/mL).

The solutions were injected into the chromatograph to observe the possible reduction or increase in the drug assay and/or formation of degradation products.

Results and discussion

Fourier-transform infrared spectroscopy (FTIR)

The spectra of clonazepam in the infrared were shown in Figure 1. The observed peaks are in agreement with the chemical structure of clonazepam, with the identification of the main functional groups in Table 3.

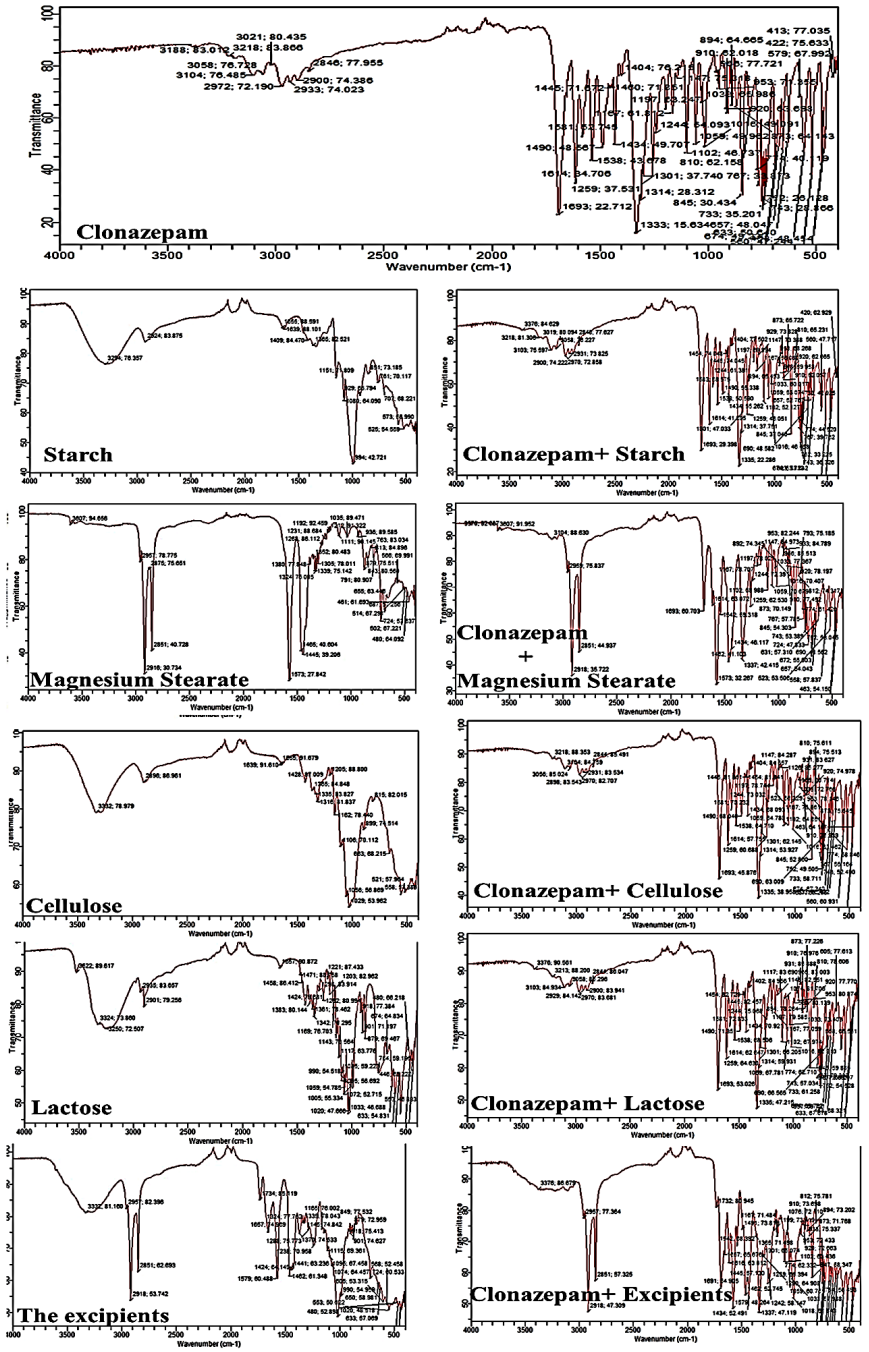

Figure 1. The individual and binary mixtures FTIR spectrum of clonazepam and excipients.

Table 3. Absorption of the main functional groups for Clonazepam.

Absorption range cm-1	Functional Group
1693	C = O deformation, amide
1614	C=N deformation
1538	C=C deformation, aromatic
1490	C=C deformation, aromatic
1445	C=C deformation, aromatic
1333	N-O_2 deformation
752	aromatic ring deformation

The spectrum obtained from the binary mixture between clonazepam and starch, the main identification bands of clonazepam are observed, 1693 cm^{-1}, 1614 cm^{-1}, 1538 cm^{-1}, 1490 cm^{-1}, 1445 cm^{-1}, 1335 cm^{-1} and 752 cm^{-1}. The starch bands are superimposed on the clonazepam bands, but it is possible to observe a widening of the bands close to 3200 cm^{-1} of the O-H bond deformation. The data obtained by FTIR for mixing clonazepam with starch are not indicative of incompatibility.

The spectrum obtained from the binary mixture between clonazepam and magnesium, the main identification bands of clonazepam are observed, 1693 cm^{-1}, 1614 cm^{-1}, 1542 cm^{-1}, 1491 cm^{-1}, 1434 cm^{-1}, 1337 cm^{-1} and 724 cm^{-1}. Magnesium stearate bands are also observed at 2918 cm^{-1} and 2851 cm^{-1}, 1573 cm^{-1}, 1462 cm^{-1}, 1102 cm^{-1}, indicating that there is no incompatibility between these studied inputs.

The spectrum obtained from the binary mixture between clonazepam and microcrystalline cellulose, the main identification bands of clonazepam are observed, 1693 cm^{-1}, 1614 cm^{-1}, 1538 cm^{-1}, 1490 cm^{-1}, 1434 cm^{-1} and 1335 cm^{-1}. The bands that identify cellulose are in overlapping regions with the clonazepam bands, and it is not possible to evaluate them individually. Data obtained by FTIR for mixing clonazepam sodium with cellulose are not indicative of incompatibility.

In the spectrum of the mixture of clonazepam with lactose monohydrate, the clonazepam identification bands are observed at 1693 cm^{-1}, 1614 cm^{-1}, 1538 cm^{-1}, 1490 cm^{-1}, 1434 cm^{-1} and 1335 cm^{-1}. The bands that identify lactose are in overlapping regions with the clonazepam bands, and it is not possible to evaluate them individually. Data obtained by FTIR for blending clonazepam with lactose are not indicative of incompatibility.

The spectrum of the mixture between clonazepam and all the excipients, some of the main identification bands of clonazepam are observed 1691 cm^{-1}, 1616 cm^{-1}, 1542 cm^{-1}, 1491 cm^{-1}, 1434 cm^{-1} and 1337 cm^{-1}. It is also possible to observe some bands of excipients, namely, 2918 cm^{-1} and 2851 cm^{-1} from magnesium stearate.

The main clonazepam bands and bands from the excipients are visualized with minimal shifts compared to the individual spectra. The data obtained by infrared spectroscopy show that the excipients are compatible with clonazepam under the conditions tested, since the similarity of the bands were in agreement with the literature and there was no formation of other bands in the spectrum.

Thermogravimetry (TG)

The clonazepam TG curve, Figure 2, shows only one event in the range of 272.83 °C to 324.54 °C due to decomposition (19.1% of mass loss) with a midpoint at 292.37 °C.

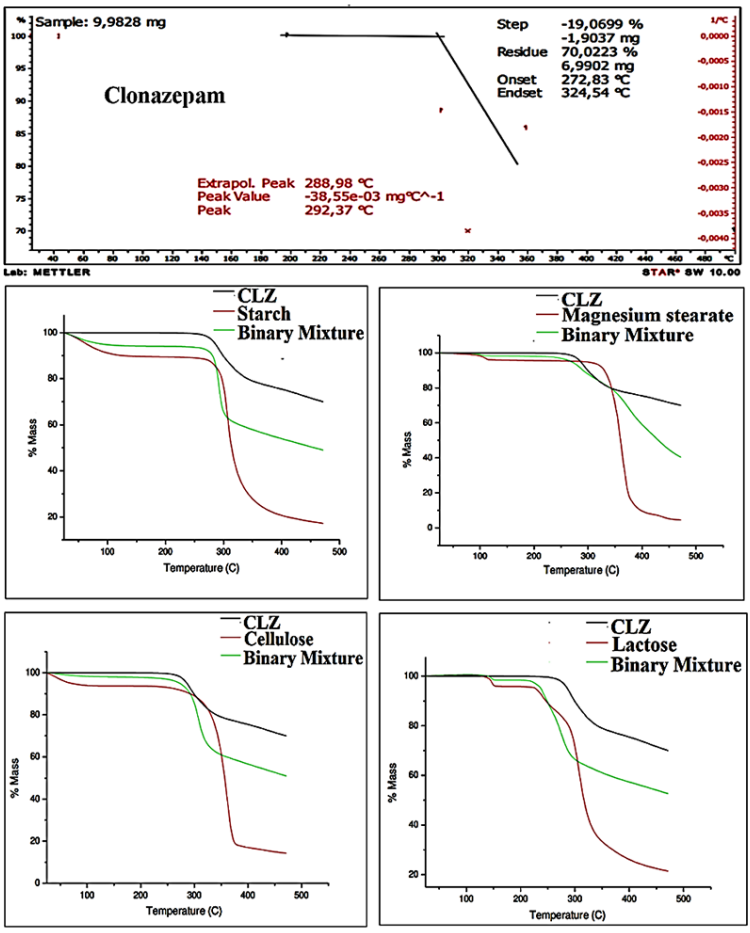

Figure 2. The individual and binary mixtures TG curves of clonazepam and excipients.

Table 4 presents a summary of the data obtained for the individual curves and binary mixtures. It is observed in the TG that the mass loss from the excipients was due to dehydration and decomposition and for clonazepam due to decomposition. The TG curves are similar to the individual curves, not indicating incompatibility.

Table 4. Discussion of results for the TG for Clonazepam and excipients.

Sample	Temperature Range (°C) Onset – Endset	Weight Loss (%)	Event
Clonazepam	272.83 – 324.54	19.1	Decomposition
Starch	35.5 °C – 96.38	9.4	Dehydration
	294.5°C – 322.5	82.6	Decomposition
Clonazepam + Starch	28.9 – 85.11°C	5.7	Dehydration
	283.82 – 300.21	32.3	Decomposition
Magnesium Stearate	99.71 – 146.0	2.48	Dehydration
	343.09 –440.0	22.89	Decomposition
	449.08 – 492.046	0.51	Decomposition
Clonazepam + Magnesium Stearate	93.73 – 109.49	1.39	Dehydration
	268.26 – 297.64	8.65	Decomposition
	353.82 – 415.85	33.46	Decomposition
Microcrystalline Cellulose	28.78 – 71.84	5.80	Dehydration
	337.24 – 371.17	85.58	Decomposition
Clonazepam + Microcrystalline Cellulose	37.7 – 96.02	1.81	Dehydration
	291.42 – 322.31	48.96	Decomposition
Lactose	140.95 – 151.24	5.80	Dehydration
	229.60 – 252.59	6.78	Decomposition
	295.06 – 332.51	52.00	Decomposition
Clonazepam + Lactose	142.75 – 150.39	1.71	Dehydration
	233.380- 253.03	9.41	Decomposition
	268.95 – 291.95	15.39	Decomposition

Differential scanning calorimetry (DSC)

The clonazepam has only one endothermic event, related to melting, with temperature ranges of T_{onset} = 237.38 °C and T_{peak} = 237.36 °C, Figure 3, followed by decomposition according to an exothermic event close to 300 °C.

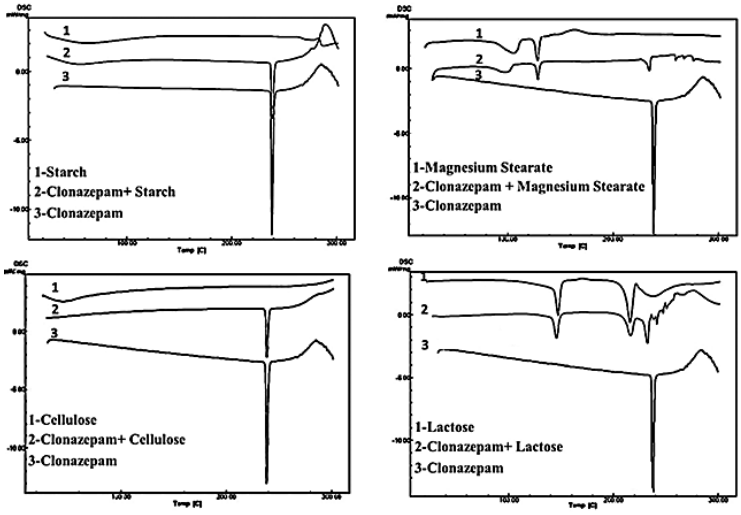

Figure 3. The individual and binary mixtures DSC curves of clonazepam and excipients.

Table 5 presents a summary of the data obtained for the individual and binary mixtures DSC curves (Figure 3). It is observed in the curve of the binary mixtures that the enthalpies for the loss of moisture from the excipients and the melting of clonazepam are proportional to the individual curves. The data indicate that the excipients and clonazepam are compatible under the conditions evaluated.

Table 5. Discussion of results for the DSC for Clonazepam and excipients.

Sample	Temperature (°C) (On set – Peak)	Enthalpy (J/g)	Event
Clonazepam	237.38 - 238.36	113.01	Fusion
Starch	22.8 - 63.29	286.43	Water Loss
Clonazepam + Starch	24.6 - 55.83	110.63	Water Loss
	237.49 - 238.96	53.51	Fusion
Magnesium Stearate	88.77 - 105.82	97.97	Water Loss
	125.35 - 128.90	42.12	Fusion
Clonazepam + Magnesium Stearate	85.55 - 97.97	57.87	Water Loss
	126.92 - 128.93	25.05	Fusion
	231.45 - 234.43	21.42	Fusion (Clonazepam)
Microcrystalline Cellulose	27.68 - 45.19	84.38	Water Loss

(*Continued*)

Table 5. *Continuation.*

Sample	Temperature (°C) (On set – Peak)	Enthalpy (J/g)	Event
Clonazepam + Microcrystalline Cellulose	31.09 - 57.84	2.36	Water Loss
	237.27 - 238.73	53.48	Fusion (Clonazepam)
Lactose	143.31 - 146.99	130.39	Water Loss
	211.62 - 215.88	128.12	Fusion
	222.27 - 237.07	101.20	Decomposition
Clonazepam + Lactose	142.08 - 145.39	51.42	Water Loss
	211.37 - 216.49	54.17	Fusion / Decomposition
	228.96 - 232.61	40.71	Fusion (Clonazepam)

High performance liquid chromatography (HPLC)

In the studies carried out, the data obtained from the thermal analysis were compared with the results of assay and related substances by HPLC (validated method) as shown in Figure 4 and Figure 5, allowing greater security in the interpretation of the data and also enriching the work with a comparative that challenges the efficiency of thermal analysis.

Through the analysis of the chromatograms of the standard solutions, sample, and placebo, it was observed that the proposed method has specificity, since it does not suffer interference from the excipients next to the clonazepam signal. In addition, the observed clonazepam peak purity is 0.99.

Peak purity calculations were performed by comparing the spectrum of the peak apex (reference spectrum, point that presents the highest concentration of the analyte and the best-defined spectrum), with several spectra sampled from the beginning to the end of the clonazepam peak, being collected a spectrum every 400 milliseconds.

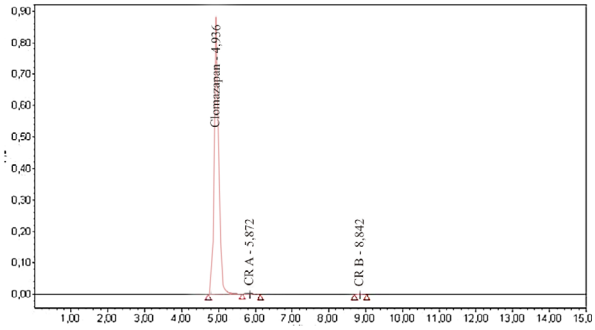

Figure 4. Chromatogram of clonazepam, CR A (related compound A), CR B (related compound B) Standards Solution.

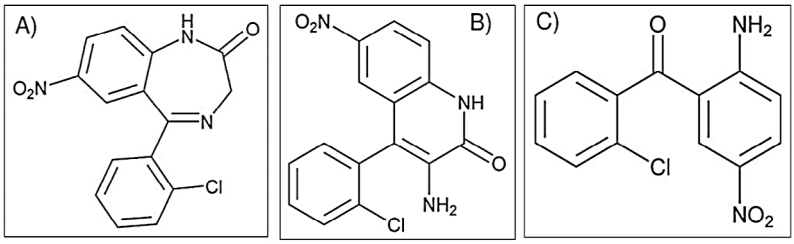

Figure 5. A: molecular structure of clonazepam. **B:** molecular structure of related compound A. **C:** molecular structure of related compound B.

From this total of spectra, an evaluation was carried out, counting all the spectra that presented at least 95% similarity with the reference spectrum and also calculating those that presented less than 95% of similarity.

When subjected to forced degradation, the chromatograms showed a reduction in clonazepam assay, and the formation of degradation peaks was observed in all types of hydrolysis (Figure 6 and Figure 7). When subjected to thermal and photolytic degradation, there was little variation in the assay for both the standard and the sample.

The compiled with the results of the forced degradation study of the standard and of the sample referring to clonazepam are shown respectively in tables 6 and 7.

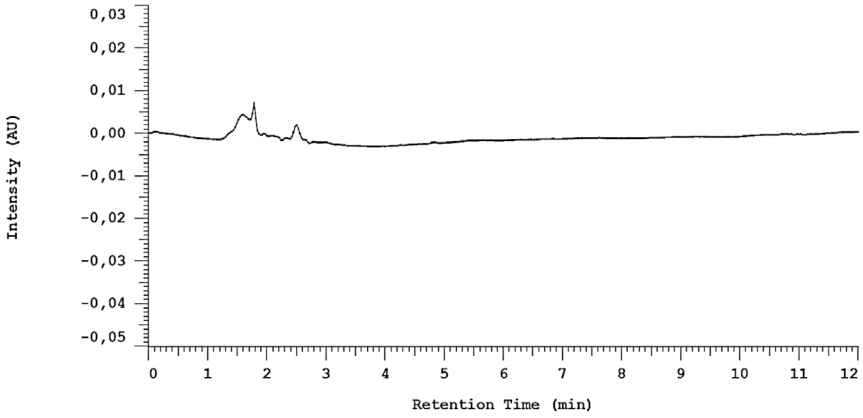

Figure 6. Chromatogram of placebo (Basic Degradation - NaOH 0.1 N).

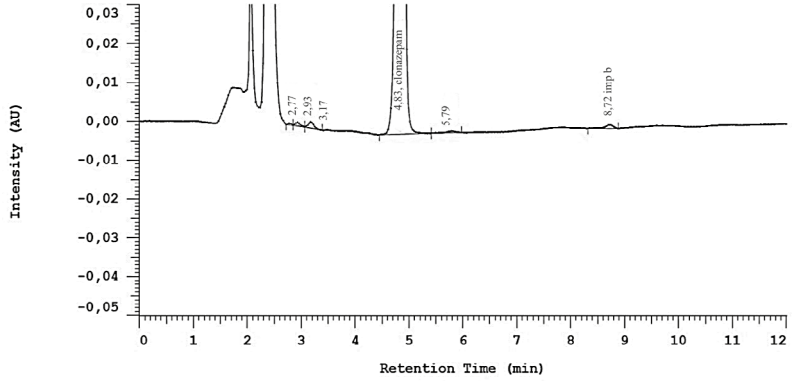

Figure 7. Chromatogram of clonazepam (Basic Degradation - NaOH 0.1 N).

Table 6. Standard forced degradation.

Conditions	Recovery (%) 24 hours	Degradation (%) 24 hours	Recovery (%) 48 hours	Degradation (%) 48 hours
Control Sample	100.0	0.0	100	0.0
Acid Degradation (HCl 0.1 N)	89.5	10.5	79.7	20.3
Basic Degradation (NaOH 0.1 N)	65.3	34.7	48.6	51.4
Oxidative Degradation (H_2O_2 3%)	94.8	5.2	93.8	6.2
Thermal Degradation (60 °C)	102.3	2.3	88.9	11.1
Photolytic Degradation (UV)	99.0	1.0	99.6	0.4

Table 7. Sample forced degradation.

Conditions	Recovery (%) 24 hours	Degradation (%) 24 hours	Recovery (%) 48 hours	Degradation (%) 48 hours
Control Sample	100.0	0.0	100	0.0
Acid Degradation (HCl 0.1 N)	91.6	8.4	79.9	20.1
Basic Degradation (NaOH 0.1 N)	64.8	35.2	48.6	51.4
Oxidative Degradation (H_2O_2 3%)	97.9	2.1	93.8	6.2
Thermal Degradation (60 °C)	103.0	3.0	98.1	1.9
Photolytic Degradation (UV)	99.7	0.3	98.9	1.1

Stability Studies

The samples submitted to temperatures of 30 °C and 40 °C did not show changes in their organoleptic and physical characteristics, when observed macroscopically. The percentage assay obtained at the temperature of 30 °C had minimal variations in the three tested periods. The observation of degradation products in this same condition was only observed in the final period of 90 days.

In samples submitted at a temperature of 40 °C, lower values of assay were observed when compared to the values obtained at a temperature of 30 °C, but without significant variations. Degradation products can be observed in the second evaluated period of 60 days.

The degradation products observed in the accelerated stability study according to Table 8 were also observed in the forced degradation study, without a considerable increase in them. There was no change or variation in the assay results (dosing) above 5% when compared to the initial result in both the 30 °C/75% of relative humidity and in the study at 40 °C/75% of relative humidity as demonstrated in Table 9.

Table 8. Formation of degradation products during the stability study.

Degradations Products	Tested Conditions		
	30 °C / 75% RH	40 °C / 75% RH	
	90 days	60 days	30 days
Related Compound A (RRT≈ 1.20)	x	x	x
Related Compound B (RRT≈1.81)	x	x	x
Unknown impurity (RRT≈0.45)	-	-	x
Unknown impurity (RRT≈0.88)	x	-	x
Unknown impurity (RRT≈0.93)	x	-	x

Table 9. Assay results during the stability study.

Tested Conditions	30°C / 75% RH	40°C / 75% RH
30 days	99%	98%
60 days	99%	97%
90 days	98%	95%

Conclusion

Based on the analyzed dataset, the drug-excipient compatibility performed through the DSC and TG techniques can be considered as the first-choice technique for evaluating compatibilities, due to its speed and versatility.

However, it must be associated with HPLC and FTIR, since these provided quantitative and qualitative information about the active ingredient, respectively. Thermal analysis provided quick results, but difficult to interpret. For this reason, the complementary techniques (FTIR, HPLC) were valid to confirm the results obtained by the thermal analysis and thus generate more reliable results.

According to the results obtained by DSC, TG, FTIR and HPLC, it was possible to identify that there were no significant changes in the isolated drug and in the 1:1 drug/excipient binary mixtures, indicating that there is no interaction between the tested excipients and clonazepam.

The HPLC method developed was optimized to obtain the best conditions for the identification and quantification of clonazepam, offering great advantages in terms of simplicity, since the mobile phase employed is easy and quick to prepare, which allows it to be used routinely in quality control analyses.

Another advantage is the fact that it does not contain salts in its composition, which could damage the column and the chromatographic system, in addition to allowing the identification and reproducible quantitative analysis of clonazepam with analytical reliability.

Through this study it was possible to unify the information about compatibility between clonazepam and excipients, highlighting the importance of these studies for the development of stable formulations.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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A modelagem matemática da detecção de furfural e ácido lático em pães fermentados

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Resumo

Introdução: A concentração de furfural e ácido lático é importante para as propriedades nutricêuticas e parafarmacéuticas dos pães. Neste trabalho, realiza-se uma análise teórica da detecção eletroanalítica de ambos os compostos em pães. **Metodologia:** O processo da detecção eletroanalítica de furfural e ácido lático em pães fermentados no ânodo, modificado pelo oxihidróxido de cobalto, tem sido desenvolvido e analisado do ponto de vista teórico. O modelo matemático correspondente foi desenvolvido e analisado mediante a teoria de estabilidade linear e análise de bifurcações. **Resultados e discussão:** Foi comprovada a eficiência do elétrodo de oxihidróxido de cobalto na detecção eletroanalítica de ambos os analitos em meio ligeiramente ácido. O oxihidróxido de cobalto pode ser estabilizado pelo polímero

condutor básico, para compensar o pH do pão. Quanto ao comportamento oscilatório, este é menos provável que nos casos semelhantes, haja vista a fraca ionização do ácido lático e do ácido furoico – produto da oxidação do furfural. **Conclusões:** Ação catalítica do composto de cobalto facilita a obtenção e manutenção do estado estacionário estável neste sistema e, por conseguinte, providencia a fácil leitura de sinal analítico.

Palavras-chave: furfural, ácido lático, pão fermentado, sensor eletroquímico, estado estacionário estável

Summary

The mathematical modeling for furfural and lactic acid determination in fermented breads

Introduction: The furfural and lactic acid concentration is important for the nutriceutical and parapharmaceutical properties of bread. In this work, a theoretical analysis of the electroanalytical detection of both compounds in bread has been given. **Methodology**: The process of electroanalytical detection of furfural and lactic acid in fermented breads at the anode, modified by cobalt oxyhydroxide, has been developed and analyzed from a theoretical point of view. The corresponding mathematical model was developed and analyzed using linear stability theory and bifurcation analysis. **Results and discussion**: The efficiency of the cobalt oxyhydroxide electrode in the electroanalytical detection of both analytes in a slightly acidic medium was proven. Cobalt oxyhydroxide can be stabilized by the basic conductive polymer to compensate for the pH of the bread. As for the oscillatory behavior, this is less likely than in similar cases, given the weak ionization of lactic acid and furoic acid – the product of furfural oxidation. **Conclusions**: Catalytic action of the cobalt compound facilitates obtaining and maintaining stable steady state in this system and, therefore, provides easy reading of analytical signal.

Keywords: furfural, lactic acid, sourdough bread, electrochemical sensor, stable steady state

Resumen

El modelado matemático de la detección de furfural y ácido láctico en panes fermentados

Introducción: La concentración de furfural y ácido láctico es esencial para las propiedades nutricéuticas y parafarmacéuticas del pan. En este trabajo se realiza un análisis teórico de la detección electroanalítica de ambos compuestos en panes. **Metodología:** Se ha desarrollado y analizado desde un punto de vista teórico el proceso de detección electroanalítica de furfural y ácido láctico en pan fermentado en el ánodo, modificado por oxihidróxido de cobalto. El modelo matemático correspondiente fue desarrollado y analizado utilizando la teoría de estabilidad lineal y análisis de bifurcaciones. **Resultados y discusión:** Se confirmó la eficiencia del electrodo de oxihidróxido de cobalto en la detección electroanalítica de ambos analitos en un medio ligeramente ácido. El oxihidróxido de cobalto puede estabilizarse mediante el polímero conductor básico para compensar el pH del pan. Ya el comportamiento oscilatorio es menos probable que en casos similares, dada la débil ionización del ácido láctico y del ácido furoico, producto de la oxidación del furfural. **Conclusiones:** La acción catalítica del compuesto de cobalto facilita la obtención y el mantenimiento de un estado estable en este sistema y, por tanto, proporciona una fácil lectura de la señal analítica.

Palabras-clave: furfural, ácido láctico, pan de masa madre, sensor electroquímico, estado estacionario estable

Introdução

O Império Otomano, que existiu entre os anos 1299 e 1922, foi um dois maiores impérios no mundo. Na sua maior extensão, em 1687, o Império controlou 5,2 milhões de quilómetros quadrados, em que viveram 30 000 000 de pessoas, ou seja, uma em cada vinte e cinco pessoas no mundo de então viveu no Império Otomano [1-3]. O auge do Império foi durante o reino do sultão Solimão I o Legislador (Kanuni). Na Turquia ele é apelidado de Legislador, por organizar o Kanunname (Código de Leis do Império). O código abrangeu quase todas as áreas, inclusive a produção de pão. A lei previu que a farinha passasse pelo coador de poro minúsculo e que o pão fosse bem passado e não desse nenhum cheiro diferente do "cheiro a pão".

O cheiro a pão é produzido pelo furfural (Fig. 1 à esquerda) – produto da fermentação das pentoses e hexoses na forma de furanose. É um aldeído, formado durante a fermentação natural e artificial da massa [4-7]. Outro produto importante da fermentação é o ácido lático [8-10], que é produto da fermentação da glicose pelas bacilas como *L. bulgaricus, L. delbrueckii, L. acidophilus,* estreptococo *S. thermophilus* (cuja atividade aumenta quando o pão se esquenta) e bifidobactério *B. infantis* e *B. lactis*. O 5-hidroximetilfurfural, produto da fermentação de hexofuranoses, aparece em méis.

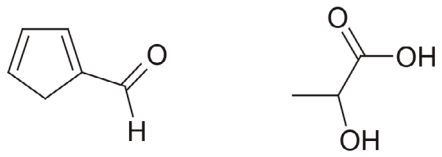

Figure 1. Furfural e ácido lático

Além da fermentação de pão, os bifidobactérios mencionados são usados em formulações farmacêuticas [11, 12] para tratamento de algumas infeções gastrointestinais e diareias.

Quanto ao furfural, ele é um nutricêutico e antioxidante, que também serve como ponto de partida para obtenção de vários fármacos (como furosemida). O ácido lático, por sua vez, além de ser um componente alimentar, é um produto de metabolismo humano. Ele também é usado em formulações farmacêuticas como substância principal corretor de metabolismo. A concentração de ambos os produtos é importante para a manutenção da homeostase. Destarte, o desenvolvimento de um método para a quantificação de ambos os compostos é realmente atual [13-15].

Ambos os compostos são eletroativos, haja vista o possuírem grupos funcionais oxidantes e redutores [14-16]. Assim, tanto os processos anódicos como catódicos são-lhe compatíveis. No caso de um processo anódico, o uso do oxihidróxido de cobalto, um material semicondutor, que se vê como alternativa ao dióxido de titânio, poderia ser usado para a detecção do furfural e ácido lático em pães fermentados.

Sem embargo, tanto a oxidação das moléculas orgânicas pequenas, como a síntese do oxihidróxido de cobalto, soem acompanhar-se pelas instabilidades eletroquímicas [17-19], que influenciam o desempenho eletroanalítico do sensor. Isto pode levar à dificuldade na interpretação do sinal analítico do sensor.

Assim, o objetivo geral deste trabalho é avaliar, do ponto de vista mecanístico teórico, o comportamento do sistema com a detecção eletroanalítica do furfural e ácido lático sobre o oxihidróxido de cobalto. O modelo matemático correspondente é analisado

mediante a teoria de estabilidade linear, para verificar as condições de estabilidade do estado estacionário (e, por conseguinte, do melhor desempenho do sensor), das instabilidades oscilatória e monotônica. Outrossim, far-se-á a comparação do comportamento deste sistema com o dos semelhantes [20, 21].

O sistema e seu modelo

Durante a análise de massa fermentada, cujo pH é levemente reduzido, o oxihidróxido de cobalto, na ausência de analito, reduz-se conforme:

$$CoO(OH) + H^+ + e^- \rightarrow CoO + H_2O \quad (1)$$

Nos valores inferiores de pH, o óxido de cobalto destrói-se, conforme:

$$CoO + 2H^+ \rightarrow Co^{2+} + H_2O \quad (2)$$

Destarte, para estabilizar o oxihidróxido de cobalto, este deve ser depositado sobre um polieletrólito de cariz básico como quitosana, polianilina ou polímeros de corantes com nitrogênio piridínico. Como se mencionará abaixo, a matriz polimérica não deve ser assaz básica para ionizar os ácidos lático, pirúvico e furoico – produto da oxidação do furfural.

A oxidação far-se-á pelo grupo aldeída no caso do furfural e pela hidroxila do ácido lático. Destarte, o processo eletroanalítico realizar-se-á conforme a Fig. 2:

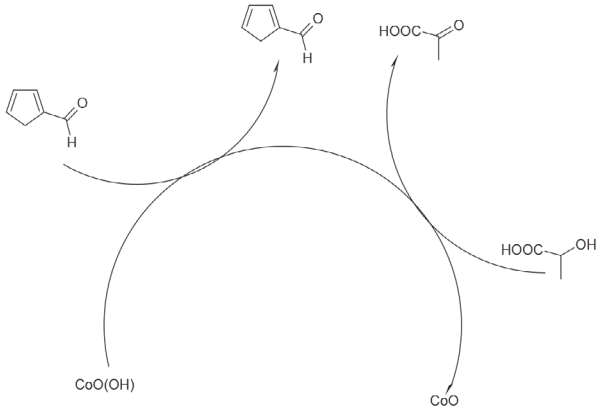

Figure 2. O processo eletroanalítico

Em meios ácido e neutro, o oxihidróxido de cobalto regenerar-se-á na etapa eletroquímica conforme (3):

$$CoO + H_2O - e^- \rightarrow CoO(OH) + H^+ \quad (3)$$

Este processo não inclui interação nem polimerização de cada um dos analitos. Outrossim, nenhuma reação lateral, capaz de comprometer a estabilidade dos analitos e(ou) modificador, se realiza. Assim, para descrever o comportamento deste sistema, assumindo umas suposições [18-21], nós descrevemos o comportamento deste sistema por um conjunto de três equações diferenciais de balanço:

$$\begin{cases} \frac{df}{dt} = \frac{2}{\delta}\left(\frac{\phi}{\delta}(f_0 - f) - f_1\right) \\ \frac{dl}{dt} = \frac{2}{\delta}\left(\frac{\Lambda}{\delta}(l_0 - l) - l_1\right) \\ \frac{dc}{dt} = \frac{1}{c}\left(\frac{1}{\delta}(f_1 + l_1 - c_1)\right) \end{cases} \quad (4)$$

Sendo f e l as concentrações pré-superficiais de furfural e ácido lático, Φ e Λ são seus coeficientes de difusão, f_0 e l_0 as concentrações deles no interior da solução, e f_1 e l_1 as velocidades da sua oxidação pelo oxihidróxido de cobalto (5 – 6), C é a concentração superficial máxima do óxido de cobalto e c_1 é a velocidade da sua eletrooxidação (3, 7).

As velocidades das reações correspondentes são descritas como (5 – 7):

$$f_1 = k_{f1} f (1 - c)^2 \quad (5)$$

$$l_1 = k_{l1} l (1 - c)^2 \quad (6)$$

$$c_1 = k_{c1} c \exp\frac{f\varphi_0}{RT} \quad (7)$$

Sendo os parâmetros k as constantes das velocidades das respectivas reações, F é número de Faraday, φ_0 é salto do potencial, relativo ao potencial de carga zero, R é a constante universal de gases e T é temperatura do vaso.

Neste caso, o meio neutro ou ligeiramente ácido favorece a leitura fácil do sinal analítico e, por conseguinte, otimização no desempenho eletroanalítico do sensor, conforme se descreverá abaixo.

Resultados e discussão

Para investigar o comportamento do sistema com a detecção eletroanalítica do furfural e ácido lático no elétrodo, modificado pelo oxihidróxido de cobalto, analisamos o conjunto de equações diferenciais (4), junto com as relações algébricas (5 – 7) mediante a teoria de estabilidade linear. Os elementos estacionários do Jacobiano podem ser descritos conforme (8):

$$\begin{pmatrix} a_{11} & a_{12} & a_{13} \\ a_{21} & a_{22} & a_{23} \\ a_{31} & a_{32} & a_{33} \end{pmatrix} \tag{8}$$

Sendo:

$$a_{11} = \frac{2}{\delta}\left(-\frac{\Phi}{\delta} - k_{f1}(1-c)^2\right) \tag{9}$$

$$a_{21} = 0 \tag{10}$$

$$a_{13} = \frac{2}{\delta}\left(2k_{f1}f(1-c)\right) \tag{11}$$

$$a_{21} = 0 \tag{12}$$

$$a_{22} = \frac{2}{\delta}\left(-\frac{\Lambda}{\delta} - k_{l1}(1-c)^2\right) \tag{13}$$

$$a_{23} = \frac{2}{\delta}\left(2k_{l1}\,f(1-c)\right) \tag{14}$$

$$a_{31} = \frac{1}{c}\left(k_{f1}(1-c)^2\right) \tag{15}$$

$$a_{32} = \frac{1}{c}\left(k_{l1}(1-c)^2\right) \tag{16}$$

$$a_{33} = \frac{1}{c}\left(-2k_{f1}f(1-c) - 2k_{l1}(1-c) - k_{c1}exp\frac{F\varphi_0}{RT} + jk_{c1}c\,exp\frac{F\varphi_0}{RT}\right) \tag{17}$$

Observando os elementos da diagonal principal (9), (13) e (17) pode-se concluir que em no meio neutro e ligeiramente ácido (correspondente ao pão fermentado), *as oscilações eletroquímicas,* embora se deem, são menos prováveis que nos casos semelhantes, haja vista o baixo grau de ionização de cada um dos analitos e seus produtos de oxidação.

O único fator responsável pelo comportamento oscilatório são as influências da etapa eletroquímica na força iônica, capacitância e impedância da dupla camada elétrica, que provocam câmbios cíclicos no valor da corrente elétrica (neste caso, trata-se de um sensor amperométrico). Embora geralmente as oscilações sejam de alta frequência e pequena amplitude, o seu formato vai depender da natureza do eletrólito de suporte e do polímero condutor usado como matriz. Esta dependência foi observada experimental [18, 19] e teoricamente [20, 21].

Para investigar a estabilidade do estado estacionário mediante a aplicação do critério Routh-Hurwitz, simplificamos o determinante, introduzindo as novas variáveis e reescrevendo o determinante como (18):

$$\frac{4}{\delta^2 c}\begin{vmatrix} -\varphi\Xi & 0 & P \\ 0 & -\lambda - \mathrm{t} & \Sigma \\ \Xi & T & -P-\Sigma-\Omega \end{vmatrix} \tag{18}$$

Abrindo os parêntesis e aplicando o requisito Det J<0, saliente do critério, obtemos, após trocar os signos pelos opostos, o requisito de estabilidade de estado estacionário como (19):

$$\varphi(\lambda \mathrm{P} + \lambda\Sigma + \lambda\Omega + TP) + \Xi(\lambda\Sigma + \lambda\Omega + \mathrm{T}\Omega) > 0 \tag{19}$$

O que define um processo eficiente, controlado pela difusão dos dois analitos. A influência do fator cinético aumenta, quando os efeitos do processo eletroanalítico na dupla camada elétrica se expressam com maior intensidão.

Como citado anteriormente, neste processo eletroanalítico a estabilidade dos analitos e do modificador não é comprometida pelas reações laterais. Assim, a estabilidade do estado estacionário corresponder-se-á à linearidade da dependência entre a concentração de cada um dos analitos e a corrente, o que permitirá a fácil leitura do sinal analítico.

Quanto ao *limite de detecção*, este é correspondente à instabilidade monotônica, que se define sob condição Det J=0, ou (20):

$$\varphi(\lambda \mathrm{P} + \lambda\Sigma + \lambda\Omega + TP + T\Omega) + \Xi(\lambda\Sigma + \lambda\Omega + \mathrm{T}\Omega) > 0 \tag{20}$$

Os ésteres do ácido lático podem ser detectadas por este meio, já que o compósito CoO(OH) – polímero condutor realiza a separação dos picos.

No caso do pH ligeiramente alcalino, a detecção de ambos os compostos pode realizar-se. Entretanto, o lactato, o piruvato como produto da oxidação do lactato e o íon furoato como produto da oxidação do furfural, ficarão presentes em forma iônica, influenciando a dupla camada elétrica. Destarte, as velocidades das reações (5 – 6) reescrever-se-ão incluindo uma parte exponencial, presente em [20, 21].

Quanto ao *processo catódico*, este é mais favorável para o meio ácido, correspondente aos pães fermentados e às formulações farmacêuticas. Neste caso, o oxihidróxido de vanádio bivalente, ou um polímero condutor são sugeridos como modificadores de elétrodo. Assim, o comportamento do sistema será descrito pelo conjunto (21):

$$\begin{cases} \frac{df}{dt} = \frac{2}{\delta}\left(\frac{\Phi}{\delta}\ f_0 - f) - f_2\right) \\ \frac{dl}{dt} = \frac{2}{\delta}\left(\frac{\Lambda}{\delta}(l_0 - l) - l_1\right) \\ \frac{dc}{dt} = \frac{1}{v}\left(f_1 + f_2 + l_1 - c_1\right) \end{cases} \tag{21}$$

Considerando as duas possibilidades de redução do furfural.

Este processo é ainda mais compatível e descrever-se-á num dos nossos próximos trabalhos.

Conclusões

Da análise do processo com a determinação do furfural e do ácido lático, foi possível concluir que a ação catalítica do composto de cobalto facilita a obtenção e manutenção do estado estacionário estável neste sistema e, por conseguinte, a fácil leitura de sinal analítico. O processo eletroanalítico é controlado pela difusão, não sendo, porém, dispensável o fator cinético. Quanto ao comportamento oscilatório, este é possível neste sistema. Ele é causado pelos efeitos periódicos na estrutura da dupla camada elétrica

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Os autores não têm conflitos de interesse a declarar.

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Efecto de la deprescripción de medicación inapropiada en adultos mayores: una revisión de literatura y meta-análisis de ensayos clínicos controlados

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Resumen

Introducción: La pluripatología en las personas mayores es un factor de riesgo para polimedicación, cascada de medicación y medicación potencialmente inapropiada. La deprescripción se plantea como estrategia para reducir la carga farmacológica y medicación inapropiada. **Objetivo:** Establecer el efecto de la deprescripción en los resultados en salud de personas mayores según lo reportado por la literatura. **Metodología:** Se desarrolló una revisión sistemática y meta-análisis hasta junio del 2020, utilizando las bases de datos MEDLINE vía PubMed, LILACS, SciELO y Epistemonikos, siguiendo la metodología PRISMA. Se incluyeron estudios experimentales y cuasi-experimentales, en idioma inglés y español. Se evaluaron los niveles de evidencia y el riesgo de sesgo para cada estudio individual. La reducción de prescripciones potencialmente inapropiadas, la mortalidad, las caídas y el número de hospitalizaciones fueron evaluadas en el meta-análisis. **Resultados:** Se identificaron un total de 1524 registros por bases de datos. Se incluyeron 24 artículos en el análisis descriptivo y 14 en el cuantitativo. El número de prescripciones potencialmente inapropiadas se redujo significativamente (OR 0,51; IC 95% 0,41 – 0,62), igual que el número de caídas (OR 0,73; IC 95% 0,57 – 0,94) después de aplicar las interven-

ciones de deprescripción de medicación inapropiada. **Conclusiones:** Se evidenció un efecto positivo en la reducción de prescripciones potencialmente inapropiadas y el número de caídas. La deprescripción parece no tener efecto significativo sobre la mortalidad y la calidad de vida.

Palabras Clave: Ancianos frágiles; polifarmacia; medicamentos potencialmente inapropiados; deprescripción.

Summary

Effect of deprescribing inappropriate medication in older adults: a literature review and meta-analysis of controlled clinical trials

Introduction: Pluripathology in older people is a risk factor for polypharmacy, medication cascade and potentially inappropriate medication. Deprescribing is proposed as a strategy to reduce pharmacological burden and inappropriate medication. **Objective:** Establish the effect of deprescription on the health outcomes of older people as reported by the literature. **Methodology:** A systematic review and meta-analysis was developed until June 2020, using the MEDLINE databases via PubMed, LILACS, SciELO and Epistemonikos, following the PRISMA methodology. Experimental and quasi-experimental studies were included, in English and Spanish. Levels of evidence and risk of bias were assessed for each individual study. The reduction of potentially inappropriate prescriptions, mortality, falls and the number of hospitalizations were evaluated in the meta-analysis. **Results:** A total of 1524 records were identified per database. 24 articles were included in the descriptive analysis and 14 in the quantitative analysis. The number of potentially inappropriate prescriptions was significantly reduced (OR 0.51; 95% CI 0.41 – 0.62), as was the number of falls (OR 0.73; 95% CI 0.57 – 0.94) after applying interventions to deprescribe inappropriate medication. **Conclusions:** A positive effect was evident in the reduction of potentially inappropriate prescriptions and the number of falls. Deprescribing appears to have no significant effect on mortality and quality of life.

Keywords: Frail Elderly; Polypharmacy; Potentially Inappropriate Medication List; Deprescriptions.

Resumo

Efeito da prescrição de medicamentos inapropriados em idosos: revisão de literatura e meta-análise de ensaios clínicos controlados

Introdução: A pluripatologia em idosos é um fator de risco para polifarmácia, cascata de medicamentos e medicamentos potencialmente inapropriados. A desprescrição é proposta como estratégia para reduzir a carga farmacológica e a medicação inadequada. **Objetivo:** Estabelecer o efeito da desprescrição nos desfechos de saúde dos idosos conforme relatado pela literatura. **Metodologia:** Foi desenvolvida uma revisão sistemática e meta-análise até junho de 2020, utilizando as bases de dados MEDLINE via PubMed, LILACS, SciELO e Epistemonikos, seguindo a metodologia PRISMA. Foram incluídos estudos experimentais e quase-experimentais, em inglês e espanhol. Os níveis de evidência e o risco de viés foram avaliados para cada estudo individual. A redução de prescrições potencialmente inapropriadas, mortalidade, quedas e número de internações foram avaliadas na metanálise. **Resultados:** Foram identificados 1.524 registros por base de dados. Foram incluídos 24 artigos na análise descritiva e 14 na análise quantitativa. O número de prescrições potencialmente inapropriadas foi significativamente reduzido (OR 0,51; IC 95% 0,41 – 0,62), assim como o número de quedas (OR 0,73; IC 95% 0,57 – 0,94) após a aplicação de intervenções para prescrever medicamentos inapropriados. **Conclusões:** Foi evidente um efeito positivo na redução de prescrições potencialmente inadequadas e no número de quedas. A prescrição parece não ter efeito significativo na mortalidade e na qualidade de vida.

Palavras-chave: Idosos frágeis; polifarmácia; medicamentos potencialmente inapropriados; descrição.

Introducción

La esperanza de vida se ha incrementado debido a las mejoras sanitarias y el desarrollo científico y tecnológico que han tenido las ciencias en las últimas décadas. Sin embargo, también se han incrementado las enfermedades crónicas no transmisibles al punto de ser la causa del 71% de las muertes a nivel mundial [1] y dentro de estas se destacan las enfermedades que implican un manejo con medicación recurrente [2-4]. Las cifras de medicamentos por pacientes a nivel mundial pueden oscilar entre 3 y 10 medicamentos por paciente [3, 5].

La polimedicación es un fenómeno común en las personas mayores por la necesidad de tratar distintas comorbilidades. Con el uso de múltiples medicamentos los riesgos de resultados negativos en salud como los efectos adversos, interacciones medicamentosas, no adherencia al tratamiento, disminución de la funcionalidad, síndromes geriátricos y costos de atención elevados, se incrementan [6].

Según algunos estudios, la polimedicación se asocia con pobres resultados en salud (deterioro de la cognición, fragilidad, caídas, morbilidad y discapacidad) y con un incremento en la mortalidad [7-10]. El riesgo de aparición de estos eventos aumenta proporcionalmente a la cantidad de medicamentos prescritos, pasando de un 13% con dos medicamentos, a un 58% al tener cinco [11]. Adicionalmente, las personas mayores son más susceptibles ante los efectos negativos de la polimedicación, debido a los cambios fisiológicos, físicos y sociales que se experimentan como parte de esta etapa de la vida [12].

En una comunidad de ancianos hospitalizados en casas de reposo, el 48,7% tiene medicaciones potencialmente inapropiadas (MPI) [13], con una prevalencia del 42% en estas comunidades [14]. En España se reporta que el 69,9% de los pacientes dados de alta en servicios de geriatría tienen polimedicación, mientras que de los otros servicios tienen un porcentaje más alto de 79,7% [15]. En Colombia, un estudio desarrollado en la ciudad de Bogotá [5] reportó que aproximadamente el 7% de los medicamentos que consumen los pacientes crónicos eran MPI. En Europa, las cifras de MPI en ancianos oscila entre el 23% y el 36% [16-18], mientras que en Asia, se encuentran valores más elevados (52,5%) [19]. En Estados Unidos afecta a un 40% de las personas mayores que residen en hogares de reposo y un 12% en aquellos que viven en la comunidad [20]. En Brasil, se reporta una prevalencia del 37,6%, siendo mayor en los ancianos entre 60-69 años de edad [21]. Por su parte, en Colombia, se reporta que aproximadamente el 21,5% de los ancianos hospitalizados tienen al menos una MPI [22].

Una revisión identificó a las benzodiacepinas, los anti-inflamatorios no esteroideos (AINE), antihistamínicos y antipsicóticos dentro de las MPI más frecuentes en personas mayores [23], y a pesar de los esfuerzos y la existencia de distintos métodos para identificarlas, el uso de herramientas y criterios que permiten una detección oportuna es limitado [24].

Ante estas cifras, se plantea la deprescripción como una estrategia para revertir los efectos iatrogénicos de las MPI, con el objetivo de mejorar el uso de los medicamentos y prevenir efectos adversos en las personas mayores [8], mejorando los resultados clínicos en salud [25], con beneficios en la adherencia terapéutica, la calidad de vida y la reducción de la mortalidad [26]. Considerando lo anterior, esta revisión sistemática de

literatura y meta-análisis tuvo como objetivo establecer el efecto de la deprescripción en los resultados en salud de personas mayores según reportes de la literatura científica.

Métodos

La presente revisión sistemática de la literatura se realizó según el protocolo estándar para revisiones sistemáticas y meta-análisis PRISMA [27]. Este estudio no fue registrado en *The International Prospective Register of Systematic Reviews* (PROSPERO) pero el protocolo fue preparado previamente y no tuvo enmiendas.

Criterios de elegibilidad (*Eligibility criteria*)

Para esta revisión sistemática se tuvieron en cuenta como estudios elegibles aquellos con diseño experimental y cuasiexperimental, que incluyeran participantes personas de ≥ 60 años de edad atendidos en diferentes servicios y niveles de atención, publicados hasta junio de 2020 en idioma inglés o español. Fueron excluidos los estudios que no cumplieron los criterios de inclusión, los protocolos de investigación, los reportes de investigaciones no concluidas y aquellos con texto completo no disponible.

Fuentes de información (*Information source*)

La búsqueda fue realizada en las bases de datos MEDLINE vía PubMed, LILACS, SciELO, Epistemonikos y CENTRAL utilizando los términos referidos para ensayos clínicos controlados aleatorizados, en el idioma inglés y español, publicados hasta junio de 2020.

Estrategia de búsqueda (*Search strategy*)

Los términos usados fueron ajustados para cada una de las bases de datos empleando MeSH (Medical Subject Headings) y Decs (Descriptores en Ciencias de la Salud) (Material suplementario; Tabla S1). Se realizaron búsquedas manuales en las listas de referencias de las publicaciones recuperadas.

Proceso de selección (*Selection process*)

Se recopilaron todas las citas identificadas y fueron transferidas al gestor bibliográfico EndNote® Web (Clarivate Analytics, Filadelfia, PA, EE. UU.), lo cual permitió la identificación y eliminación de los textos duplicados. Posteriormente, dos autores revisaron los títulos y resúmenes (KO y RD), evaluándose por separado los artículos con alta probabilidad de inclusión a texto completo.

Extracción y síntesis de datos (*Data collection process*)

La extracción de datos fue realizada de forma independiente en una plantilla estandarizada que incluyó información referente a las características iniciales y duración del estudio, la medicación objeto de deprescripción, los tipos de intervención, los proveedores de las mismas y los resultados reportados. Adicionalmente, se extrajo de los estudios la información relacionada con la población de estudio, país, tipo de estudio, muestra, sexo, edad promedio, tipo de intervención, duración de la intervención (meses) y un breve resumen del resultado del estudio. Los artículos con alta elegibilidad fueron evaluados a texto completo por dos investigadores de forma independiente. Se utilizó Revman versión 5.4.1 para la consolidación de los datos de los estudios de la revisión sistemática y meta-análisis.

Datos (*Data items*) / Resultados (*Outcomes*)

En los estudios, se buscaron resultados que permitieran evaluar la eficacia de las intervenciones de deprescripción relacionados con los siguientes resultados: reducción de la prescripción potencialmente inapropiada, mortalidad, caídas y hospitalizaciones. Lo anterior, a partir de las intervenciones referidas a prácticas de deprescripción de medicamentos relacionadas con la revisión de medicación, recomendaciones de deprescripción, intervenciones educativas, soporte en recursos electrónicos o webs para toma de decisiones en medicación. Lo anterior, comparado con la revisión estándar en la medicación, entendida como a aquella que se realiza de manera habitual durante la atención del paciente por parte del profesional de la salud (médico, enfermera o farmacéutico).

Evaluación del riesgo de sesgo (*Study risk of bias assessment*)

Los estudios elegibles fueron evaluados de forma independiente usando la herramienta de valoración de riesgo de sesgo de Cochrane [28] y el nivel de evidencia con la herramienta Sackett [29]. Para cada estudio se evaluaron los cinco dominios del riesgo de sesgo (sesgo de selección, de rendimiento, detección, desgaste, notificación y otros sesgos). Las categorías de evaluación de sesgo fueron bajo riesgo, alto riesgo y no es claro. Los desacuerdos fueron resueltos mediante la discusión con un tercer revisor (JB).

Medición del efecto (*Effect measures*)

El análisis primario se efectuó a partir de la comparación del efecto de las intervenciones de deprescripción y los resultados evaluados. Para las variables dicotómicas, se estimó el *Odd Ratio* (*OR*) con sus intervalos de confianza (IC) del 95%. Adicionalmente se realizó medición de la heterogeneidad mediante el estadístico I^2. Los análisis se realizaron con la herramienta Review Manager (RevMan) versión 5.4.1 del Grupo Cochrane.

Síntesis de métodos (*Synthesis methods*)

El meta-análisis incluyó un análisis de subgrupos de los resultados incluidos, se incluyó una valoración de la heterogeneidad estadística por los grupos y general. Además se realizó la valoración visual del modelo meta-analítico mediante la inspección del *forest plot* para cada resultado. Se realizó un análisis de sensibilidad utilizando el método "Excluir resultados de estudios específicos" para evaluar el impacto de cada estudio en el efecto combinado.

Resultados

Descripción de los estudios de la búsqueda

En la búsqueda se identificaron un total de 1524 registros por base de datos y se adicionaron 10 registros obtenidos por búsqueda manual. Después de evaluar los títulos y resúmenes, se evaluaron 64 artículos a texto con alta probabilidad de elegibilidad Al finalizar el proceso, un total de 24 artículos se incluyeron en la revisión descriptiva y 14 en el meta-análisis (Figura 1). Las razones por las cuales los demás se excluyeron fueron: duplicidad, otra metodología (cualitativo), idioma diferente al inglés o al español, no especificaba la edad.

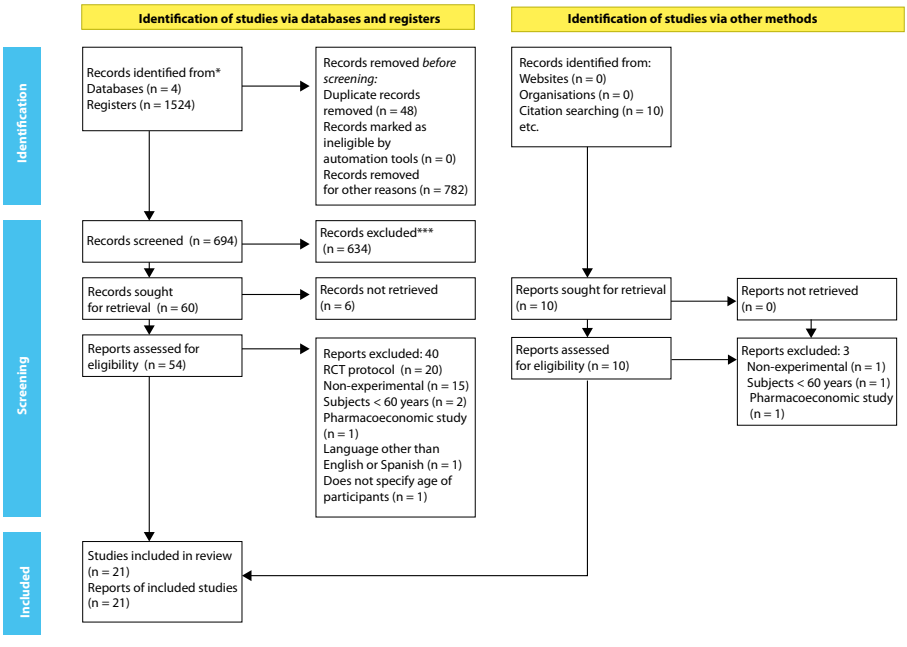

Figura 1. Diagrama PRISMA.

Características de los estudios

El 83,3% (n = 20) de los estudios incluidos en la revisión eran de tipo experimental, específicamente ensayos clínicos aleatorizados. En relación a la edad de los participantes, todos incluyeron participantes con edades iguales o superiores a 60 años. Dos de los artículos reportaron las edades en términos de media y no por rango: Ballard *et al.* [30], con 85,26 ± 7,02 años y Lang *et al.* [31], con 80 ± 8,1 años respectivamente (Tabla 1).

La intervención de deprescripción más utilizada fue la revisión de medicación reportada en un 58,3% de los trabajos analizados, seguida de las intervenciones de tipo educativo con un 20,8%. Diferentes profesionales fueron los proveedores de estas, destacando los farmacéuticos con un 62,5%, seguido por los médicos y las enfermeras con un 58,3% y 20,8%, respectivamente. Siete de los artículos se referían a equipos multidisciplinarios.

El 33,3% de los estudios no reportó una herramienta específica que guiara el proceso de deprescripción. El instrumento mayormente utilizado fue la lista de criterios STOPP/ START (*Screening Tool of Older Person's Prescriptions/ Screening Tool to Alert doctors to the Right Treatment*) en un 25%, seguidos por los Criterios Beers y otras herramientas (Tabla 2).

Resultados de salud de las intervenciones de deprescripción

Reducción de la medicación potencialmente inapropiada (MPI)

El análisis de las intervenciones usadas para reducir la MPI incluyó tres subgrupos: revisión de la medicación, conferencia de caso y recomendaciones de deprescripción. Los hallazgos del meta-análisis para este resultado indican un OR = 0,51 (IC95% 0,41–0,62) y una heterogeneidad del 0%, es decir, los resultados mostraron que la deprescripción tiene un efecto significativo en la reducción de MPI (Figura 2).

Mortalidad

El análisis del subgrupo de las intervenciones de revisión de la medicación incluyó seis estudios con un OR= 0,83 (IC95% 0,66-1,04) y una heterogeneidad del 24. El subgrupo de intervención educativa incluyó tres estudios con un OR=1,58 (IC95% 0,99-2,52) y una heterogeneidad del 0%. De acuerdo con los resultados del meta-análisis (Figura 3), para este resultado se obtuvo un OR=0,91 (IC95% 0,76 – 1,09) y una heterogeneidad de 47,2%. Lo anterior indica que la deprescripción no tiene un efecto significativo en la reducción de la mortalidad.

Tabla 1. Intervenciones para la deprescripción de medicación potencialmente inapropiada en personas mayores: revisión sistemática de la literatura

N°	Referencia y año	País	Tipo de estudio	Duración[a]	Edad[b]	Muestra	Escenario	Intervención	Proveedor	Herramienta de Deprescripción	Medicación Objeto
1	Ailabouni *et al.* (2019) [32]	Nueva Zelanda	Antes-Después	6	≥ 65	46	Asilo de Ancianos	Revisión de Medicación	Farmacéutico y Médico general	Drug Burden Index	Anticolinérgicos y Sedantes
2	Allard *et al.* (2001) [33]	Canadá	ECA	12	>75	266	Comunidad	Conferencia de Caso	Médico, Farmacéutico y Enfermera	Quebec Committee on Drug Use in the Elderly	Polifarmacia
3	Anderson *et al.* (2020) [34]	Australia	EC	18	≥ 70	145	Centros de Atención Primaria	Revisión de Medicación	Médicos	No reportada	Polifarmacia
4	Ballard *et al.* (2016) [30]	Reino Unido	ECA	9	85,26 ± 7,02	277	Asilo de Ancianos	Revisión de Medicación	Médicos	Guía NICE de demencia	Antipsicóticos
5	Boersma *et al.* (2019) [35]	Países Bajos	ECA	12	≥ 70	124	Clínica geriátrica ambulatoria	Recomendaciones de Deprescripción	Médicos	STRIP Asistant: STOPP/START	Polifarmacia
6	Bregnhøj *et al.* (2009) [36]	Dinamarca	ECA	3	≥ 65	166	Comunidad	Entrenamiento Educativo Interactivo	Médicos	Medication Appropriateness Index	Polifarmacia
7	Clyne *et al.* (2015) [37]	Irlanda	ECA	12	≥ 70	196	Centros de Atención Primaria	Revisión de Medicación	Médicos, Farmacéuticos	No reportada	Polifarmacia
8	Frankenthal *et al.* (2014) [38]	Israel	ECA	12	≥ 65	359	Asilo de Ancianos	Revisión de Medicación	Farmacéuticos	STOPP/START	Polifarmacia

Table 1. *Continuation.*

N°	Referencia y año	País	Tipo de estudio	Duración[a]	Edad[b]	Muestra	Escenario	Intervención	Proveedor	Herramienta de Deprescripción	Medicación Objeto
9	García-Gollarte *et al.* (2014) [39]	España	ECA	3	> 65	1.018	Asilo de Ancianos	Revisión de Medicación/ Intervención Educativa	Médicos	STOPP/ START	Polifarmacia
10	Geurts *et al.* (2016) [40]	Paises Bajos	ECA	4	≥ 60	512	Comunidad	Revisión de Medicación	Farmacéuticos y Médicos	No reportada	Polifarmacia
11	Gutiérrez-Valencia *et al.* (2019) [41]	España	Antes-Después	6	≥ 75	234	Hospital	Revisión de Medicación	Farmacéuticos	STOPP/ START	Polifarmacia
12	Hanlon *et al.* (1996) [42]	Estados Unidos	ECA	12	≥ 65	288	Comunidad	Deprescripción liderada por farmacéutico	Farmacéutico Clínico	Medication Appropriateness Index	Polifarmacia
13	Lang *et al.* (2012) [31]	Suiza	Prospectivo de intervención	6	80 ± 8,1	150	Hospital	Intervención educativa	Médicos Geriatras y Psiquiatras	STOPP/ START	Polifarmacia
14	Lenander *et al.* (2014) [43]	Suecia	ECA	15	≥ 65	209	Centro de Atención Primaria	Revisión de Medicación	Farmacéuticos	Criterios Beers	Polifarmacia
15	Lisby *et al.* (2010) [44]	Dinamarca	ECA	3	≥ 70	99	Hospital	Revisión Sistemática de Medicación	Farmacéuticos	No reportada	Polifarmacia
16	Martin *et al.* (2018) [45]	Canadá	ECA	6	≥ 65	489	Comunidad	Intervención Educativa	Farmacéuticos	No reportada	Sedantes-hipnóticos, Antihistamínicos, Glibenclamida y AINEs

Table 1. *Continuation.*

N°	Referencia y año	País	Tipo de estudio	Duración[a]	Edad[b]	Muestra	Escenario	Intervención	Proveedor	Herramienta de Deprescripción	Medicación Objeto
17	McDonald, *et al.* (2019) [46]	Canadá	EC	12	≥ 65	873	Hospital	Soporte informático para toma de decisiones	Médicos, Farmacéuticos y Enfermeras	STOPP, Beers y Choosing Wisely list	Polifarmacia
18	Olsson *et al.* (2012) [47]	Suecia	ECA	12	≥ 75	150	Comunidad	Revisión de Medicación	Médicos, Enfermeras	No descrita	Polifarmacia
19	Pitkäla *et al.* (2014) [48]	Finlandia	ECA	12	≥ 65	227	Asilo de Ancianos	Intervención Educativa	Enfermeras	Criterios Beers	Polifarmacia
20	Pope *et al.*, 2011 [49]	Reino Unido	ECA	6	≥ 60	225	Hospital Residencial	Revisión de Medicación	Médicos, Farmacéuticos, Enfermeras	Criterios Beers	Polifarmacia
21	Potter *et al.* (2016) [50]	Australia	ECA	12	≥ 65	95	Asilo de Ancianos	Revisión de Medicación	Farmacéuticos, Médicos	Algoritmo de Deprescripción	Polifarmacia
22	Tamblyn *et al.* (2003) [51]	Canadá	ECA	13	> 65	12.560	Comunidad	Soporte informático para toma de decisiones	Médico	No reportada	Polifarmacia
23	Tannenbaum *et al.* (2014) [52]	Canadá	ECA	6	≥ 65	261	Comunidad	Intervención Educativa	Farmacéuticos	No reportada	Benzodiacepinas
24	Verdoorn *et al.* (2019) [53]	Países Bajos	ECA	6	≥ 70	629	Comunidad	Revisión de Medicación	Farmacéuticos	No reportada	Polifarmacia

ECA= Ensayo clínico aleatorizado, NICE= National Institute for Health and Care Excellence, STRIP= Systematic Tool to Reduce Inappropriate Prescribing.

[a] en meses.

[b] en años.

Tabla 2. Características resumidas de los estudios

Variable		N	%
Tipo de Estudio			
	Experimental	20	83,3
	Cuasiexperimental	4	16,7
Edad de los Participantes			
	≥ 60 años	14	58,3
	≥ 70 años	8	33,3
	≥ 80 años	0*	0
Ubicación			
	Europa	14	58,3
	América	6	25,0
	Asia	1	4,2
	Oceanía	3	12,5
Duración del Estudio			
	3 – 6 meses	11	45,8
	7 – 12 meses	10	41,7
	> 12 meses	3	12,5
Escenario			
	Comunidad	9	37,5
	Asilo de ancianos	6	25,0
	Hospital	5	20,8
	Clínica geriátrica ambulatoria	1	4,2
	Centro de atención primaria	3	12,5
Medicación Objeto			
	Polifarmacia	20	83,3
	Sedantes-hipnóticos-BZD	3	12,5
	Antipsicóticos	1	4,2
	AINEs	1	4,2
	Otros fármacos	2	8,3
Intervención			
	Revisión de Medicación	12	57,1
	Intervención educativa	5	19,0
	Conferencia de caso	1	4,8
	Recomendaciones de Deprescripción	1	4,8
	Deprescripción directa	1	4,8
	Soporte informático	2	9,5

Tabla 2. *Continuation.*

Proveedor de la Intervención			
	Farmacéutico	13	61,9
	Médico	12	57,1
	Enfermeras	5	23,8
Herramienta de Deprescripción			
	Criterios Beers	4	16,7
	STOPP/START	6	25,0
	Índices (DBI/MAI)	3	12,5
	Otras herramientas	4	16,7
	No reportada	8	33,3

BZD= Benzodiacepinas, AINEs= Analgésicos Antinflamatorios No Esteroideos, DBI= Drug Burden Index, MAI= Medication Appropiateness Index.
*Dos trabajos presentaron la edad de sus participantes en valor de medias y no por rangos.

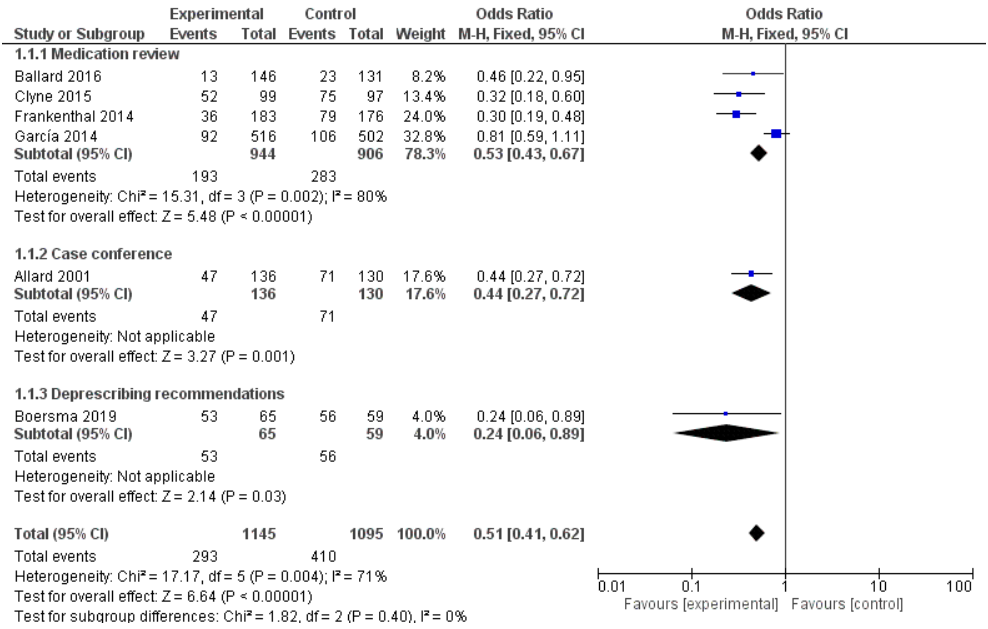

Figura 2. Efecto de la deprescripción en la reducción de medicación potencialmente inapropiada.

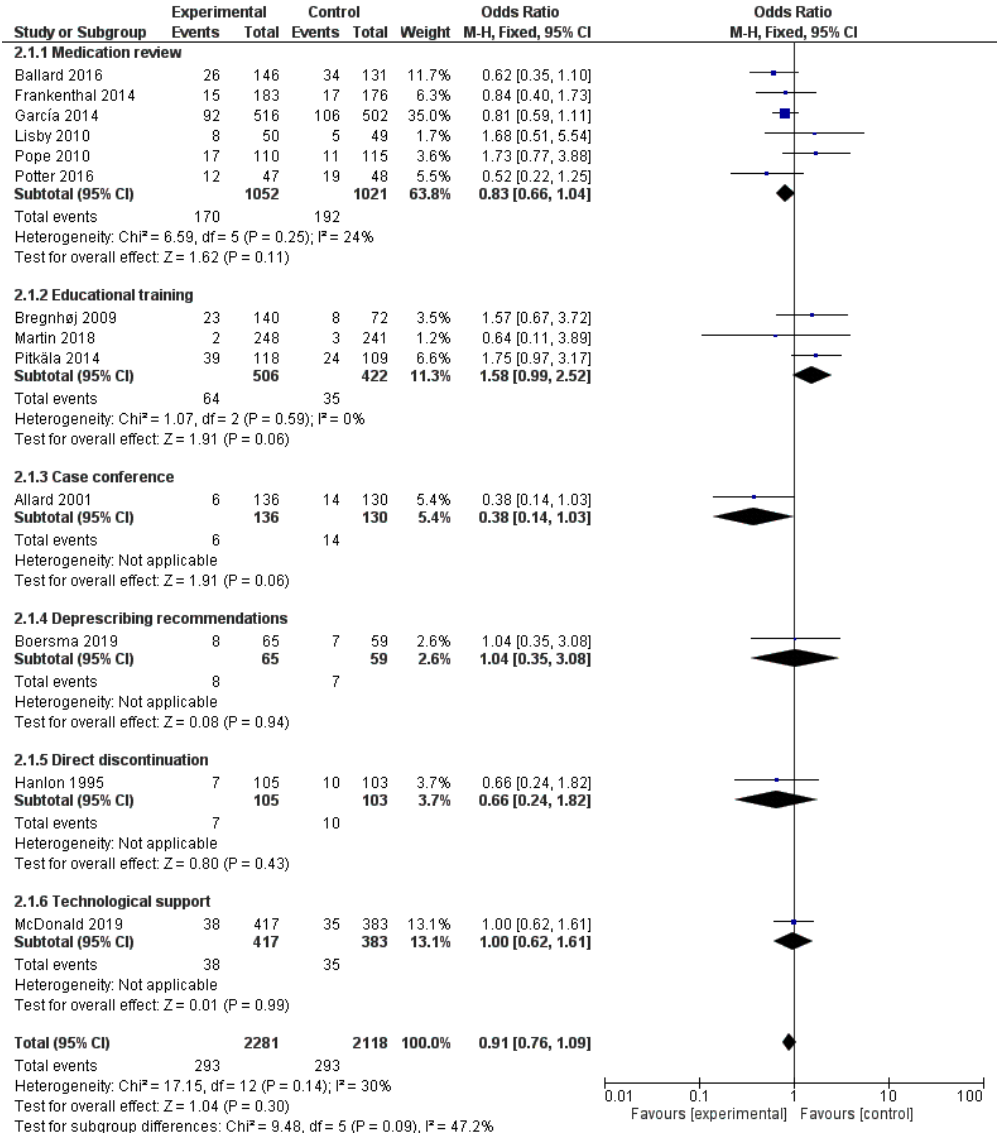

Figura 3. Efecto de la deprescripción en la mortalidad

Caídas

Cuatro estudios [32, 39, 46, 50] reportaron resultados en relación a las caídas o el riesgo de las mismas. Todos mostraron reducción en su número luego de las intervenciones de

deprescripción, sin embargo, en uno de ellos [50], esta no mostró significancia estadística. La agrupación de tres estudios analizables (Figura 4) mostró que la deprescripción reduce de forma significativa las caídas (OR 0,73; IC 95% 0,57 – 0,94).

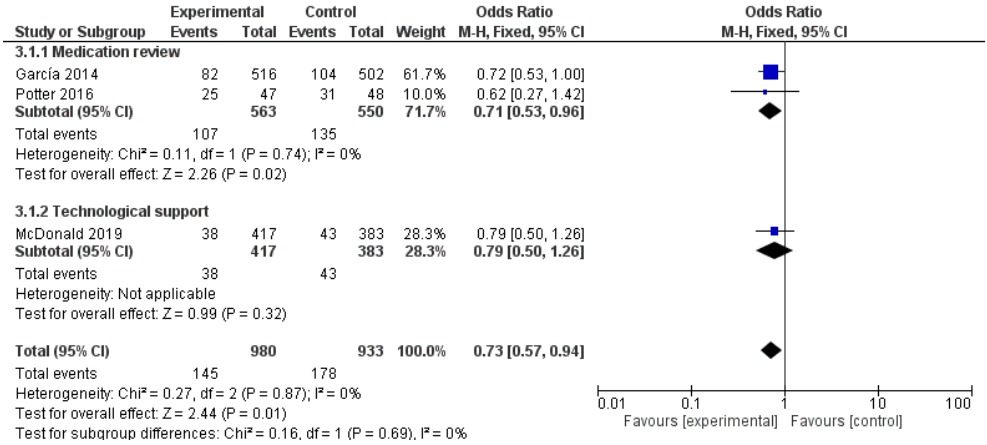

Figura 4. Efecto de la deprescripción en las caídas

Hospitalización

Cinco estudios [39, 44, 48-50] analizaron el requerimiento de hospitalización después de las intervenciones. Tres de ellos no evidenciaron diferencia significativa al comparar el grupo experimental con el grupo control. Un solo estudio [48] mostró disminución del número de hospitalizaciones en los individuos del grupo experimental con significancia estadística; mientras que Pope *et al.* [49], evidenciaron lo contrario al presentarse un aumento en el número de hospitalizaciones en el grupo experimental, también sin significancia estadística.

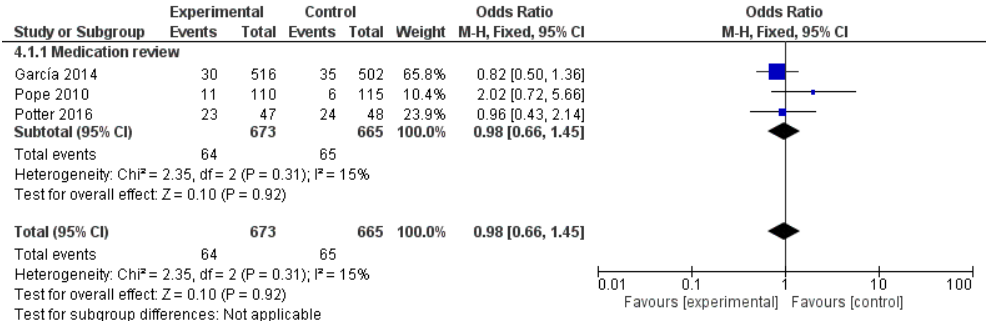

Figura 5. Efecto de las intervenciones de deprescripción en el número de hospitalizaciones.

En relación a la estancia hospitalaria, tres de estos artículos reportaron un menor número de días en el hospital en el grupo experimental frente a los controles.

El meta-análisis de tres artículos analizables (Figura 5) muestra que las intervenciones de deprescripción no tienen un efecto significativo en la reducción del número de hospitalizaciones (OR 0,98; IC 95% 0,66 – 1,45).

Calidad de vida

Cinco artículos [32, 42, 47, 48, 54] reportaron resultados en relación a la calidad de vida, cuatro sin evidenciar diferencia alguna entre ambos grupos. Por otro lado, Pitkäla *et al.* [48], aseguran una disminución significativa en la calidad de vida autorreferida por los individuos del grupo experimental después de haberse realizado la deprescripción en comparación con los controles. No hubo artículos con resultados que pudieran aportar al meta-análisis.

Calidad de la información

En relación a la calidad de la información reportada por las investigaciones analizadas, la mayor proporción (58,3%) se ubicó en el nivel de evidencia 1b con un grado de recomendación A, seguido por el nivel 2b con grado de recomendación B en un 41,7%.

El mayor riesgo de sesgo se presentó en el ocultamiento de la asignación de los participantes a los grupos como experimental y control, con un total de 41,7% de los artículos ubicados en riesgo alto. Así mismo, el cegamiento de los participantes y el personal mostró un gran porcentaje de riesgo poco claro y la menor tasa de riesgo bajo (Figura 3).

El menor riesgo de sesgo se evidenció en referencia al reporte de los resultados, con la mayor tasa de riesgo bajo en el reporte de los resultados incompletos, seguido por la notificación selectiva de los resultados donde ningún artículo evidenció riesgo alto.

Discusión

Aunque la deprescripción surge en la literatura a partir del 2003, se han desarrollado varias revisiones que contemplan la deprescripción y analizan diferentes aspectos sobre ella, incluyendo el efecto que esta tiene sobre algunos resultados en salud [7, 8, 55, 56].

Huiskes *et al.* [55], Gutiérrez-Valencia *et al.* [57] y Bülow *et al.* [58], reportan resultados similares en sus revisiones en relación a la ubicación geográfica de los estudios; mientras que otros tres estudios [8, 56, 59] muestran un mayor número de investigaciones en los Estados Unidos seguidos del continente europeo.

Dos estudios [8, 58], al igual que esta revisión, reportan como el tipo de medicación más prevalente como objeto de deprescripción a la polifarmacia, a diferencia de Page *et al.* [7] reporta un mayor número de estudios que realizaron deprescripción de una sola medicación como fármaco individual, situación que no fue evidenciada en ninguno de los estudios analizados en la presente investigación.

El escenario más frecuente en el que se realizó la deprescripción en esta revisión fue el ámbito comunitario, resultado obtenido también por Page *et al.* [7] seguido del hospital y los asilos. Una de las revisiones sólo incluyó estudios realizados en hogares para ancianos [8]. Lo anterior puede deberse a que estos escenarios agrupan una mayor cantidad de personas adultas mayores en el contexto poblacional.

En lo que respecta a los profesionales que proveen las intervenciones de deprescripción, se encontraron resultados similares encabezando a los farmacéuticos como el principal actor a cargo del proceso [8, 58], aunque dos estudios especifican que estos hacían parte de equipos multidisciplinarios [57, 59]. Por otro lado, una revisión encabeza a los médicos como principales proveedores de la deprescripción [7].

Esta última muestra como la intervención más frecuente a las revisiones de medicación, así como Kua *et al.* [8] con un 26,8% y Gutiérrez-Valencia *et al.* [57] con un 72%, resultado similar al obtenido en la presente revisión. Mientras que el estudio de Thompson-Coon *et al.* [56] contrasta con las intervenciones educativas como las más frecuentes.

Adentrándose en el análisis de los resultados en salud, varios estudios [8, 56-59] reportan similares resultados en cuanto a la reducción significativa del número de medicamentos, con la consecuente disminución de las prescripciones potencialmente inapropiadas.

Uno de los estudios [7] muestra una reducción significativa de la mortalidad cuando la deprescripción se aplica a través de intervenciones específicas para cada paciente en contraste cuando estas son de carácter educativo con una tasa mayor de mortalidad, contrastando con nuestro resultado donde el efecto positivo se vio cuando se aplica la revisión de medicación. Por otro lado, Huiskes *et al.* [55] y Gutiérrez-Valencia *et al.* [57], obtuvieron resultados no concluyentes respecto, aunque Kua *et al.* [8] sugieren que esta podría reducir el riesgo de muerte en un 26%.

Una de las revisiones reporta una reducción efectiva del número de caídas en los individuos a quienes se realizó la deprescripción [55], y otra sugiere que esta podría reducir el riesgo en un 24% [8]. Sin embargo, otra [57] no es concluyente al respecto. Page *et al.* [7] agrega que si bien el número de personas que experimentan caídas no se reduce con la deprescripción, si se modifica el número de caídas que experimenta cada individuo siendo menor después de la deprescripción.

Varias revisiones [8, 55, 56, 58, 59] reportan que los resultados no son significativos en relación al efecto de la deprescripción en la reducción de las hospitalizaciones o readmisiones al hospital.

Cuatro investigaciones [7, 55, 57, 59] reportan resultados similares en relación al efecto de la deprescripción en la calidad de vida, sin evidenciar diferencias significativas.

Conclusión

La deprescripción mostró reducción en la tasa de PPI, las caídas, la estancia hospitalaria, la calidad de vida y la mortalidad. De las anteriores, se evidenció un efecto positivo en la reducción de las medicaciones potencialmente inapropiadas y el número de caídas. La mortalidad no tuvo efecto significativo después de las intervenciones de deprescripción. No se puede establecer el efecto de la deprescripción en la mortalidad por causa cardiovascular debido a que los resultados no evidencian un análisis individual de las causas de mortalidad específica. Por su parte, la deprescripción parece no tener ningún efecto en relación a la calidad de vida autorreferida por las personas intervenidas. Es necesario realizar mayores investigaciones para generar resultados que sean más concluyentes al respecto.

Conflicto de interés

Los autores no declaran conflicto de interés

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Supplementary Material

Table S1: Search strategies by database and results

DATABASE	SEARCH STRATEGY	RESULTS
MEDLINE	((((((aged[Title/Abstract]) OR elderly[Title/Abstract]) OR frail elderly[Title/Abstract]) OR older adults[Title/Abstract]) OR frail older adults[Title/Abstract]) AND ((((((deprescriptions[Title/Abstract]) OR inappropriate prescribing[Title/Abstract]) OR polypharmacy[Title/Abstract]) OR over prescribing[Title/Abstract]) OR withholding treatment[Title/Abstract]) OR withdrawing treatment[Title/Abstract]) AND (((((mortality) OR death rate) OR mortality rate) OR health outcome) OR outcome assessment (health care)))	438
LILACS	((tw:(aged)) OR (tw:(elderly)) OR (tw:(frail elderly)) OR (tw:(older adults))) AND ((tw:(deprescriptions)) OR (tw:(polypharmacy)) OR (tw:(inappropriate prescribing)) OR (tw:(withholding treatment))) AND (tw:(mortality))	30
SciELO	(aged) OR (frail elderly) OR (older adult) AND (deprescriptions) OR (polypharmacy) OR (inappropriate prescribing) OR (withholding treatment) OR (mortality) OR (health outcomes)	1048
Epistemonikos	(title:((title:(aged) OR abstract:(aged)) OR (title:(frail elderly) OR abstract:(frail elderly)) OR (title:(older adults) OR abstract:(older adults)) AND (title:(deprescriptions) OR abstract:(deprescriptions)) OR (title:(polypharmacy) OR abstract:(polypharmacy)) OR (title:(inappropriate prescribing) OR abstract:(inappropriate prescribing)) AND (title:(mortality) OR abstract:(mortality))) OR abstract:((title:(aged) OR abstract:(aged)) OR (title:(frail elderly) OR abstract:(frail elderly)) OR (title:(older adults) OR abstract:(older adults)) AND (title:(deprescriptions) OR abstract:(deprescriptions)) OR (title:(polypharmacy) OR abstract:(polypharmacy)) OR (title:(inappropriate prescribing) OR abstract:(inappropriate prescribing)) AND (title:(mortality) OR abstract:(mortality))))	8
TOTAL		**1524**

Artículo de investigación clínica / https://doi.org/10.15446/rcciquifa.v53n3.119172

Prescripción de melagenina loción para el tratamiento del vitíligo en atención primaria de salud

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Resumen

Introducción: el vitíligo se considera como un trastorno de susceptibilidad genética, caracterizada por la despigmentación de la piel a causa le deficiencia de melanocitos funcionales, dando lugar a la formación de manchas despigmentadas, especialmente en el rostro, axilas y áreas expuestas. **Objetivo:** el objetivo del estudio fue caracterizar la población en el municipio Santa Clara, que recibía para el manejo del vitíligo, el medicamento melagenina loción. **Metodología:** se realizó una investigación descriptiva transversal, que se corresponde con un estudio de uso de medicamentos del tipo prescripción-indicación de melagenina loción en marzo 2024. El universo estuvo conformado por 105188 pacientes y la muestra quedó conformada por 673 pacientes. El tipo de muestreo fue aleatorio simple y los resultados se presentaron en tablas y gráficos mediante números absolutos y porcentaje. Se cumplieron los principios éticos de la investigación científica. **Resultados:** predominó el sexo femenino (63,15 %) y grupo de edad entre 51 y 60 años (21,84 %). **Conclusión:** la prevalencia, predominio del sexo femenino y pacientes mayores de 30 años ratifica los resultados obtenidos en algunos estudios previos.

Palabras clave: Vitíligo, tratamiento, despigmentación, ancianos, estudio de prescripción-indicación.

Summary

Melagenin lotion prescription for vitiligo treatment in primary health care

Introduction: Vitiligo is considered a genetic susceptibility disorder, characterized by skin depigmentation due to a deficiency of functional melanocytes, giving rise to the formation of depigmented spots, especially on the face, armpits, and exposed areas. **Aim:** The objective of study to characterize a population of Santa Clara municipality, which received a melagenin lotion for management of vitiligo. **Methodology:** a descriptive and cross-sectional investigation that squared with a medications use study, of prescription-indication type, was conducted aimed at assessing the prescription of melagenin lotion in March 2024. The universe was 105188 patients and the sample was 673 patients, the sampling was simple random and the results were presented in tables and graphics using absolute numbers and percentages. The principle of confidentiality of the reviewed information was respected. **Results:** predominated the female sex (55.36 %) and patients between 51 to 60 years old (21.84 %). Conclusion: the prevalence, female sex and patients over 30 year old predominance ratify the results obtained in some previous studies.

Keywords: Vitiligo, treatment, depigmentation, elderly, prescription-indication study.

Resumo

Prescrição de loção de melagenina para tratamento de vitiligo na atenção primária à saúde

Introdução: o vitiligo é considerado um distúrbio de suscetibilidade genética, caracterizado pela despigmentação da pele devido à deficiência de melanócitos funcionais, levando à formação de manchas despigmentadas, principalmente na face, axilas e áreas expostas. **Objetivo:** o objetivo do estudo foi caracterizar a população do município de Santa Clara que recebeu o medicamento loção de melagenina para tratamento do vitiligo. **Metodologia:** foi realizada investigação descritiva transversal, que corresponde a um estudo de uso de medicamentos do tipo prescrição-indicação loção de melagenina em março de 2024. O universo foi composto por 105.188 pacientes e a amostra foi composta por 673 pacientes. O tipo de amostragem foi aleatória simples e os resultados foram apresentados em tabelas e

gráficos utilizando números absolutos e percentuais. Os princípios éticos da pesquisa científica foram respeitados. **Resultados:** predominou o sexo feminino (63,15%) e a faixa etária entre 51 e 60 anos (21,84%). **Conclusão:** a prevalência, o predomínio do sexo feminino e de pacientes com mais de 30 anos confirmam os resultados obtidos em alguns estudos anteriores.

Palavras-chave: Vitiligo, tratamento, despigmentação, idoso, estudo de prescrição-indicação.

Introducción

El vitíligo es una enfermedad autoinmune de la piel que se caracteriza por la presencia de manchas y áreas despigmentadas circunscritas debido a la destrucción de los melanocitos [1, 2]. Se considera como una enfermedad relativamente común y es un trastorno adquirido que ataca, específicamente, a la epidermis [3, 4].

Existen dos formas bien conocidas el vitíligo segmentario (VS), el cual es el menos común y el vitíligo no segmentario (VNS), que se caracteriza por parches hipocrómicos bilaterales [5]. Las opciones de tratamiento son varias y debe seleccionarse individualmente según el tipo de vitíligo, lo extenso del daño y la actividad de la enfermedad [6].

En Cuba, se dispone en las unidades de farmacia comunitaria (atención primaria de salud) del medicamento melagenina loción. Dicho medicamento fue descubierto y desarrollado por el Dr. Carlos Manuel Miyares Cao. Es un extracto alcohólico de placenta humana al 50 % que se produce en el Centro de Histoterapia Placentaria Dr. Carlos Manuel Miyares Cao y que tiene como indicaciones terapéuticas el tratamiento del vitiligo y en la despigmentación de la piel originada por quemaduras de cualquier origen. El extracto alcohólico de placenta humana al 50 % contiene un principio activo capaz de acelerar la síntesis melánica y la reproducción de los melanocitos. El medicamento se aplicará frotándolo con los dedos en las zonas despigmentadas, hasta cubrir la lesión, todos los días, en una ocasión diaria, con intervalos de 24 horas y sin exposición a la lámpara infrarroja o luz solar [7].

En Cuba la prescripción de melagenina loción se realiza a través de certificado médico para medicamentos controlados, restringida su prescripción para la especialidad de Dermatología para el tratamiento del vitíligo avalado por la Instrucción 1/2000 emitida por el Centro para el Desarrollo de la Farmacoepidemiología.

Teniendo en cuenta los planteamientos anteriores, se decide realizar la presente investigación con el objetivo de caracterizar la población con prescripción de melagenina loción en el municipio de Santa Clara, Cuba.

Metodología

Se realizó una investigación descriptiva y transversal, que se corresponde con un estudio de uso de medicamentos del tipo prescripción-indicación, dirigido a valorar la prescripción de melagenina loción en el municipio de Santa Clara al cierre de marzo de 2024. Se definieron como participantes en la investigación todos los pacientes ambulatorios con prescripción de certificado médico vigentes al momento del estudio con dicha prescripción.

El universo fue de 105 188 pacientes, que tenían prescripción por certificado para medicamentos controlados vigentes y la muestra quedó conformada por 673 pacientes de este universo (muestreo intencional) los que presentaban prescripción por certificado médico de melagenina loción en el municipio de Santa Clara al cierre de marzo de 2024. Se excluyeron los pacientes con información incompleta. Se analizaron las variables edad y sexo. Además, la muestra se estratificó por 10 grupos de edad: 0-10, 11-20, 21-30, 31-40, 41-50, 51-60, 61-70, 71-80, 81-90 y más de 90 años de edad.

Para obtener la información se revisaron los certificados médicos de melagenina loción en el municipio de Santa Clara, los datos fueron registrados en una base de datos prediseñada en MS Excel(R) y se utilizó como procedimiento estadístico el cálculo porcentual. Se realizó una lectura crítica de la información presentada en tablas. Los resultados se presentan en tablas y gráficos mediante números absolutos y porcentaje.

Se aplicaron métodos teóricos y empíricos. Dentro de los teóricos se utilizaron histórico-lógico, analítico–sintético y el enfoque sistémico mientras que dentro de los empíricos se emplearon observación, modelo de recogida de datos y matemáticos.

La integridad de los datos obtenidos en el estudio a partir de los certificados para medicamentos controlados se realizó de acuerdo con los principios éticos para la investigación médica en humanos, establecidos en la *Declaración de Helsinki* [8].

Resultados

Tras realizar la revisión del comportamiento histórico de la prescripción de melagenina loción durante el período de 2015 a 2024 (en el mes de marzo), se pudo comprobar que mostró el mayor valor durante el año 2024 (Figura 1).

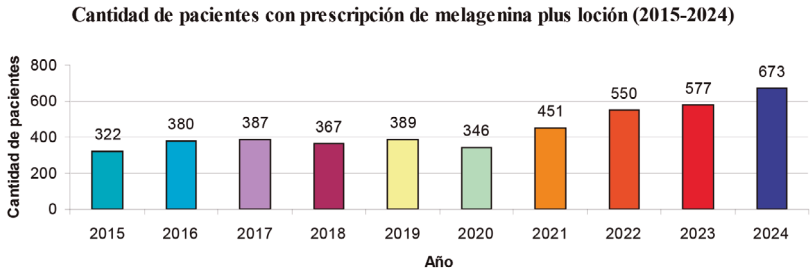

Figura 1. Total de pacientes con prescripción de melagenina loción. Período 2015-2024.
Fuente: Informe mensual de pacientes con medicamentos controlados con certificado médico.

Mensualmente se registra, por el área de epidemiología, en 4 grupos de edad la cantidad de medicamentos que su prescripción es a través de certificado médicos en todas las farmacias comunitarias. El comportamiento mostrado por la melagenina loción durante el período 2015-2024 (en el mes de marzo) reflejó un incremento más significativo en los grupos de 16-39, 40-59 y 60 ó más años de edad. (Tabla 1).

Tabla 1. Total de pacientes por grupo de edad con prescripción de melagenina loción. Período 2015-2024.

Año	Grupos de edad				Total
	0-15	16-39	40-59	60	
2015	50	70	101	101	322
2016	54	85	120	121	380
2017	54	88	123	122	387
2018	54	85	115	113	367
2019	54	88	125	122	389
2020	54	75	105	112	346
2021	54	113	143	141	451
2022	55	132	193	170	550
2023	55	141	197	184	577
2024	67	187	219	200	673

Fuente: Informe mensual de pacientes con medicamentos controlados con certificado médico.

La distribución global de pacientes con prescripción de melagenina loción por sexo mostró un predominio del sexo femenino (Tabla 2).

Tabla 2. Distribución global de pacientes con prescripción de melagenina loción por sexo.

Sexo	Cantidad	%
Femenino	425	63,15
Masculino	248	36,85

Fuente: Certificados Médicos para Medicamentos Controlados de pacientes con prescripción de melagenina loción.

La distribución global de pacientes con prescripción de melagenina loción por grupo de edad mostró los mayores valores para los pacientes entre 50 y 60 años (21,84 %). La media de edad fue de 46,3 y la mediana 50 años (Tabla 3).

Tabla 3. Distribución global de pacientes con prescripción de melagenina loción por grupo de edad.

Grupos de Edad	Cantidad	%
0-10	27	4,01
11-20	77	11,44
21-30	65	9,66
31-40	93	13,82
41-50	80	11,89
51-60	147	21,84
61-70	102	15,16
71-80	56	8,32
81-90	23	3,42
Más de 90	3	0,45

Fuente: Certificados Médicos para Medicamentos Controlados de pacientes con prescripción de melagenina loción.

Discusión

Este estudio tuvo como objetivo caracterizar la prescripción de melagenina loción en el municipio Santa Clara, el cual serviría de punto de partida para un estudio más detallado en la provincia de Villa Clara.

Al analizar la cantidad global de pacientes con prescripción de melagenina loción en el municipio Santa Clara (673 pacientes) y teniendo en cuenta que la población estimada, al cierre de 2022 [9], era de 245 959 habitantes lo cual correspondía al 0,27 % de la misma. Existen diversos criterios acerca de la prevalencia del vitíligo a nivel mundial aunque las cifras son muy similares. Autores como Ezzedine et al. [3], y Grimes [4] refieren que su prevalencia es de 0,1 - 2 % en la población general [3, 4] aunque otros autores como Iannella *et al.* [10], describen que afecta a un 0,5 - 1% de la población mundial. Por otra parte, Castro Caicedo et al. [11], argumentan que se presenta en

el 1 % de la población. Silverberg [12] reseña que oscila entre 0,4 – 2 % mientras que Kruger y Schallreuter [13] y Zhang *et al.* [14], argumentan que la prevalencia está entre 0,2 – 2 %.

Se pudo comprobar un predominio del sexo femenino, casi 2/3 partes de la muestra (63,15 %). Iannella *et al.* [10], consideran que el vitíligo afecta con independencia de raza o sexo, corroborado por Speeckaert *et al.* [15]. Autores como Grimes [4] y Fernández Paniagua *et al.* [5], describen que esta enfermedad se da equitativamente en hombres y mujeres y sin preferencia de raza o estado socioeconómico. En un estudio realizado en el Centro Nacional de Vitíligo y Psoriasis en el centro de Arabia Saudita desde agosto 2002 hasta agosto 2006, Alissa *et al.* [16], comprobó que de los 4134 casos estudiados el 53,5 % correspondían al sexo femenino. Abolafia *et al.* [17], refieren que el padecimiento de esta enfermedad trae consigo un deterioro de la calidad de vida, el cual es mayor en mujeres que en hombres. En muchos pacientes la despigmentación conlleva a una baja aceptabilidad social del vitíligo y se sienten incómodos por su imagen corporal especialmente cuando se manifiesta en zonas del cuerpo más visibles.

En cuanto a la edad, se evidenció que el 62,71 % se encontraba entre los 30 y 70 años de edad. Grimes [4] y Fernández-Paniagua *et al.* [5], detallan que puede aparecer a cualquier edad, pero usualmente se presenta entre los 10 y 30 años de edad. El 70-80 % de los adultos lo presentan antes de los 30 años de edad, siendo su presentación en la edad avanzada relacionada con enfermedad tiroidea, diabetes mellitus, artritis reumatoide y alopecia areata mientras que, un tercio de los pacientes con esta enfermedad presentan historia familiar de vitíligo o de alguna otra enfermedad autoinmune. En otro estudio realizado desde 2010 hasta 2014 por Kong *et al.* [18], para pacientes con vitíligo de aparición tardía contó con más mujeres (56,4 %).

Rashighi *et al.* [19], argumentan que, aunque es de distribución mundial y se observa su presentación en todas las edades, parece predominar en climas cálidos. Presentando mayor prevalencia en mujeres jóvenes, aunque afecta a ambos sexos por igual. Aproximadamente el 50% del total de los casos comienza su presentación en un rango de edad entre los 10 años y 20 años, habiéndose reportado casos desde las seis semanas de edad. Además, un tercio de los casos, los pacientes con vitíligo tienen antecedentes familiares o, de alguna otra enfermedad autoinmune.

En Cuba, Pernas [20], realizó en 2014 un estudio descriptivo retrospectivo en pacientes con diagnóstico clínico de vitíligo moderado o intenso que acudieron al Centro de Histoterapia Placentaria de Cuba entre agosto de 2008 y agosto de 2010, la muestra

estuvo constituida por 70 pacientes. Se observó una media de 36,4 años, el grupo etario más representado fue el de 40 a 49 años (28, 6 %) y el de menor representación fue el de 60 a 69 años (5,7%) y predominó el sexo femenino (61,4 %).

Se considera que el medicamento melagenina loción ha demostrado su eficacia, lo cual está avalado por un uso sostenido en las farmacias comunitarias de Cuba durante más de 20 años y sin reporte de reacciones adversas producto de su aplicación. Este último aspecto también es reflejado por Miyares *et al.* [21], en un estudio realizado en los Servicios Clínicos del Centro de Histoterapia Placentaria entre los años 1998 hasta el 2003, con 300 pacientes de vitíligo, arribando al final del estudio que 231 pacientes mostraron repigmentación completa o notable, 28 repigmentación parcial, 24 ninguna repigmentación y 17 nuevas lesiones además de no observarse efectos adversos ni locales ni sistémicos en ninguno de los pacientes sometidos al tratamiento.

Además, este estudio nos brinda una información útil relacionada con el comportamiento del uso de la melagenina loción en la atención primaria de salud, que a pesar de presentar pocas variables sociodemográficas, pudiera ser ampliada con variables tales como raza, tiempo de evolución de la enfermedad, antecedentes familiares de vitíligo, respuesta obtenida después de la aplicación del medicamento lo cual nos enriquecería la información relacionada con la eficacia del mismo.

Conclusiones

El estudio presentó como principal limitación la pobre presencia de estudios de prescripción de melagenina loción en el ámbito de la farmacia comunitaria. Finalmente, se concluyó que el grupo etáreo de 30 años o más son los más propensos al uso de melagenina loción mientras que se mostró un predominio del sexo femenino. Sería muy provechoso ampliar este estudio a otros municipios de la provincia, lo cual permitiría ampliar la muestra estudiada y recopilar mayor información al respecto.

Conflicto de intereses

El autor declara no tener conflicto de intereses.

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Formulación de una emulsión cosmética fotoprotectora usando extractos de la cianobacteria *Nostoc sphaericum*

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Resumen

Introducción: Las cianobacterias son uno de los pocos grupos de organismos que han desarrollado una amplia gama de adaptaciones evolutivas que garantizan mecanismos de fotoprotección. Entre ellas, la síntesis de metabolitos secundarios específicos que funcionan como filtros solares y antioxidantes. En la zona altoandina, específicamente en los cuerpos de agua, se observan colonias de cianobacterias de consistencia gelatinosa de forma esférica, de color verde azulado, una de ellas es el *Nostoc sphaericum,* cuya adaptación a las condiciones extremas ha permitido la biosíntesis de sustancias con potencial actividad fotoprotectora. **Objetivo: El principal objetivo de la presente investigación fue** formular una emulsión cosmética

fotoprotectora usando los extractos de *N. sphaericum*. **Metodología:** Las muestras de *N. sphaericum* se secaron por liofilización. Se realizó la extracción usando diferentes solventes por maceración. Se realizó un estudio de preformulación para elegir la emulsión más estable y con mejor compatibilidad con el extracto y la actividad fotoprotectora se midió a través del método espectrofotométrico de Mansur para establecer el FPS. **Resultados**: El solvente con mayor porcentaje de extracción fue el etanol de 70°, logrando 2,6 %. La formulación base de polawax presentó mejor estabilidad y compatibilidad con el extracto etanólico de 70° a las concentraciones de 1,25 %, 2,5 % y 5 %, cumpliendo con mantener sus características organolépticas y fisicoquímicas por un periodo de 7 días. El FPS calculado para las emulsiones fueron 0,572; 0,651 y 1,096 respectivamente. **Conclusiones**: Las emulsiones cosméticas fotoprotectoras formuladas presentan FPS dependientes de la concentración y similares a las de otras especies de cianobacterias previamente estudiadas.

Palabras clave: Cianobacteria, radiación solar, concentración, fotoprotección

Summary

Formulation of a photoprotective cosmetic emulsion using extracts from the cyanobacteria *Nostoc sphaericum*

Introduction: Cyanobacteria are among the few organisms for which many evolutionary adaptations for photoprotection have evolved. Among them is synthesizing specific secondary metabolites that function as sunscreens and antioxidants. In our high Andean zone, specifically in bodies of water, it is widespread to observe colonies of gelatinous consistency of spherical or lobular shape, of bluish-green color; these are cyanobacteria type algae, one of them is *Nostoc sphaericum*, whose adaptation to the extreme conditions has allowed the biosynthesis of substances with potential photoprotective activity. **Objective:** The main aim of the present investigation was to formulate a photoprotective cosmetic emulsion using *N. sphaericum* extracts. **Materials and methods:** Samples of *N. sphaericum* were dried by lyophilization. Extraction was performed using different solvents by maceration. A preformulation study was carried out to choose the most stable emulsion with the best compatibility with the extract. Mansur's spectrophotometric method measured the photoprotective activity to establish the SPF. **Results:** The solvent with the highest extraction percentage was ethanol 70°, achieving 2.6 %. The polawax formulation was the one that presented the best stability and compatibility with the 70° ethanolic extract at concentrations of 1.25 %, 2.5 %, and 5 %, maintaining its organoleptic and physico-

chemical characteristics for 7 days. The SPF calculated for the emulsions were 0.572, 0.651, and 1.096 at 1.25 %, 2.5 %, and 5 %, respectively. **Conclusions:** Photoprotective cosmetic emulsions were formulated using the 70° ethanolic extract of *N. sphaericum* with concentration-dependent SPFs similar to those of other species of cyanobacteria previously studied.

Keywords: Cyanobacteria, Solar Radiation, Concentration, Photoprotection

Resumo

Formulação de emulsão cosmética fotoprotetora a partir de extratos da cianobactéria *Nostoc sphaericum*

Introdução: As cianobactérias são um dos poucos grupos de organismos que desenvolveram uma ampla gama de adaptações evolutivas que garantem mecanismos de fotoproteção. Entre eles, a síntese de metabólitos secundários específicos que funcionam como filtros solares e antioxidantes. Na região alta andina, especificamente em corpos d'água, observam-se colônias de cianobactérias de consistência gelatinosa e formato esférico, de cor azul esverdeada. Uma delas é *Nostoc sphaericum*, cuja adaptação a condições extremas permitiu a biossíntese de. substâncias com potencial atividade fotoprotetora. **Objetivo:** O objetivo principal da presente pesquisa foi formular uma emulsão cosmética fotoprotetora utilizando extratos de *N. sphaericum*. **Metodologia:** Amostras de N. sphaericum foram secas por liofilização. A extração foi realizada com diferentes solventes por maceração. Foi realizado um estudo de pré-formulação para escolher a emulsão mais estável e com melhor compatibilidade com o extrato e a atividade fotoprotetora foi medida através do método espectrofotométrico de Mansur para estabelecer o FPS. **Resultados:** O solvente com maior percentual de extração foi o etanol 70°, atingindo 2,6%. A formulação base de polawax apresentou melhor estabilidade e compatibilidade com o extrato etanólico 70° nas concentrações de 1,25%, 2,5% e 5%, mantendo suas características organolépticas e físico-químicas por um período de 7 dias. O FPS calculado para as emulsões foi de 0,572; 0,651 e 1,096 respectivamente. **Conclusões:** As emulsões cosméticas fotoprotetoras formuladas apresentam FPS dependentes de concentração semelhantes aos de outras espécies de cianobactérias previamente estudadas.

Palavras-chave: Cianobactérias, radiação solar, concentração, fotoproteção

Introducción

Las cianobacterias son procariotas Gram negativas fotosintéticas, consideradas una de las formas de vida autótrofa más antiguas del planeta. Se utilizan tradicionalmente en distintas partes del mundo y recientemente han ganado interés científico por su capacidad de producir compuestos con actividad antioxidante [1-3]. Se trata de una variedad de algas macroscópicas de color verde azul, que forma parte del género Nostoc, se encuentran en lagos y lagunas de las zonas andinas a una altitud de alrededor de 3 000 metros sobre el nivel del mar [4].

Las colonias de cianobacterias verde azuladas, verde oliva o marrón forman el nostoc. El contenido de clorofila las hace verdes, mientras que el azul proviene de un pigmento llamado Ficocianina, que está relacionado con la fotosíntesis [5]. Las cianobacterias presentan características fotosintéticas similares a las de las algas eucariotas y las plantas superiores, a pesar de tener una organización estructural y bioquímica que se asemeja a las bacterias gramnegativas [6]. Estas características peculiares de las cianobacterias, de presentar complejos sistemas fotosintéticos, sistemas de adaptación y defensa, permite la producción de metabolitos importantes, como flavonoides, pigmentos (b-caroteno, c-ficoestrina, ficobiliproteínas), fenoles, saponinas, esteroides, taninos, terpenos y vitaminas [7, 8].

En los numerosos depósitos de lagunas, arroyos, manantiales y en diversos ambientes acuáticos de los Andes, es muy común observar colonias de consistencia gelatinosa de forma esférica o lobular, de color verde azulado, se trata de algas del tipo *Nostoc sphaericum*, comúnmente llamadas murmunta, cushuro, llullucha, crespito, nostoc, yurupa, uva de río y cochayuyo [9, 10]. El primer informe botánico sobre estas algas fue en 1892 por Nils Gustaf Lagerheim, quien las descubrió en Bolivia, Perú y Ecuador. Se utiliza en la alimentación tradicional en guisos y ensaladas o se consume directamente [4] debido a su alto contenido en fibra, aminoácidos, proteínas, vitaminas e hidratos de carbono [11, 12].

La cianobacteria *Nostoc sphaericum* es considerada como un suplemento nutricional económico, además es una fuente valiosa para el desarrollo de alimentos y medicamentos [13].

La radiación solar es uno de los factores ambientales que más contribuyen al envejecimiento de la piel y a la carcinogénesis y aunque actualmente el mercado ofrece protectores solares que contienen filtros UV inorgánicos y orgánicos, que absorben considerable radiación UV, sin embargo, presentan toxicidad, se pueden absorber sistémicamente, tienen un alto potencial alergénico, carcinogénico y otros efectos secun-

darios específicos como la inhibición de la biosíntesis de la vitamina D [14]. Uno de los filtros solares más fotoalergénicos es el filtro UVB PABA, que ha atraído la atención de la comunidad científica, porque parece ser cancerígeno in vitro a través de la fotosensibilización de los dímeros de timidina [15, 16]. Por este motivo, se suele sustituir por el padimato-O, que es más seguro, pero menos eficaz, y que no está exento de controversia [17]. Otros compuestos capaces de provocar fotoalergias incluyen las benzofenonas, de las cuales la oxibenzona tiene absorción sistémica y también afecta los niveles de hormonas reproductivas endógenas [18, 19]. Entre los filtros UVA, la avobenzona también puede causar fotoalergia [20]. Los filtros reflectantes inorgánicos ZnO y TiO_2 absorben considerablemente la radiación UV, pero también producen radicales altamente oxidantes y generan efectos adversos, pero al tener un efecto blanco sobre la piel, son poco atractivos para los consumidores, por lo que los fabricantes, para mejorar su potencial estético, utilizan nanopartículas microfinas de estos óxidos (<100 nm), que son transparentes en la piel, sin embargo, con este tamaño de partícula pierden eficacia y pueden ser absorbidas por los tejidos del huésped generando serias reacciones adversas [21].

Dados los problemas asociados a los protectores solares, es importante desde el punto de vista médico y medioambiental identificar un protector solar con alta eficacia, fotoestabilidad, baja toxicidad y respetuoso con el medio ambiente. Por tanto, es necesario investigar posibles formulaciones cosméticas que contengan activos naturales como los que están presentes en los extractos de *Nostoc sphaericum* de las lagunas altoandinas de la región Cusco.

Los extractos de las cianobacterias son una nueva opción para utilizarlos como fotoprotectores naturales ampliando la variedad de protectores solares. Se ha demostrado en investigaciones previas que, los extractos obtenidos de ciertas cianobacterias pueden contener componentes que absorben la luz UV de forma eficaz [22].

Considerando dicha problemática y el interés de la industria cosmética en la búsqueda de nuevas opciones naturales para el cuidado de la piel y protección frente a los daños ocasionados por la radiación solar, que presenten menos efectos adversos, es importante desarrollar el presente estudio, cuyo objetivo es elaborar una emulsión cosmética fotoprotectora a base de los extractos de *Nostoc sphaericum*, así como determinar sus principales metabolitos y el factor de protección solar (SPF).

Materiales y métodos

Recolección de la muestra

La muestra se recolectó de la laguna de Uma cocha (-13.321880, -71.836754), en la localidad de Pisac (Figura 1), usando envases de plástico. Las muestras fueron llevadas al Laboratorio de Tecnología Farmacéutica de la Universidad Nacional de San Antonio Abad del Cusco (UNSAAC), y fueron colocadas en refrigeración hasta su liofilización.

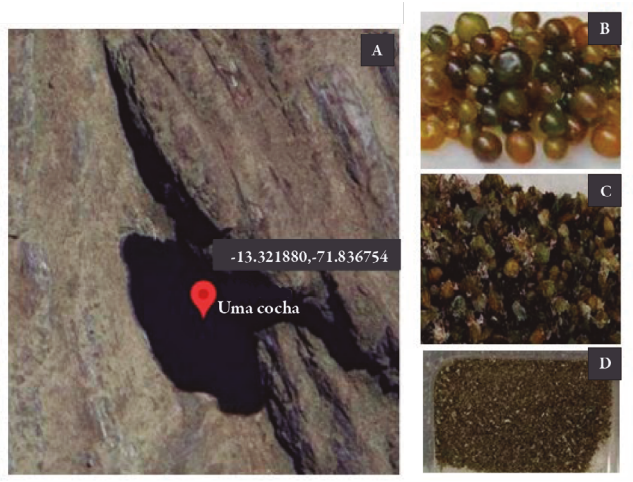

Figura 1. **(a)** Localización de la laguna Uma cocha (Pisac); **(b)** Muestra fresca de *N. sphaericum*; **(c)** Muestra liofilizada de *N. sphaericum*; **(d)** Muestra liofilizada molida de *N. sphaericum*

Identificación taxonómica

La muestra fue identificada en el Herbario Vargas Cuz por el Mgt. Alfredo Tupayachi usando claves dicotómicas, consulta de bibliografía especializada y comparación con muestras del herbario, correspondiendo a la especie *Nostoc sphaericum* Born ex Flah.

Liofilización

Las muestras previamente congeladas fueron liofilizadas usando el equipo Ilshin Biobase (TFD5503, Benchtop Model Freeze Dryers). La temperatura estuvo entre -31 y -60 °C y el vacío final fue de 0,020 mBar.

Molienda de la muestra

La muestra liofilizada de *Nostoc sphaericum* fue molida usando un molino de granos, hasta obtener una muestra finamente pulverizada.

Extracción usando diferentes solventes

Se pesaron 4 porciones de 1 g de muestra pulverizada cada una y se colocaron en envases de vidrio color ámbar. Posteriormente se añadieron 30 mL del solvente de extracción a cada frasco (agua, etanol de 70°, etanol absoluto y metanol) hasta cubrir totalmente el pulverizado. Se dejó macerando protegido de la luz por 15 días, se filtró y se concentró en baño maría (30-35 °C). El porcentaje de extracción se determinó utilizando la siguiente fórmula:

$$\%\mathrm{EES} = \left(\frac{\mathrm{Pf}}{\mathrm{Pi}}\right) \cdot 100 \quad (1)$$

Donde: %EES es el porcentaje de extracción del extracto seco; Pi es el peso inicial de la muestra pulverizada; y Pf es el peso final del extracto seco

Prueba de determinación de flavonoides

Se realizó la prueba de Shinoda a cada uno de los extractos obtenidos a fin de determinar cuál de estos presentaba mayor cantidad de flavonoides, debido a que consideramos que estos son los metabolitos responsables del efecto fotoprotector. Se redisolvieron los extractos secos en etanol de 70° y se añadieron dos gotas de HCl(c) y 0,5 g de Magnesio metálico [23]. La presencia de una coloración amarilla a rojo es indicativo de flavonas y flavonoles; los flavononoles desarrollan colores rojo a magenta, las flavanonas, chalconas y auronas no dan coloración [24, 25].

Formulación de la emulsión cosmética fotoprotectora

Se desarrolló un estudio de preformulación con diferentes bases cosméticas, entre ellas Beeler, Cold cream, Lanette y Polawax. La incorporación del extracto etanólico de 70° de *Nostoc sphaericum* se realizó a las concentraciones de 1,25 %; 2,5 % y 5 % en cada una de las bases cosméticas. Estas pre-formulaciones fueron observadas durante 7 días bajo condiciones de temperatura ambiente y humedad de 45 %, durante todo el tiempo de observación estuvieron mantenidas herméticamente cerradas y protegidas de la luz. La elección de la emulsión cosmética final se realizó en base a la homogeneidad, extensibilidad y compatibilidad de la base con el extracto a las diferentes concentraciones. A la emulsión elegida se la sometió a pruebas de estabilidad organoléptica y fisicoquímica. Habiéndose evaluado consistencia, color, aspecto y olor como parámetros organolépticos y pH, separación de fases (centrifugación), textura y distribución y tamaño de los glóbulos de la fase interna de la emulsión como parámetros fisicoquímicos.

Determinación de la actividad fotoprotectora *in vitro*

La actividad fotoprotectora de las emulsiones formuladas con el extracto etanólico 70° de *Nostoc sphaericum* fue evaluada de acuerdo con el método de Mansur *et al.* [26] y adaptado por Gutiérrez *et al.* [27].

Se disolvieron 5 mg de cada emulsión (1,25 %; 2,5 % y 5 %) en etanol absoluto, aforando a 25 mL, para obtener soluciones de 0,2 mg/mL; se llevó a sonicar por 5 minutos y se filtraron usando un filtro de 0,22 μm descartándose los 10 primeros mililitros. Se leyeron las absorbancias de cada una de las soluciones en el espectrofotómetro UV-Vis a λ = 290; 295; 300; 305; 310; 315 y 320 nm. Para el fotoprotector patrón (ENX FPS 50), se realizó un paso adicional con el fin de obtener lecturas por debajo de 1 nm, y sean confiables de acuerdo con lo recomendado por Gutiérrez *et al.* [27], se hizo una nueva dilución tomando 0,1 mL de la solución de 0,2 mg/mL y aforando hasta 10 mL con etanol. Con esta disolución se realiza las lecturas espectrofotométricas, siendo 100 el factor de dilución en este caso. Para calcular el FPS se usó la siguiente fórmula:

$$\text{FPS espectrofotométrico} = \text{FC} \cdot \sum_{290}^{320} \text{EE}(\lambda) \cdot \text{I}(\lambda) \cdot \text{Abs}(\lambda) \qquad (2)$$

Donde, FPS es el factor de protección solar; FC es 10 (factor de corrección); EE(λ) es el efecto eritemogénico de la radiación de longitud de onda λ; I(λ) es la intensidad del sol en la longitud de onda λ; y Abs(λ) es la absorbancia de la solución en la longitud de onda λ.

Resultados y discusión

Extracción usando diferentes solventes

La Tabla 1 muestra el porcentaje de extracción obtenido para cada uno de los solventes usados.

Tabla 1. Porcentaje de extracción con diferentes solventes

Solvente	% de extracción
Agua	N. D
Etanol absoluto	1,4[a]
Etanol 70°	2,6[b]
Metanol	1,9[a]

N.D.: No determinado; Diferentes letras significa que existen diferencias significativas ($p<0,05$, según la prueba deTukey)

El mayor porcentaje de extracción fue obtenido con etanol de 70°, habiendo diferencias significativas con los porcentajes de extracción de los otros solventes.

De acuerdo con Ignat *et al.* [28], el tipo de solvente y el método de extracción son factores que afectan el porcentaje de extracción especialmente de compuestos fenólicos, dentro de ellos los flavonoides, esto último concuerda con lo obtenido en el presente estudio, porque se determinó que el extracto obtenido con etanol de 70° presentó mayor presencia de flavonoides (flavonas y flavonoles, por el color desarrollado), en comparación con los otros solventes. En ese sentido, la polaridad del solvente es considerada de suma importancia por otros autores quienes plantean que los solventes más polares suelen arrastrar más metabolitos secundarios que los solventes apolares [29].

Los flavonoides suelen ser extraídos en mayor cuantía con solventes hidroalcohólicos a diferencia de los acuosos. Esto debido a que la extracción de flavonoides con la mezcla etanol-agua es beneficiosa, porque el agua provoca la hidratación de la muestra, permitiendo que el etanol debilite con más facilidad el enlace entre el soluto y la matriz vegetal. En ese sentido, los solventes hidroalcohólicos aumentan la permeabilidad de la pared celular y facilitan la extracción de flavonoides de baja, mediana y elevada polaridad [30].

Como se muestra en la Tabla 2, a través de una marcha fitoquímica del extracto obtenido con etanol de 70° se pudo determinar la presencia de metabolitos secundarios como glicósidos fenólicos, flavonoides, quinonas y lactonas sesquiterpénicas en abundante cantidad, en tanto que alcaloides, saponinas y taninos se detectaron de forma poco abundante.

Tabla 2. Metabolitos secundarios identificados en el extracto etanólico de 70° de *Nostoc sphaericum*

Metabolitos Secundarios	Reactivo	Resultado
Glicósidos Fenólicos	$FeCl_3$	++
Flavonoides	Shinoda	++
	Vapores de NH_3 + luz UV	++
Taninos	$FeCl_3$ 1%	+
Quinonas	Bornträguer	++
Alcaloides	Dragendorff	+
	Wagner	+
Lactonas Sesquiterpénicas	Baljet	++
Esteroides	Liebermann-Burchard	-
Saponinas	Prueba de espuma	+

Leyenda: Muy abundante +++; abundante ++; poco abundante +; negativo (-)

En el trabajo desarrollado por Sánchez-Sebastián [31] se reportó la presencia de Triterpenos y esteroides, Compuestos fenólicos y flavonoides en el extracto etanólico de *Nostoc commune*, una especie relacionada a la nuestra.

Asimismo, en el estudio realizado por Baldotano-Torres [32], se pudo verificar la presencia de alcaloides, saponinas, flavonoides y compuestos fenólicos, resultados muy parecidos a los nuestros.

En el trabajo desarrollado por Chuquilín-Goicochea y Rosales-Laguna [33], se reportó la presencia de saponinas, azúcares reductores y aminoácidos en el extracto acuoso de *Nostoc sphaericum* Vaucher.

El medio ambiente en el cual se desarrollan las especies vegetales es crucial para la biosíntesis de metabolitos secundarios, así como la etapa de desarrollo y los factores del lugar donde se desarrollan, todas estas condiciones repercuten directamente en la producción de los metabolitos secundarios y permite explicar las diferencias encontradas en los distintos estudios sobre la presencia de metabolitos secundarios.

Formulación de la emulsión cosmética fotoprotectora

El proceso de pre-formulación sirvió para elegir a la emulsión base de Polawax como la emulsión que demostró compatibilidad entre sus componentes, en la Tabla 3, se muestra la composición de la emulsión:

Tabla 3. Fórmula cuali-cuantitativa de la emulsión cosmética fotoprotectora elegida

Componentes	INCI	Porcentaje	Función
Extracto de *Nostoc sphaericum*	---------	1,25 %, 2,5 % y 5 %	Activo
Polawax	Emulsifying Wax NF	10,0 %	Emulsionante
Alcohol cetoestearílico	Cetoestearyl alcohol	3,5 %	Tensoactivo no iónico Emulsionante
Propilenglicol	Propylene glicol	5,0 %	Humectante
EDTA Na_2	Disodium EDTA	0,1 %	Agente quelante
Butil Hidroxi Tolueno	BHT	0,05 %	Antioxidante
Propilparabeno+Metilparabeno	Propylparaben+Methylparaben	0,5 %	Preservante
Agua purificada	Aqua	c.s.p 100 %	Vehículo

Las características finales de la emulsión fueron consistencia semisólida, aspecto homogéneo y olor característico a *Nostoc* (*sui generis*), el color varió de acuerdo con el porcentaje del extracto añadido.

Características organolépticas

En la Tabla 4 se observan los resultados del análisis organoléptico realizado a las emulsiones elaboradas a base del extracto etanólico de 70° a concentraciones 1,25 %, 2,5 % y 5 %, el análisis se realizó al día 1 y 7.

Tabla 4. Características organolépticas de las emulsiones formuladas con el extracto etanólico 70°

	Características	**1 día**	**7 días**
Emulsión al 1,25 %	Consistencia	Semisólido	Semisólido
	Color	Verde claro	Verde claro
	Aspecto	Homogéneo	Homogéneo
	Olor	*Sui generis* a *Nostoc*	*Sui generis* a *Nostoc*
Emulsión al 2,5 %	Consistencia	Semisólido	Semisólido
	Color	Verde	Verde
	Aspecto	Homogéneo	Homogéneo
	Olor	*Sui generis* a *Nostoc*	*Sui generis* a *Nostoc*
Emulsión al 5 %	Consistencia	Semisólido	Semisólido
	Color	Verde oscuro	Verde oscuro
	Aspecto	Homogéneo	Homogéneo
	Olor	*Sui generis* a *Nostoc*	*Sui generis* a *Nostoc*

Como se aprecia en la Tabla 4, el color de la emulsión concuerda con el verde claro para la emulsión al 1,25 %. Para la emulsión al 2,5 % se tuvo un verde más acentuado. Para la emulsión al 5 % se tuvo un verde oscuro. Esta variación de color se puede apreciar en la Figura 2.

El aspecto de las emulsiones se estableció mediante observación visual directa, observando la ausencia de grumos, separación de fases o variaciones de color dentro de la emulsión. Se observó que todas las emulsiones mantuvieron un aspecto homogéneo desde el día 1 hasta el día 7.

En cuanto a la consistencia en todas las emulsiones se tuvo una consistencia semisólida sin separación de sus componentes, lo que demuestra la estabilidad de todas las emulsiones formuladas independientemente del porcentaje de extracto etanólico de 70° incorporado.

La evaluación del olor se realizó directamente con el olfato, todas las emulsiones exhibieron un agradable olor característico a Nostoc, aunque el olor percibido en la emulsión al 1,25 % fue más tenue que el de la emulsión al 5 %.

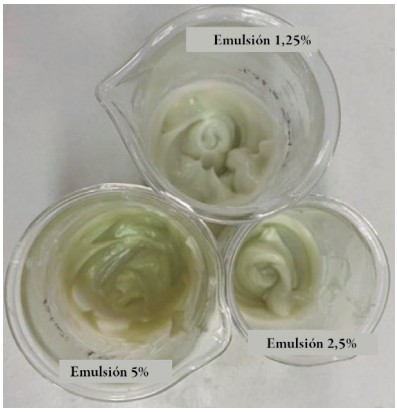

Figura 2. Color, aspecto homogéneo y consistencia semisólida de las emulsiones formuladas con diferentes porcentajes de extracto.

Características fisicoquímicas

En la Tabla 5 se muestran los resultados del análisis fisicoquímico realizado a las emulsiones elaboradas a base del extracto etanólico de 70° a las concentraciones de 1,25 %, 2,5 % y 5 %, el análisis se realizó al día 1 y 7.

Tabla 5. Características fisicoquímicas de las emulsiones formuladas con el extracto etanólico 70°

	Características	**1 día**	**7 días**
Emulsión al 0,25 %	pH	6,5	6,5
	Centrifugación	Sin separación ni sedimentación	Sin separación ni sedimentación
	Textura	Poco grasosa	Poco grasosa
	Distribución y tamaño de los glóbulos de la fase interna	Sin aglomeración ni coalescencia	Sin aglomeración ni coalescencia
Emulsión al 0,5 %	pH	6,5	6,5
	Centrifugación	Sin separación ni sedimentación	Sin separación ni sedimentación
	Textura	Poco grasosa	Poco grasosa
	Distribución y tamaño de los glóbulos de la fase interna	Sin aglomeración ni coalescencia	Sin aglomeración ni coalescencia

(*Continúa*)

Tabla 5. *Continuación.*

	Características	1 día	7 días
Emulsión al 1 %	pH	6,5	6,5
	Centrifugación	Sin separación ni sedimentación	Sin separación ni sedimentación
	Textura	Poco grasosa	Poco grasosa
	Distribución y tamaño de los glóbulos de la fase interna	Sin aglomeración ni coalescencia	Sin aglomeración ni coalescencia

El pH determinado para todas las emulsiones evaluadas estuvo en 6,5 tanto en el día 1 como en el día 7, lo que sugiere que no ha habido ningún cambio que afecte el pH durante el período de evaluación, lo que podría sugerir una buena estabilidad de las emulsiones [34]. El pH de la piel es un factor importante que se debe tener en cuenta al formular emulsiones cosméticas, debido a que variaciones de éste podrían generar algunas infecciones, por lo que estas emulsiones deben presentar un pH ácido entre 5,5 y 6,5 para no comprometer las funciones de la piel. En nuestro caso, observamos que las emulsiones formuladas están en el rango recomendado.

La evaluación de la separación de fases a través de la centrifugación se fundamenta en la generación de una tensión sobre la muestra simulando un aumento de la movilidad de las partículas y prediciendo una posible inestabilidad [35]. En nuestra evaluación, las emulsiones con diferentes concentraciones del extracto etanólico de 70° de *N. sphaericum* no mostraron separación de fases, no se observó precipitaciones ni coalescencia (Figura 3).

La textura en todas las emulsiones se determinó como poco grasosa, debido a que la formulación corresponde a la de una emulsión de fase interna oleosa y fase externa acuosa, por lo que no presenta capacidad oclusiva, pero si es evanescente.

La observación microscópica permitió verificar la homogénea distribución de las gotículas de aceite dispersadas en la fase acuosa, asimismo se observó el tamaño homogéneo de las gotículas y la ausencia de aglomeración de las gotículas, lo que demuestra la estabilidad de las emulsiones, pues no se evidenció fenómenos de coalescencia (Figura 3).

De acuerdo con Florence y Whitehill [36], el aumento o disminución del tamaño de los glóbulos indica un proceso de inestabilidad, por lo que en nuestra investigación tomamos en cuenta la ausencia de gotículas de aceite fusionadas entre sí, así como la ausencia de gotículas rotas.

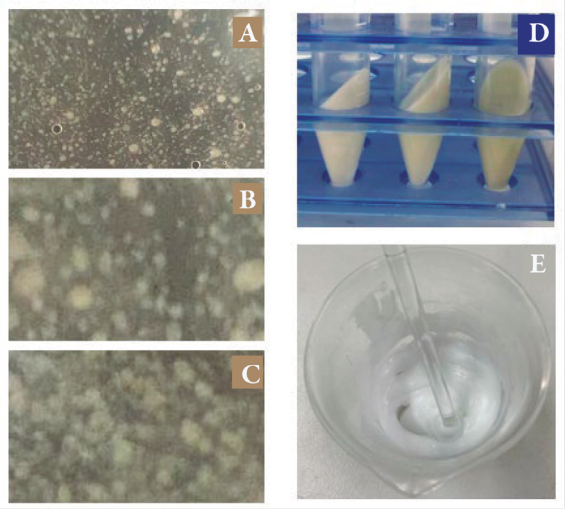

Figura 3. Resultados del análisis fisicoquímico: Observación microscópica (a) Emulsión al 1,25 %; (b) Emulsión al 2,5 %; (c) Emulsión al 5 %; (d) Centrifugación sin separación de fases; (e) Emulsión base homogénea.

Actividad fotoprotectora de las emulsiones

En la Tabla 6 se observa el cálculo del FPS de las emulsiones formuladas con el extracto etanólico de 70° a las concentraciones de 1,25 %; 2,5 % y 5 % y del fotoprotector patrón (ENX) usando el método de De Souza-Mansur *et al.* [26]

Como se muestra en la Tabla 6, el FPS calculado para las emulsiones de *N. sphaericum* al 1,25 %; 2,5 % y 5 % fueron 0,572; 0,651; 1,096 respectivamente, existiendo una relación directa entre la concentración y el FPS calculado, es decir a mayor concentración mayor FPS. En tanto que para el fotoprotector patrón ENX se calculó un FPS de 48,892, el cual es muy cercano al FPS 50 declarado.

Vega *et al.* [22] formularon una emulsión aceite-agua con el extracto hidroalcohólico de la cianobacteria *Scytonema sp.* (25 % p/p), y el FPS calculado fue 1,9.

En el estudio realizado por Bowange *et al.* [37] se determinó el FPS de los extractos etanólicos de varias especies de cianobacterias, entre ellas *Limnothrix sp*.; *Geitlerinema sp*. y *Synechocystis sp*. con un rango de SPF entre 0,17 y 0,24, todos los cuales mostraron un SPF medio comparativamente muy bajo; *Calothrix sp*. con 1,23 y *Oscillatoriales* con 1,57, ambos comparativamente más altos, pero sin diferencias significativas entre ellos. Según estos autores, estos resultados sugieren que estas cianobacterias podrían ser más eficaces que muchos otros extractos de plantas.

Tabla 6. Cálculo del FPS de las emulsiones formuladas con con el extracto etanólico de 70° a las concentraciones de 1,25 %; 2,5 % y 5 % y del fotoprotector patrón (ENX)

		Absorbancias				EExl *ABS			
nm	**EExl**	**1,25 %**	**2,5 %**	**5 %**	**ENX**	**1,25 %**	**2,5 %**	**5 %**	**ENX**
290	**0,0150**	0,159	0,173	0,227	0,393	0,002	0,003	0,003	0,006
295	**0,0817**	0,141	0,146	0,218	0,465	0,012	0,012	0,018	0,038
300	**0,2874**	0,042	0,048	0,087	0,427	0,012	0,014	0,025	0,123
305	**0,3278**	0,045	0,053	0,094	0,626	0,015	0,017	0,031	0,205
310	**0,1864**	0,049	0,057	0,097	0,330	0,009	0,011	0,018	0,062
315	**0,0839**	0,052	0,062	0,099	0,470	0,004	0,005	0,008	0,039
320	**0,0180**	0,163	0,200	0,341	0,898	0,003	0,004	0,006	0,016
					SUMA	0,057	0,065	0,110	0,489
					x 10	**0,572[a]**	**0,651[a]**	**1,096[b]**	
					x100				**48,892[c]**

Diferentes letras significan que existen diferencias significativas (p<0,05, según la prueba de Tukey)

Hossain *et al.* [38] calcularon un valor de FPS de 2,37 ± 0,755 para la cianobacteria *Cephalothrix komarekiana*. Asimismo, en el estudio realizado por Favas *et al.* [39] se reportaron diferentes valores de FPS dependiendo del tipo de extracto y la concentración para cuatro especies de cianobacterias, así para los extractos de acetona, el valor más prometedor (19,2) se encontró para *Leptolyngbya boryana*, para *Leptolyngbya cf. ectocarpi* (10,7), para *Cephalothrix lacustris* (7,79) y para *Nodosilinea nodulosa* (3,50) todos a la concentración de 200 μg/mL. En tanto que para los extractos acuosos a la concentración de 1000 μg/mL se reportaron FPS de 17.16 para *Leptolyngbya boryana*, 14,86 para *Cephalothrix lacustris,* 11,54 para *Leptolyngbya cf. ectocarpi* y 7,27 para *Nodosilinea nodulosa.*

Considerando los resultados mostrados, podemos concluir que el FPS determinado para muchas cianobacterias, incluyendo el calculado para la emulsión al 5% del extracto etanólico de 70° de *Nostoc sphaericum* en el presente trabajo de investigación, en comparación con los valores SPF obtenidos para extractos de frutas y vegetales muy utilizados como alimentos, entre ellos sandía (0,97 ± 0,41), *Aloe vera* (1,28 ± 0,02), zanahoria (1,34 ± 0,13), pepino (1,45 ± 0,35), fresa (1,63 ± 0,34) y papaya (1,75 ± 0,26), resultan siendo altos y promisorios [40].

Cabe resaltar que la presente investigación fue desarrollada con concentraciones bajas del extracto etanólico de *N. sphaericum* (1,25 %, 2,5 % y 5 %), en comparación con el desarrollado por Vega *et al.* [22], quienes elaboraron la emulsión con 25 % del extracto

hidroalcohólico de la cianobacteria *Scytonema sp*., por lo que consideramos que a mayor concentración del extracto etanólico de 70° de *N. sphaericum* se podría tener un mayor FPS.

Aunque cada vez más empresas cosméticas incorporan bloqueadores solares a sus productos, continúa siendo complicado convencer a los consumidores de los beneficios de su uso diario para retrasar el envejecimiento prematuro de la piel. Si, por un lado, la aplicación diaria de bloqueadores solares es un hábito poco arraigado, por otro lado, existe cierto temor en el uso de sustancias sintéticas, debido a sus inoportunos riesgos asociados [41]. A raíz de esto, la investigación sobre fotoprotectores naturales ha aumentado considerablemente en los últimos años, su potencial biodegradabilidad y menor toxicidad, los hace más beneficiosos para el ser humano y el medio ambiente. En ese sentido, el estudio de los extractos derivados de las cianobacterias constituye una estrategia muy importante para encontrar potenciales moléculas fotoprotectoras.

Conclusiones

N. sphaericum es una cianobacteria altoandina cuyo extracto etanólico de 70° presenta metabolitos secundarios como flavonoides y compuestos fenólicos que incorporados a una emulsión cosmética en porcentajes de 1,25 %; 2,5 % y 5 % ha demostrado tener un FPS dependiente de la concentración habiendo alcanzado valores de 0,572, 0,651 y 1,096 respectivamente y en comparación con los extractos de otras cianobacterias presenta una adecuada actividad fotoprotectora. Las características finales de las emulsiones mostraron consistencia semisólida, aspecto homogéneo y olor característico a *Nostoc* (*sui generis*), el color varió de acuerdo con el porcentaje del extracto añadido. El pH determinado para todas las emulsiones evaluadas fue de 6,5. No hubo separación de fases, precipitaciones ni coalescencia, en tanto que, al microscopio se observó gotículas de tamaño homogéneo y ausencia de aglomeración. Sin embargo, teniendo en cuenta estudios previos en los cuales se han formulado emulsiones con porcentajes de extractos de cianobacterias por encima del 20 %, consideramos que se deben estudiar emulsiones con extractos de *N. sphaericum* con porcentajes más altos para evidenciar un mayor SPF. Asimismo, es esencial realizar estudios de eficacia y de seguridad para confirmar y consolidar las propiedades fotoprotectoras en los pacientes.

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Conflictos de interés

Los autores declaran no tener ningún conflicto de interés.

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Chitosan cosmetic cream: stability and non-clinical studies

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Summary

Introduction: Chitosan has been used in various products for skin care, hair care, cleaning and other products. These not only provide health benefits, but also excellent cosmetic qualities and efficacy. **Objective**: To evaluate the stability of a 1.0 % chitosan cosmetic cream, as well as its non-clinical effects. **Methods**: Three pilot batches of the cosmetic formulation were prepared by the melting method. Physical, chemical and microbiological evaluation was performed for 365 days, under shelf life conditions. The studied parameters were organoleptic characteristics, pH, extensibility, degree of deacetylation and microbiological count. Non-clinical evaluations of the cream included dermal and ophthalmic irritability. The dermoregenerative effect was evaluated in a photoaging model in the skin of mice photosensitized with ultraviolet radiation. **Results**: The cream was stable for 12 months, packaged in high-density polyethylene bottles at room temperature, and by predictive analysis until to 24 months. The formulated semisolid is safe, being considered a system of low toxicity to the organism, with moderate dermoregenerative activity. **Conclusions**: The pilot batches maintained their stability for 12 months at 30 ± 2 °C and 70 % relative humidity. The 1.0 % chitosan cream is non-irritating dermally and ophthalmically and has a moderate dermoregenerative effect.

*Keywords***:** chitosan; cream; dermoregenerative effect, non-clinic study; stability

Resumen

Crema cosmética de quitosano: estudios de estabilidad y no-clínicos

Introducción: El quitosano se ha empleado en diversos productos para el cuidado de la piel, el cabello, de limpieza, entre otros. Los mismos no solo brindan beneficios para la salud, sino también excelentes cualidades cosméticas y eficacia. **Objetivo**: Evaluar la estabilidad de una crema cosmética de quitosano al 1,0 %, así como sus efectos no clínicos. **Métodos**: Se elaboraron tres lotes pilotos de la formulación cosmética por el método de fusión. Se realizó la evaluación física, química y microbiológica por 365 días, en condiciones de vida de estante. Los parámetros estudiados fueron las características organolépticas, pH, extensibilidad, grado de desacetilación y conteo microbiológico. Las evaluaciones no clínicas de la crema incluyeron la irritabilidad dérmica y oftálmica. El efecto dermorregenarador se evaluó en un modelo de fotoenvejecimiento en la piel de ratones fotosensibilizados con radiaciones ultravioletas. **Resultados**: La crema resultó estable durante 12 meses, envasada en frascos de polietileno de alta densidad y a temperatura ambiente, y por el análisis predictivo hasta 24 meses. El cosmético formulado es estable, seguro y de baja toxicidad al organismo, con actividad dermorregeneradora moderada. **Conclusiones**: Los lotes pilotos mantuvieron su estabilidad por 12 meses a 30 ± 2 °C y 70 % de humedad relativa. La crema de quitosano al 1,0 % no es irritante dérmico, ni oftálmico y tiene efecto dermorregenerador moderado.

Palabras claves: efecto dermorregenerador; estabilidad; estudios no clínicos; quitosano.

Resumo

Creme cosmético de quitosana: estabilidade e estudos não clínicos

Introdução: A quitosana tem sido utilizada em diversos produtos para cuidados com a pele, cabelos, limpeza, entre outros. Eles não só proporcionam benefícios à saúde, mas também excelentes qualidades cosméticas e eficácia. **Objetivo:** Avaliar a estabilidade de um creme cosmético de quitosana a 1,0 %, bem como seus efeitos não clínicos. **Métodos:** Três lotes piloto da formulação cosmética foram preparados pelo método de fusão. A avaliação física, química e microbiológica foi realizada durante 365 dias, nas condições de vida útil. Os parâmetros estudados foram características

organolépticas, pH, extensibilidade, grau de desacetilação e contagem microbiológica. As avaliações não clínicas do creme incluíram irritabilidade dérmica e oftálmica. O efeito dermorregenerativo foi avaliado em modelo de fotoenvelhecimento na pele de camundongos fotossensibilizados com radiação ultravioleta. **Resultados:** O creme manteve-se estável por 12 meses, acondicionado em frascos de polietileno de alta densidade e em temperatura ambiente, e por análise preditiva até 24 meses. O cosmético formulado é estável, seguro e de baixa toxicidade ao organismo, com moderada atividade dermorregenerativa. Conclusões: Os lotes piloto mantiveram sua estabilidade por 12 meses a 30 ± 2 °C e 70 % de umidade relativa. O creme de quitosana a 1,0% não é irritante dérmico ou oftálmico e tem efeito dermorregenerativo moderado.

Palavras-chave: efeito dermorregenerativo; estabilidade; estudos não clínicos; quitosana.

Introduction

Chitosan, obtained by deacetylation of chitin, is a polymer with a helix structure with reactive amino groups, which offers many possibilities for modification and ionic interactions. Being one of the few cationic polysaccharides, this gives it unique properties with respect to the rest of the polysaccharides, which are generally neutral or negatively charged [1].

It is characterized by being biodegradable, biocompatible and of low toxicity. It is attributed antibacterial, antifungal, surfactant, antitumor, mucoadhesive, healing, antioxidant and other properties [2, 3]. It has as other advantages that it is renewable and economically feasible.

Due to these properties, its field of use is very varied and includes the pharmaceutical, food, cosmetics, paper, textile, water treatment and other industries. Among the most important international markets are Japan, the United States, Europe and some Asian countries, mainly China and Korea [1].

Among the routes of administration of the biopolymer, the parenteral and oral routes (frequently as excipient) have been used, but the topical route (as an active pharmaceutical ingredient) is definitely the most represented. The most studied systems by this route are hydrogels, films, dressings, ointments, creams, among others [4-7].

It has been described that the wound-healing effect of chitosan is attributed to accelerating the infiltration of polymorphonuclear cells into the injury area, increasing the

effusion of dense forms of fibrin, activating migration and stimulating fibroblast proliferation in the wound, with the production of collagen [8-10].

Chitosan and its derivatives are included in skin and hair care products, oral care, perfumery, as well as dental care. They have been used to maintain skin moisture, treat acne, improve hair flexibility, tone the skin, and in tooth care products in toothpastes, tooth whiteners and chewing gums [1].

Other essential applications of chitosan are in hair care products such as shampoos, hairsprays and dyes. Also in the manufacture of face, hand and body creams; in cleaning products such as cleansing milks, tonics, facial peels, soaps and bath agents. It is also added in the production of color cosmetic products such as lipsticks, eye shadow makeup, nail polish and deodorizing products [11].

Among the essential advantages of cosmetics using chitosan are their efficacy and the adequate hydration they achieve in the skin. They provide, therefore, not only a health benefit, but also excellent cosmetic qualities due to their antimicrobial and antioxidant properties, especially against pathogenic fungi, as well as their stabilizing role in formulations [11].

Several works have been published on the obtaining, characterization, application of this biopolymer, derived from common lobster (*Panulirus aurgus*) chitin, and its salts [12-14], as well as the antioxidant activity of its solutions [15].

Most cosmetic skin care formulations are oil/water, water/oil or double emulsions. From a dermatological point of view, the water/oil emulsion is a better choice, as the lipid film formed on the skin favors the oil-soluble active ingredients. The emulsions oil/water are also more appreciated by the consumer as they are less greasy [16]. On the other hand, they can cause a cooling sensation due to water evaporation [17]. Basic formulations of creams with skin moisturizing function are typically composed of ingredients such as emollients, humectants, thickeners, emulsifiers, stabilizers, preservatives and neutralizers [18, 19].

The purpose of the stability study of cosmetic products is to provide information on their quality over a given time, under the influence of a variety of environmental factors such as temperature, light and humidity. Other factors are related with the product, like the physical and chemical properties of the active ingredient and excipients, the type of cosmetic product and its composition, the manufacturing processes and the nature and properties of the packaging used [20].

The stability of a cosmetic is the property of the product, preserved in a specific container, to retain within a period of time, and during its shelf life, the properties it had

at the time it was manufactured by a standardized procedure. This includes chemical, physical, microbiological, toxicological and functionality evaluation [20].

Considering the need to deepen the properties of the biopolymer derived from lobster chitin (*Panulirus aurgus*), which allows the development of safe pharmaceutical and cosmetic products, the stability of a 1.0 % chitosan cosmetic cream was evaluated in the present work, as well as its non-clinical effects (dermal and ophthalmic irritability and dermoregenerative effect).

Material and y methods

Chemical products and reagents

Chitosan, supplied by the Natural and Synthetic Products Production Plant (CIDEM, Cuba), batch 11002 with 9.2 % moisture, 0.8 % sulfated ash, 0.4 % insoluble material, 78.5 % degree of deacetylation and 310 000 g/mol molecular mass, was used as active ingredient [13]. The following were used as excipients in the formulation: isopropyl myristate (Henkel, Germany), glacial acetic acid and sodium hydroxide (Panreac, Spain), propylene glycol (BASF We Create, Germany), cetyl alcohol (Ecogreen, Malaysia), stearic acid (Renichem S.L., Malaysia), sodium benzoate (Panreac, Spain) and floral fragrance (Robertet, France).

Pilot-scale production of the cream

Based on the optimized formulation [21], three 10.0 kg batches identified as L-21001, L-21002, L-21003 were prepared, packaged in bottles (F-14), high-density polyethylene caps (T-14) and low-density polyethylene liner (L-14) with tamper-evident seal, nominal capacity of 250 mL (Frasplast, Cuba).

The composition of the batches of cream was similar, and the components were: sodium hydroxide (0.60 %), cetyl alcohol (4.50 %) and stearic acid (7.40 %). It was established that stearic acid should be maximum and cetyl alcohol minimum, thus achieving the best organoleptic characteristics. Based on the results obtained in the formulation and design stage, the numerical optimization of the formulation was developed [21].

The final homogenization of the product was carried out in a colloidal mill (Probts and Class, Germany). Packaging was carried out semi-automatically, using a filling machine model AR/403 (ERWEKA, Germany).

Cream evaluation

The batches were periodically evaluated according to organoleptic characteristics, pH, extensibility and degree of deacetylation. The methodologies used for each test are described below:

Organoleptic characteristics: appearance, color and odor were determined. Samples were observed for visible modifications and instabilities like: coalescence, creaming and presence of lumps. The existence of sandiness was evaluated by touch. Acceptance criteria: Semisolid product of uniform appearance, odor and characteristic color.

Area of extensibility: 2.0 g of the semisolid was weighed on an analytical balance (SARTORIUS model ENTRIS 6202-1S, Germany) and placed in the center of a glass plate with a millimeter paper adhered to the bottom, so that the point of application of the semisolid coincided with the axes of the coordinates drawn on it. Subsequently, a 292 g glass plate was homogeneously placed on the semisolid. After 5 min, the radii corresponding to the circle formed by the sample, determined from the point of application in four directions, were measured. The total area of extension of sample was calculated reporting the average value and the standard deviation, resulting from three determinations per sample. Acceptance criteria: 25.50 - 36.20 cm^2.

Determination of pH: A Seven Compact TM S220 pH meter (Mettler Toledo, Switzerland) calibrated with buffer solutions of pH 4.01 and 7.00, respectively was used. Approximately 20.0 g of sample was transferred to a 50 mL beaker. Three replicates were performed for each sample and the mean value and standard deviation were reported. Acceptance criteria: 6.50 - 7.25.

Degree of deacetylation: For the determination of the content of amino groups, 5.0 g of the cream sample was weighed, transferred to a plastic tube, added 5 mL of chloroform (Sigma-Aldrich, Germany) and subjected to vigorous vortexing (SeouLin, Korea). Then 25 mL of 0.3 N hydrochloric acid (37 %, Merck, Germany) was added and shaken in a sieve for 1 h. Subsequently, it was centrifuged (SORVALL RC 3B PLUS, USA) at 4000 s^{-1} for 15 min at 30 °C. Then, 20 mL of aqueous phase was extracted and 10 mL of purified water was added. It was titrated (Mettler Toledo, Switzerland) with 0.3 mol/L sodium hydroxide (Merck, Germany) slowly and with continuous stirring. The degree of deacetylation was determined using the following equations:

$$MQC = Pm / 100$$
$$\%NH_2 = 16.1(Y - X) / (MQC \cdot f)$$

Where: MQC: chitosan in cream sample weighed (mg); Pm: cream weighed (mg); 100: mathematical factor for calculation; $\%NH_2$: percentage of free amino groups; 16.1: milliequivalent value between hydrochloric acid and chitosan; X: major inflection point (mL); Y: minor inflection point (mL); f: factor of sodium hydroxide dissolution.

To calculate the degree of deacetylation/g of the cream was carried out using the following formula: Degree of deacetylation (%) = ($\%NH_2 \cdot 100$) / 9. Acceptance criteria: 70-95 %.

Stability of pilot batches

Pilot batches were stored at 30 ± 2 °C and 70 % relative humidity, for 12 months. The parameters described above were periodically determined at initial, 1, 3, 6 and 12 months [20]. The stability of the product was predicted from the results of the variables pH, extensibility and degree of deacetylation using the Statagraphics® Centurion 19 program (USA, 2022).

Microbiological stability was determined through the microbial limit test for non-sterile products, at initial and at 12 months [22]. Acceptance criteria: total aerobic microorganism count (bacterial count, BC) $<10^2$ cfu/g, combined total count of filamentous fungi and yeasts (FC and Y) <10 cfu/g, absence of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.

Non-clinical studies

Dermal and ophthalmic irritancy tests were performed on the cream formulation at the Department of Pharmacological and Toxicological Research (CIDEM, Cuba). Male New Zealand albino rabbits of 1.5 to 2.0 kg body weight with lot number 0516, from the National Center for the Production of Laboratory Animals (CENPALAB, Cuba), were used and were kept during the assay in a room with controlled temperature of 22 ± 2 °C and 12/12 h light-dark cycle. Feeding consisted of standard diet for rabbits CMO 1400 from CENPALAB and acidulated water on demand.

Dermal irritancy

The rabbits, 24 h before the start of the trial, were depilated and shaved on the dorsal area of the back at a sufficient distance from the spine to proceed with the application and observation of the test sites. Three rabbits with intact skin (three sites per product) were selected and 0.5 g of the cream was applied to the skin for 4 h. The application sites were covered with 2.5 cm^2 gauze patches, which were attached to the skin with

hypoallergenic tape. At the end of this period, the patches were removed and once the application site was marked, the remnants of the test substance were removed using sterile water [23].

Observations were made at 0.5, 1, 1, 24, 48 and 72 h after patch removal. Skin reactions for erythema and edema were established according to the Evaluation System for Skin Irritation [24]. Only observations made at 24, 48 and 72 h after application were taken into account to calculate the Primary Irritation Index, which was compared with the Response Classification System for dermal irritation testing to give the test results and classify the test substance [25].

Ophthalmic irritancy

Three rabbits were taken and subjected, in the 24 h prior to the assay, to a rigorous examination of their ocular structures (cornea, iris and conjunctiva) to rule out the presence of any underlying damage. On the day of the trial, 0.1 g of the cream was instilled in the conjunctival sac fundus of the right eye; the left eye was taken as a control. Both eyelids were closed for 15 seconds to ensure maximum contact of the test substance with the ocular structures. Both eyes were examined at approximately 1, 24, 48 and 72 h after the initial application in order to detect damage to the mentioned ocular structures. Observations were made with white light to detect the presence of erythema, edema and abnormal secretions, in addition to the reaction of the iris to light, followed by examination of the cornea by fluorescein staining and observation with UV lamp.

The ocular reactions observed were evaluated according to the scale for classifying ocular lesions. Once both eyes were examined during the established hours, the Ocular Irritation Index was determined [24, 25].

Determination of the dermoregenerative effect

To test the dermoregenerative effect, chitosan creams were tested at concentrations of 0.25 %, 0.5 % and 1.0 % (w/w), in a model of photoaging in the skin of mice photosensitized with ultraviolet radiation (UVB), supported by standard histological techniques [26].

Male Balb/C mice of 20 to 25 g body weight, batch number 1811, from CENPALAB (Cuba) were used. These were maintained during the trial in a room with controlled temperature of 22 ± 2 °C and 12/12 h light-dark cycle. Feeding consisted of standard diet for rodents CMO 1000 from CENPALAB and acidified water on demand. The

animals remained in quarantine for seven days, before starting the trial, for their adaptation to the laboratory conditions. Five groups of five animals were formed: I: control without radiation, II: control radiation, III: treated low dose (0.25 %), IV: treated medium dose (0.5 %), V: treated high dose (1.0 %), VI-treated placebo.

All animals, except the controls (group I), were subjected to UVB radiation using a lamp (Spectroline®, USA), at a wavelength of 312 nm. The radiation was directed to the dorsal area of the animal at a distance of 15 cm, for 30 min. Immediately after the radiation, the respective treatments were applied, which were repeated every 24 h for a period of six days. Each substance was applied to the skin of the dorsal area in an amount of 0.1 g spread over the skin surface [27].

Daily, before each application, the appearance of the skin was evaluated to detect the presence of erythema, bedsores and skin wrinkling, using a scale from zero to four, according to the degree of damage observed. At the end of the treatment, 24 h after the last administration, the animals were sacrificed by cervical traction and the skin was removed from the dorsal area for histopathological analysis. Skin samples were processed by traditional histological procedures. Aspects such as the appearance or not of an inflammatory infiltrate, presence or not of hyperkeratosis and acanthosis, disposition of the elastic and collagen fibers and other alterations that could appear, characteristics of photoaging, as well as their possible recovery or not due to the use of the cosmetic under study were analyzed [28].

Results and discussion

Stability of the optimized formulation at pilot scale

The composition of the three batches was similar, during the elaboration process of those no difficulties were manifested, obtaining a homogeneous product, which demonstrated the reproducibility of the developed methodology.

Table 1 shows the average results of the analyses carried out on the pilot batches during 12 months, under shelf-life conditions. In all cases, the criteria established in the quality specifications of the finished product were met [21].

The organoleptic properties of the three batches remained unchanged during the study period. The preparation presented a white color, floral odor, a homogeneous appearance with brightness, free of lumps and sandiness.

Table 1. Stability of pilot batches

Parameter	Batch	Time (month)					Acceptance criteria
		Initial	1	3	6	12	
Organoleptic characteristics	21001	Accepted	Accepted	Accepted	Accepted	Accepted	Semisolid product of uniform appearance, odor and characteristic color
	21002	Accepted	Accepted	Accepted	Accepted	Accepted	
	21003	Accepted	Accepted	Accepted	Accepted	Accepted	
pH	21001	6.93/0.10	6.86/0.08	6.86/0.08	6.81/0.10	6.79/0.05	6.50 – 7.25
	21002	6.95/0.05	6.90/0.10	6.87/0.08	6.86/0.04	6.81/0.05	
	21003	6.91/0.07	6.87/0.08	6.85/0.09	6.83/0.07	6.80/0.05	
Extensibility (cm^2)	21001	31.12/1.81	29.55/0.11	30.25/3.32	29.68/4.81	27.70/2.82	25.50 – 36.20
	21002	30.21/1.95	30.53/1.50	28.80/2.84	29.59/2.92	29.00/3.91	
	21003	31.68/2.81	30.34/5.05	29.59/2.92	30.21/1.95	29.59/2.92	
Degree of deacetylation (%)	21001	78.20/2.30	78.10/2.55	78.14/2.14	78.10/2.24	78.00/2.54	70 – 95
	21002	77.80/2.35	77.90/2.60	77.76/2.55	77.50/2.10	77.40/2.75	
	21003	78.00/2.55	77.95/2.32	77.85/2.75	77.65/2.56	77.00/2.85	
Microbiological test	21001	Accepted	-	-	-	Accepted	BC: 102 cfu/g; FC and Y: 10 cfu/g; Absence of *P. aeruginosa* and *S. aureus*
	21002	Accepted	-	-	-	Accepted	
	21003	Accepted	-	-	-	Accepted	

(X/DS) Mean/Standard Deviation: n=3; BC: total count of aerobic microorganisms, FC and Y: combined total count of filamentous fungi and yeasts.

The pH of a cosmetic formulation in the form of an emulsion is of vital importance because it can influence its stability, causing its breakdown, affecting the efficacy of the preservatives, as well as the chemical stability of other components of the formulation. The pilot batches maintained a pH between 6.50 and 7.25 during the study period, with no significant differences ($p > 0.05$) between the reported values. No significant variations were observed between batches or during the study period, evidencing the stability of the formulation, which allows guaranteeing the safety of the cosmetic.

The values of the extensibility area remained in accordance with the characteristics reported during the optimization of the formulation [21]. All the above evidenced the reproducibility of the formulation and of the elaboration process developed, under the conditions used.

The extensibility of the batches showed a general tendency to decrease as storage time progressed, an expected result, considering the effect of temperature on the viscosity of semisolids. It is known that semisolids experience a structuring of the system during storage time, which implies an increase in consistency causing a decrease in the extensibility of the product [12].

Regarding the percentage of the degree of deacetylation, the values showed a tendency to decrease with time, but within the established range [21], which shows that the chitosan in the cream maintains this property during the 12 months of study.

The results of the microbiological study satisfactorily met the established limits, which demonstrate the use of quality raw materials and compliance with Good Manufacturing Practices.

The results obtained as a whole allow affirming that the 1.0 % chitosan cream maintains its physical, chemical and microbiological stability during 12 months, packed in high-density polyethylene bottles and stored at 30 ± 2 °C and 70 % relative humidity, therefore these results approve the reproducibility of the three batches.

Statistical analysis of the experimental values of pH, extensibility and degree of deacetylation demonstrated their fit to linear models (Table 2), with probability values less than 0.05. The variation of the three parameters was linearly dependent with time, with very low degradation rates, which explains the low correlation coefficients. These models were used to predict the stability by extrapolation of the three batches of the cosmetic cream, proving that it can have a stability of approximately 24 months (Figure 1).

Table 2. Analysis of mathematical models for the prediction of cream stability by extrapolation.

Parameter	Model	Fit (p)	Correlation coefficient (r^2)
pH	Linear	0.000	77.50
Degree of deacetylation	Linear	0.0115	39.40
Extensibility	Linear	0.0039	48.61

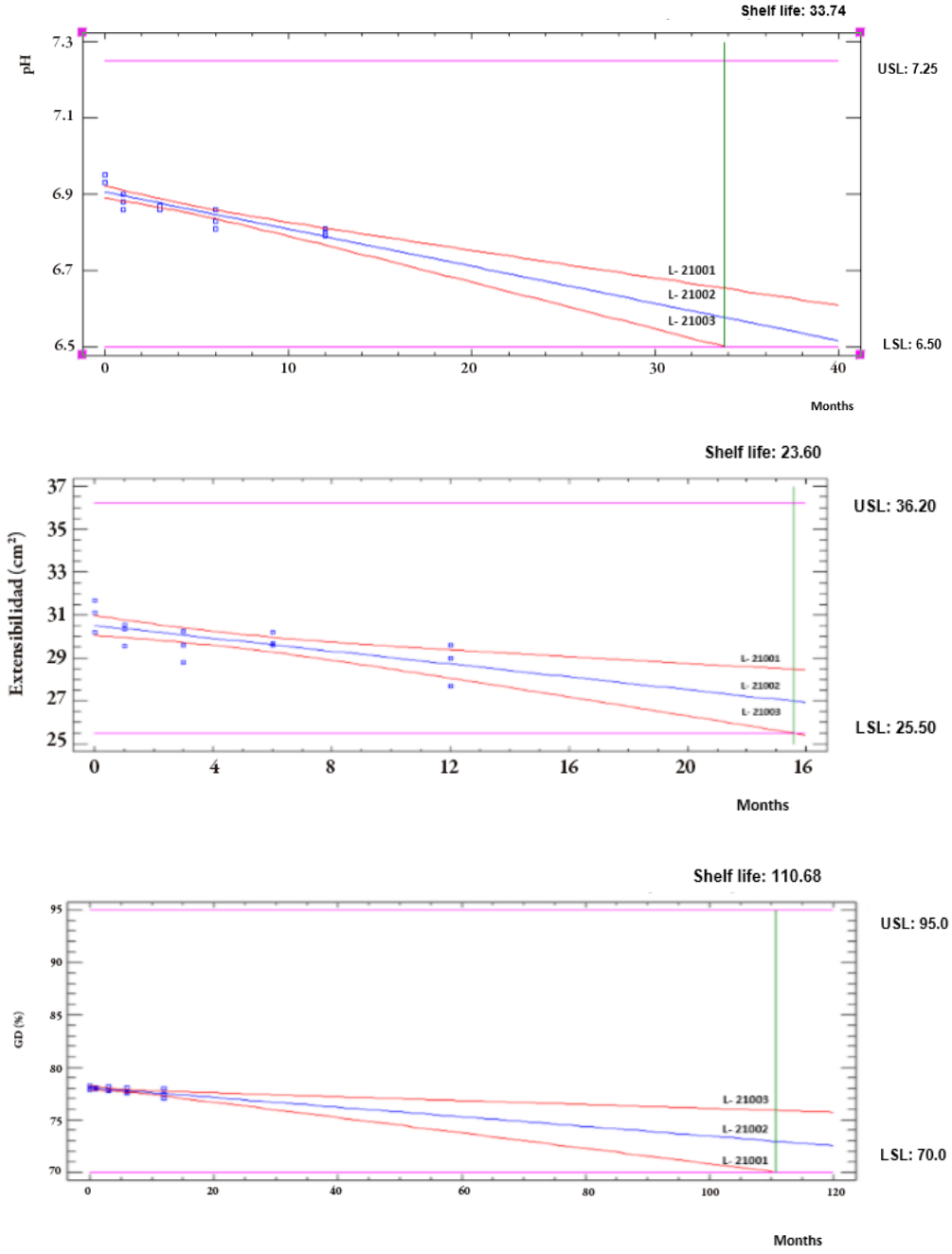

Figure 1. Extrapolation prediction of cream stability (Batch: 21001, 21002, 21003), using data of pH, extensibility and degree of deacetylation (DD).

Non-clinical evaluations

Dermal and ophthalmic irritancy tests were performed to determine the possible effects of the cream, taking into account that the product is generally intended for facial use.

During the evaluation of dermal irritability, erythema was observed in some animals. These animals did not show alterations in their behavior or weight. The irritability index was 0.32 points, which classifies the product evaluated as non-irritating.

Regarding ophthalmic irritability, during the first hour after the application of the product, there were erythematous and edematous reactions and secretions in the conjunctiva of the treated animals, but not in the iris. The cornea was affected during the first 24 h and from the clinical point of view there were no alterations in the experimental animals. From these results, the irritability index was calculated, reaching a value of 8.33 points, which allowed classifying the 1.0 % chitosan cream as non-irritant.

Considering these results, it was demonstrated that the formulated semisolid is a product that offers good safety, since it is considered a system of low toxicity to the organism.

Dermoregenerative effect of the chitosan cream

According to macroscopic observations, in group I (control without radiation) there were no alterations, serving as a comparison of the normal structures of the skin. In groups II (radiation control) and VI (placebo treated) the characteristic alterations of photoaging appeared: erythema, wrinkling of the skin and crustal-desquamative lesions. In groups III (chitosan 0.25 %), IV (chitosan 0.50 %) and V (chitosan 1.0 %), damage to dermal and epidermal structures was observed, as well as recovery from post-treatment damage.

According to microscopic observations, there were characteristic alterations of photoaging, with marked significance in the disorganization of the collagen fiber in the control group (II) and in the group treated with placebo (VI). In groups III (chitosan 0.25 %) and group IV (chitosan 0.50 %), photoaging alterations were observed, but less marked than in groups II and VI, after treatment, and average grading values of 2 were reached for both cases (Table 3), indicating that the 0.25 and 0.50 % chitosan creams have a low dermoregenerative effect.

According to the average values of graduation of histological damage observed in the skin sections of the different groups, after treatment, the recovery is appreciated in group V, treated with 1.0 % chitosan cream, reaching an average graduation value of 1 (Table 3), which indicates a moderate dermoregenerative effect.

Table 3. Grading values in the evaluation of histological damage.

Animal number	Histological scoring in the animal groups					
	I	II	III	IV	V	VI
I	0	3	1	1	1	3
II	0	3	2	2	1	3
III	0	3	3	2	1	3
IV	0	3	2	2	1	3
VI	0	3	2	3	1	3
Average graduation	0	3	2	2	1	3

Histological score: (0) normal; (1) mild; (2) moderate; (3) severe.
I: control without radiation, II: control radiation, III: treated low dose (0.25 %), IV: treated medium dose (0.50 %), V: treated high dose (1.0 %), VI: treated placebo.

The average results and standard deviation in each group of animals, showed that there are no significant differences between the groups treated with 0.25 % and 0.5 % chitosan cream, with respect to the irradiated control group, however, between the placebo and irradiated groups and the 1.0% chitosan cream group, significant differences were found (Figure 2).

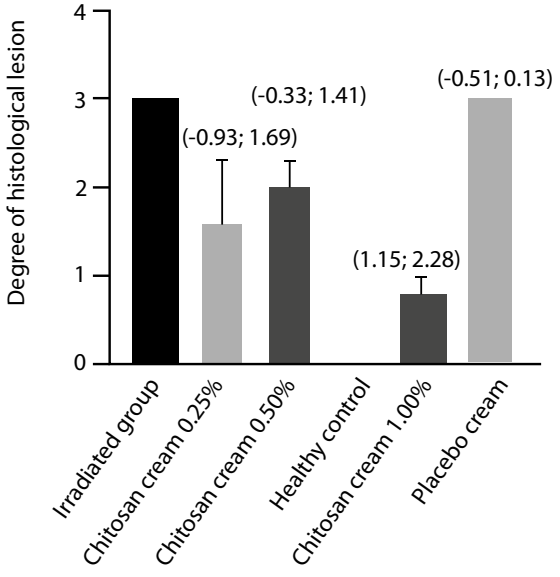

Figure 2. Microscopic evaluation of the skin of irradiated animals treated with 0.25 %, 0.50 %, 1.0 % and placebo chitosan cream. The confidence interval (σ =95 %) is shown for each of the treated groups with respect to the irradiated group.

The dermoregenerative activity of the cosmetic cream was evaluated in the photodamaged skin of mice by histological sections of the different skin layers. The skin condition was good for 100 % of group I (mice depilated without irradiation) and group V (mice depilated, irradiated and treated with 1.0 % chitosan cream). The recovery of the normal properties of the epidermis and dermis, previously damaged by radiation, was verified, particularly in the arrangement and organization of the collagen fibers. Chitosan proved to be the compound responsible for the dermoregenerative effect after application of the cream.

Conclusions

The pilot batches of the formulation maintained their stability for 12 months, packaged in high-density polyethylene bottles at room temperature. The 1.0 % chitosan cream is non-irritating dermally and ophthalmically and has a moderate dermoregenerative effect.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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Chemical-pharmaceutical application of carnitine palmitoyltransferase-2 (CPT-II) through regulating mitochondrial against cancer tumoric cells

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Summary

Background: Carnitine palmitoyltransferase II deficiency is an inherited disorder of long-chain fatty acid oxidation characterized by hypoketotic hypoglycemia, cardiomyopathy, seizures, muscle pain and weakness, and myoglobin. Individuals with carnitine palmitoyltransferase II deficiency have a defect in the production of the enzyme carnitine palmitoyltransferase-II, which plays an important role in fatty acid oxidation. Signs and symptoms of carnitine palmitoyltransferase II deficiency are due to the buildup of fatty acids and long-chain acyl-carnitine as well as reduced energy production in cells. Carnitine palmitoyltransferase II deficiency is an autosomal recessive disease caused by mutations in the CPT2 gene. During changing Nonalcoholic fatty liver disease (NAFLD) to the cirrhosis, the probability of cancer is high that should be considered as a dangerous situation. **Methods**: SDS-PAGE system of polyacrylamide gel electrophoresis through analytical method for separating charged molecules in mitochondrial mixtures according to their molecular mass in the presence of electrical fields was used. The Invitrogen® Bright Imaging (IBI) system provides was applied for the imaging and analysis of protein imprints. **Results**: Since currently no effective treatment for CPT-II deficiency, prevention of liver failure is a proper way of treatment through controlling mitochondria without affecting CPT-II potency. We discussed about the severe infantile hepatocardiac

muscular position of CPT II deficiency affects the liver heart, and muscles. **Conclusions:** Through this work, we discussed and characterized the pathophysiological function in several tissues such as liver, Kidney cancers.

Keywords: Liver cancer; Kidny failure; Hepatocellular carcinoma; Nonalcoholic fatty liver disease; CPT-I; CPT-II; SDS-PAGE system.

Resumen

Aplicación químico-farmacéutica de la carnitina palmitoiltransferasa-2 (CPT-II) mediante la regulación mitocondrial frente a células tumorales cancerosas

Antecedentes: La deficiencia de carnitina palmitoiltransferasa II es un trastorno hereditario de la oxidación de ácidos grasos de cadena larga caracterizado por hipoglucemia hipocetósica, miocardiopatía, convulsiones, dolor y debilidad muscular y mioglobina. Las personas con deficiencia de carnitina palmitoiltransferasa II tienen un defecto en la producción de la enzima carnitina palmitoiltransferasa-II, que desempeña un papel importante en la oxidación de los ácidos grasos. Los signos y síntomas de la deficiencia de carnitina palmitoiltransferasa II se deben a la acumulación de ácidos grasos y acilcarnitina de cadena larga, así como a la reducción de la producción de energía en las células. La deficiencia de carnitina palmitoiltransferasa II es una enfermedad autosómica recesiva causada por mutaciones en el gen CPT2. Durante el cambio de la enfermedad del hígado graso no alcohólico (NAFLD) a la cirrosis, la probabilidad de cáncer es alta y debe considerarse una situación peligrosa. **Métodos:** Se utilizó el sistema SDS-PAGE de electroforesis en gel de poliacrilamida como método analítico para separar moléculas cargadas en mezclas mitocondriales según su masa molar en presencia de campos eléctricos. El sistema Invitrogen® Bright Imaging (IBI) se utilizó para la obtención de imágenes y el análisis de huellas de proteínas. **Resultados:** Dado que actualmente no existe un tratamiento eficaz para la deficiencia de CPT-II, la prevención de la insuficiencia hepática es una forma adecuada de tratamiento mediante el control de las mitocondrias sin afectar la potencia de CPT-II. Aquí se discute acerca de la posición muscular hepatocardíaca

infantil grave a causa de la deficiencia de CPT II que afecta el hígado, el corazón y los músculos. **Conclusiones:** En este trabajo, se discute y caracteriza la función fisiopatológica en varios tejidos como el cáncer de hígado y riñón.

Palabras clave: Cáncer de hígado; Insuficiencia renal; Carcinoma hepatocelular; Enfermedad del hígado graso no alcohólico; CPT-I; CPT-II; Sistema SDS-PAGE.

Resumo

Aplicação químico-farmacêutica da carnitina palmitoiltransferase-2 (CPT-II) através da regulação mitocondrial contra células tumorais cancerígenas

Antecedentes: A deficiência de carnitina palmitoiltransferase II é um distúrbio hereditário da oxidação de ácidos graxos de cadeia longa, caracterizado por hipoglicemia hipocetótica, cardiomiopatia, convulsões, dor e fraqueza muscular e mioglobina. Indivíduos com deficiência de carnitina palmitoiltransferase II apresentam um defeito na produção da enzima carnitina palmitoiltransferase-II, que desempenha um papel importante na oxidação de ácidos graxos. Os sinais e sintomas da deficiência de carnitina palmitoiltransferase II são devidos ao acúmulo de ácidos graxos e acil-carnitina de cadeia longa, bem como à redução da produção de energia nas células. A deficiência de carnitina palmitoiltransferase II é uma doença autossômica recessiva causada por mutações no gene CPT2. Durante a mudança da doença hepática gordurosa não alcoólica (DHGNA) para cirrose, a probabilidade de câncer é alta, o que deve ser considerado uma situação perigosa. **Métodos:** Foi utilizado o sistema SDS-PAGE de eletroforese em gel de poliacrilamida através de método analítico para separação de moléculas carregadas em misturas mitocondriais de acordo com sua massa molecular na presença de campos elétricos. O sistema Invitrogen® Bright Imaging (IBI) fornecido foi aplicado para a geração de imagens e análise de impressões de proteínas. **Resultados:** Como atualmente não há tratamento eficaz para a deficiência de CPT-II, a prevenção da insuficiência hepática é uma forma adequada de tratamento através do controle das mitocôndrias sem afetar a potência do CPT-II. Discutimos sobre a posição muscular hepatocardíaca infantil grave da deficiência de CPT II que afeta o fígado, o coração e os músculos. **Conclusões:** Através deste trabalho, discutimos e caracterizamos a função fisiopatológica em diversos tecidos, como câncer de fígado e rim.

Palavras-chave: Câncer de fígado; Falência renal; carcinoma hepatocelular; Doença hepática gordurosa não alcoólica; CPT-I; CPT-II; Sistema SDS-PAGE.

Introduction

Carnitine palmitoyltransferase specification (CPT)

Carnitine palmitoyltransferase II (CPT II) deficiency is a condition that prevents the body from using certain fats for energy, particularly during periods without food (fasting). There are three main types of CPT II deficiency: a lethal neonatal form, a severe infantile cardio muscular form, and a myopathy form. The lethal neonatal form of CPT II deficiency becomes apparent soon after birth. Infants with this form of the disorder develop respiratory failure, seizures, liver failure, a weakened heart muscle (cardiomyopathy), and an irregular heartbeat (arrhythmia). Affected individuals also have low blood glucose (hypoglycemia) and a low level of ketones, which are produced during the breakdown of fats and used for energy. Together these signs are called hypoketotic hypoglycemia. In many cases, the brain and kidneys are also structurally abnormal. Infants with the lethal neonatal form of CPT II deficiency usually live for a few days to a few months [1-6]. Since there is a mechanism of oxidation of phospholipids in tumors, these disorders may be caused by the regulation of fatty acids in the mitochondrial matrix [7-9]. The outer membrane transferase CPT-I is generally recognized as the main regulatory site of lipid peroxidation in mitochondria, and for this reason, its regulation has been extensively studied, as has the regulation of the outer membrane transferase CPT-II. Problems related to this form of CPT II deficiency can be triggered by periods of fasting or by illnesses such as viral infections. Individuals with the severe infantile hepato-cardio-muscular form of CPT-II deficiency are at risk for liver failure, nervous system damage, coma, and sudden death. During treatment of the latent status of cancer, several changes occur, such as a striking enhancement in PGE2 concentration [13-15]. It is clear that inhibition of PGE2 reduces tumor growth in both *in vivo* and *in vitro* studies [16-20]. We exhibited the translational activities of CPT-I or CPT-II can also influence the anti-keto genic creation of extra hepatic tumor growth in human liver [10-12]. It is a question whether inhibition of prostaglandin synthesis could impair the tumor growth effects of liver mitochondrial CPTs. CPT-II deficiency is one of the most uncommon inherited traits caused by the lipid chain and also the most common cause of recurrent rhabdomyolysis in adults. It is noteable CPT-I or CPT-II are proteins that help transport fatty chains from the cytoplasm to the mitochondria during beta oxidation of fatty acids for energy production. Fatty acid oxidation (FAO) (Figure 1) is suitable for producing energy during stress, strong sports, long time exercise, fasting, and also cold climate. Consequently through a huge stress, the concentration of CPT-II will be decreased due to transition of acyl-carnitine across the inner mitochondrial membrane as a result oxidation is reduced.

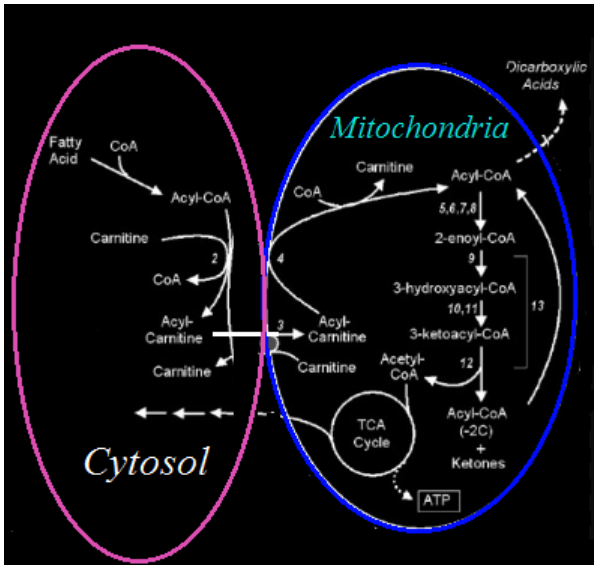

Figure 1. Esterification of fatty acids across mitochondrial.

The lethal neonatal form of CPT II deficiency becomes apparent soon after birth. Infants with this form of the disorder develop respiratory failure, seizures, liver failure, a weakened heart muscle (cardiomyopathy), and an irregular heartbeat (arrhythmia). Affected individuals also have low blood glucose (hypoglycemia) and a low level of ketones, which are produced during the breakdown of fats and used for energy. Together these signs are called hypoketotic hypoglycemia. In many cases, the brain and kidneys are also structurally abnormal. Infants with the lethal neonatal form of CPT II deficiency usually live for a few days to a few months. CPT-II is divided into three main phenotypes, two of which cause hypoglycemia due to hypoglycemia and are fatal in utero or in the first days of life. The third type, which attacks until puberty, is the typical muscle type, characterized by muscle pain, muscle weakness, and rhabdomyolysis due to vigorous exercise. Although for some sports its effect is hidden, in some other sports myalgia appears with frequent attacks of rhabdomyolysis. Mutations in the CPT2 gene cause CPT II deficiency. This gene provides instructions for making an enzyme called carnitine palmitoyltransferase 2. This enzyme is essential for fatty acid oxidation, which is the multistep process that breaks down (metabolizes) fats and converts them into energy. Fatty acid oxidation takes place within mitochondria, which are the energy-producing centers in cells. A group of fats called long-chain fatty acids must be attached to a substance known as carnitine to enter mitochondria. Once these fatty acids are inside mitochondria, carnitine palmitoyltransferase 2 removes the carni-

tine and prepares them for fatty acid oxidation. Fatty acids are a major source of energy for the heart and muscles. During periods of fasting, fatty acids are also an important energy source for the liver and other tissues

Decreasing HCC under NAFLD *in vivo*

HCC often arises in patients with liver cirrhosis caused by chronic hepatitis B or C virus infection. However, recent epidemiology studies found that non-alcoholic fatty liver disease (NAFLD) is also a high-risk factor for HCC . In humans, adoptive transfer of tumor-specific CD4+ T cells caused a complete tumor eradication in a patient bearing cholangiocarcinoma, another primary liver cancer. Furthermore, immunotherapy is becoming standard of care for the treatment of advanced HCC [21,22]. To address this question, a better understanding of the influences of fatty liver environment on T-cell metabolism is required. This may also shed light on the design of a targeted therapy and potentially a combined immunotherapy for HCC. [23–25]. Most humans have no explicit signs and cannot be recognized before liver cirrhosis or hepatocellular carcinoma (HCC) ,because the effect of early clinical screening is negligible [26]. During changing NAFLD to the cirrhosis, the probability of reverse situation has high risk that HCC should be considered as a dangerous position. As a result, many systemic diseases appear in humans by HCC, such as cardiovascular diseases, chronic alcoholic diseases, and colon and rectal tumors, all of which threaten human health [27-29]. As CPT1a up regulation leads to greater ROS and CD4+ T cell apoptosis, we sought to test whether blocking CPT-1 affects CD4+ T cells and HCC development in the context of NAFLD. As we found the CPT inhibitor perhexiline rescued murine CD4+ T cell and Jurkat cell apoptosis when cultured with C18:2 *in vitro*. Liver-specific inducible MYC oncogene transgenic mice, which spontaneously develops HCC after turning on MYC gene (MYC-ON), were fed with the MCD diet and injected with perhexiline. The liver is a central organ for lipid metabolism. With the prevalence of NAFLD in Western countries and the significant risk of NAFLD patients to develop HCC, critical components in lipid metabolism could be potential targets for the treatment of NAFLD-induced HCC. PPARs and CPT proteins are among those potential targets. However, the exact role of PPAR-α in HCC has been controversial on whether PPAR-α promotes or suppresses tumor growth [30]. NAFLD and another type known as nonalcoholic steatohepatitis (NASH) are associated with liver cancer, which causes fatty particles to build up in liver cells. The epidemic of NAFLD is rapidly increasing with the prevalence of diabetes and appears to be present in a quarter of the world's population [31,32]. In addition, this issue has been evaluated in 2011 and diagnosed that from each one per five liver cancers worldwide one of them is related to diabetes. NAFLD can be considered a dire and dangerous world subjects; nevertheless, there is no useful treatment and no mechanism of NAFLD decreasing for this disease up

to now. There are huge information indicating that metabolic conversions in the cancer tumors might be converted to anti-immune metabolism and consequently ruin antitumor immunity [33-35]. Although the anti-tumor effects of CD4 + T cells in various forms of cancers such as liver are the main target for diagnosis by researchers [36], there is no further research except using the diethylnitrosamine (DEN)-primed murine HCC model. Working on a mouse model, it was found that CD4+ T cells are able to prevent tumor initiation and remove malignant liver cells [37-40]. There are 3 particular subunits of CPT-I known as a, b and c [33,34]. CPT-Ia is the first subunit in lymphocytes, liver, kidney, spleen, lung, intestine, and pancreas. CPT-Ib is mostly translated in muscles, heart, and adipose tissue, while CPT-II is mainly translated in the brain [41-42]. Peroxisome proliferator-activated receptor (PPARs) is a category of phospholipids of RNA transcription agents that affects to metabolic systems. They can be separated into three subunits including: alpha, gamma and beta/delta .Although PPAR-α and PPAR-γ is translated in lymphocytes, finally PPAR-α is a major part in the liver for attaching to fatty acids. It has been exhibited CPT-I trough activating PPAR-α15 is considerably affected by fatty acids, , moreover several works confirm the role of both PPAR-α and PPAR-γ as activated receptors to adjustment the transcription of the CPT-Ia gene directly [43-45].

Although these changes can be tested from liver CD4+ T cells through nutrition rat with linoleic acid or NAFLD diets, more research exhibited that PPAR-α is the reason the turbulent of CPTs genes. Introduction of CPTs genes can increase ROS and lead to apoptosis of CD4 + T cells. *In vivo* treatment of mice with CPTs inhibitor in addition to reducing apoptosis of hepatic CD4 + T cells and controlling HCC growth in NAFLD system provides useful data, such as identify genes for CPTs such as liver cancer therapy promoted by NAFLD [45-50].

CPT-II genes

The carnitine palmitoyltransferase (CPT) system is responsible for transporting long-chain fatty acids from the cytoplasm into the mitochondria where the fatty acids undergo β-oxidation. Transport of long-chain fatty acids into the mitochondrial matrix requires both CPT1 and CPT2, with CPT1 being the rate limiting step. This CPT system contains two separate proteins localized in the outer (CPT1) and the inner (CPT2) mitochondrial membrane. The most suitable action of the CPTs might be mentioned as an ability for helping fatty acids to enter inside the mitochondria for any further oxidation. For this purpose, trans-membrane protein as known CPT-I is placed in OMM, as well as, CPT-II genome is located in IMM. CPT-II genome appears on chromosomes1 (1p32) and consist of 3092 nucleotides in five exons that

can encode the enzyme chain containing 660 amino acids (table 1)[50]. The expression of CPT1b and CPT2, suggesting that other lipids may also contribute to the induction of CPT genes in NAFLD.

Table 1. Structure of CP-II genome

Exson Size (bp)	Nucleotides	Amino acids	Amino acid substitution in mutations of five exons
106	756-857	80-112	Cys84Arg; Ala101Val; Ser113Leu
1303	858-2163	113-549	Met120Cys; Arg121Gln; Asn124Gln; Arg124Ter; Asn146Thr; Ala151Gln; Arg151Gly; Arg161Trp; Ile164Ter; Arg167Gln; Asp173Ser; Glu174Lys; Tyr210Ala; Asp213Trp; Met214Arg; Gln216Arg; Pro227Leu; Ala231Ser; Arg247Trp; Gln274Met; Arg296Ser; Arg296Leu; Ala296Ter; Gly310Gly;Cys326Tyr; Lys328Gly; Met342Asp; Phe352Met; Val368Ile; His369Arg; Arg382Lys; Phe383Tyr;Gln413Gln; Phe448Ala; Arg450Ter; Gln451Glu; Gln454Ter; Lys457Trp; Tyr479Phe; Tyr479Cys;Glu480Arg; Gln487Lys; Gly497Ser; Ile502Thr; Arg503Ser; Phe504Leu; Asn516Ser; Gln545Ala
934	2164-3092	550-660	Ala560Gln; Leu575Pro; Arg576Gly; Ser588Gln; Ser590Asn; Gly600Met; Pro604Ser; Val605Leu;Asp608Gly; Tyr628Ser; Arg631Cys; Lys644Ser
667	1-669	1-50	Pro41Leu; Pro50His
80	669-750	51-79	Pro55Arg; Ala67Gly

Intermediate role of PPAR-α for CPT gene

Previously, it was reported that PPAR-α can directly up regulate CPT1a expression [50, 51], so the hypothesized that the induction of CPT genes observed in CD4+ T cells treatment is mediated by PPAR-α. To test this, we used the PPAR-α agonist bezafibrate and monitored CPT gene expression .CPT-I and CPT-II contributed to the oxidation of long-chain fatty acids in mitochondria as well as their transport across the mitochondrial membrane for β-oxidation [51]. From a genetic concept point of view, CPT-II is identified by seventy percent of mutant alleles, which plays an important role in fatty acid entry into mitochondrial fatty acid oxidation as well as cellular metabolic homeostasis [52]. Oxaliplatin as an effective anti-cancer drug is used for increasing the CPT-II activation in cancer tumors and enhancing the catabolism of fatty acids. Structural amino acids evaluation from Exon 4 and Exon 5 and also its

activities indicating, which CPT-II might be became instable due to heterogeneous mutations , exogenous carcinogens, endogenous perturbation and huge mutation phenomenon [53]. Since the liver cells should control their metabolic systems in any usage of variety of feeds and ATP necessities [52-55], CPT-II must be well catalyzes Trans esterified acylcarnitine's transferred from cytosol into the inter-membrane space (IMS) [55-57]. CPTs act in the oxidation of LCFAs containing CPT-I and CPT-II in the OMM, catalyze fatty acids to form fatty acids with the participation of ATP and CoA, and then transport long-chain acyl-CoA into the system via the delivery system. Mitochondria many genes encoding CPT-II are known to be recessive genetic defects and clinical situations of associated diseases such as hypoglycemia, cardiomyopathy, arrhythmias, and rhabdomyolysis can be considered [58-68].

Materials and methods

Naphthylene was obtained from Aldrich Chemical Co; PPO, Palmitoyl CoA, protein G, carnitine, POPOP, sepharose-protein G, FMP, Indomethacin was prepared from Sigma, and dioxan from Brazil. [3H]-methylcarnitine, nitrocellulose (Highbong extra), Hypercassette and Hyperscreen were purchased from Amersham (UK). Mice were divided into four groups: 1-tumor-bearing group (TB), 2-control group (C), 3-control treated with indomethacin, and 4-tumor-bearing group treated with indomethacin. The livers of rat embryos (gestational days 10-18) were divided into pieces in Dulbecco's modified essential medium (DMEM). The pieces were placed in 0.25% collagenase solution for half hour at 37 °C. Again it centrifuged by speed of 1500 r/min for 8 minutes. Collagen enzyme potential was neutralized by adding 25% fetal serum to the mixture. They were suspended in DMEM and 12% fetal serum and cultured in a plastic thermos at -70°C. This method is based on the method of reference 63 [63] and after killing the mice, their livers were cleaned and homogenized with a buffer solution (250 mM mannitol, 70 mM sucrose, 3 mM HEPES, 0.3 mM EDTA, pH 7.2). In the same buffer, the homogenate is filtered and centrifuged twice at 1200 rpm for 10 minutes. Finally, the above part of the mixture was centrifuged three times at 8000 rpm for 15 minutes (Sorvall RC2B centrifuge). The maximum activity of CPT-I and CPT-II was measured in isolated mitochondria using detergents according to number 64 [64]. Finally, they were re-suspended in buffer containing 0.12 mM KC1, 5.0 mM Tris-HC1 (pH 7.3) and centrifuged (15,000 rpm, 10 min). They were then re-suspended in 15 mM phosphate buffer (pH 7.3), frozen in liquid nitrogen, and then thawed at room temperature. They were again ultra-centrifuged.

PCR for RNA application

RNA was extracted from cell pellets or frozen tissue with QIAshredder (Qiagen) and RNeasy MiniKit . The sequence of primers used for quantitative RT-PCR have been estimated from RNA isolation . All reactions of quantitative RT-PCR were run in triplicates using iQTM SYBR Green Super mix and performed on the ViiATM Real-Time PCR System.

Mitochondrial coloring

Mitochondrial-associated ROS was detected by mitoSOXstaining according to the manufacturer's protocol. Briefly, treated cells were stained with 3 μM mitoSOX for half hour in a CO2 incubator at 38 °C. After washing twice, the cells were processed.

Fluorescent coloring

Fibroblast cells were seeded several times by NuncTM Lab-TekTM Chambered Cover glass wells. After half day, cells were washed with phosphate-buffered saline (PBS) three times and incubated with 2 μM BODIPY probe with 10 nM MitoTracker Deep Red in PBS at 38 °C for half hour min. Cells cleaned by PBS and was kept for imaging. Live cells were imaged using a Zeiss LSM880 laser (Figure 2).

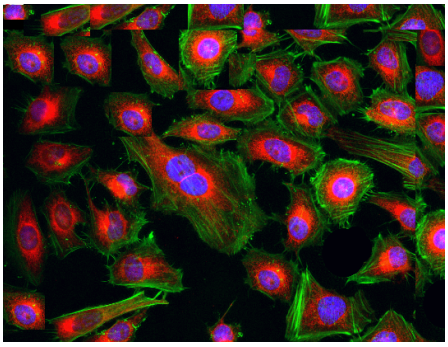

Figure 2. Mitochondrion fluorescent coloring.

Results

We found that intrahepatic CD4+ T cells are an indispensable component of antitumor surveillance in NAFLD, and linoleic acid (C18:2) causes CD4+ T cell apoptosis by impairing electron transport chain (ETC) function and generating ROS8. During this process, CPT1 catalyzes the transfer of the acyl group of a long chain fatty

acyl-CoA from coenzyme A to carnitine so the resulting acylcarnitine can cross the mitochondrial outer membrane, while CPT2 reverses this reaction inside mitochondria. Table 2 exhibits the optimal potentials and activities of CPT-I and CPT-II in mitochondria provided by the livers of TB and control mice. For CPT-I, no significant differences emerged between the two groups. However, the CPT-II potential in liver mitochondria of TB-infected rats was 55% lower than that of control rats.

Table 2. Enzymatic potential

	Liver	
Samples	**CPT-I**	**CPT-II**
C (n=15)	3.25	2.22
TB (n=6)	3.85	1.01
	Tumor	
TB (n=8)	2.12	3.43
TB-ind (n=15)	3.09	5.21

Figure 3 exhibits the schematic of proteins separated by SDS-PAGE of mitochondrial samples obtained from tuberculosis and control mice. The only distinct difference from the more abundant proteins was a small translation of the corresponding proteins into the mitochondria of TB mice.

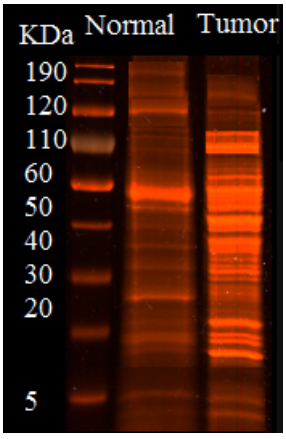

Figure 3. SDS/PAGE segregation of mitochondrial from the rats, including normal and tumors.

In Figure 4, Western blot positions for CPT-I and CPT-II immunoreactivity in mitochondria from control mice and *M. tuberculosis* are shown. Although the difference between these two patterns in CPT-I amounts in mitochondria isolated from control

as well as TB mice was not significant, TB mitochondria showed little CPT-II activity at 69,000. In addition, in these mitochondria, a second band was also detected by the rat liver CPT-II antibody exhibited.

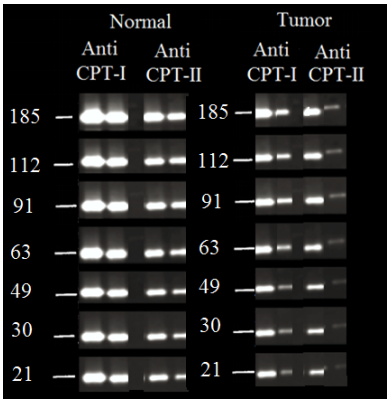

Figure 4. Western blot detection of immunoreactive CPT-I and CPT-II proteins in liver mitochondria isolated.

These effects were not found *in vitro* when embryonic hepatocytes were cultured in the presence of 12 g of indomethacin because there was no induction of CPT-I or CPT-II in indomethacin-treated cells compared with control cells (Table 3).

Table 3. Effects of indomethacin *in vivo* and *in vitro* on CPT I and CPT II from liver mitochondria.

Enzymatic potential (nmol/min.mg)		
Items	CPT-I	CPT-II
control (n=4)	2.99	3.12
control –ind (n=6)	5.85	3.11
TB (n=3)	3.02	1.12
TB-ind (N=12)	4.15	4.05
Cultured hepatocytes (n=5)	3.21	4.41
Cultured hepatocytes –ind (n=5)	3.22	4.21

Discussion

In vivo feeding and *in vitro* culture experiments showed that induced CPT gene up regulation. Using either an agonist or antagonist, it confirms that PPAR-α mediates the induction of CPT genes, as well as targeting of CPT inhibits HCC development in the context of NAFLD. Since fatty acid diffusion to cytoplasm does not stop in

the livers of TB rats, it can be interpreted that the metabolic destroying creates during the transport of fatty acids into the mitochondrial matrix. Contrary to any previous hypothesis and prediction, CPT-I is capable of controlling FAO in all metabolic states. To date, neither the activity of this enzyme nor the translation of the corresponding protein has been affected in the liver mitochondria of TB mice. However, CPT-II potency was halved, and Western blotting using an anti-CPT-II polyclonal antibody revealed the source of the additional reactive enzyme in liver mitochondria obtained from TB mice. These considerations add to our knowledge that the less likely isoform of CPT-II is translated either through transcription of different genes or through post-transcriptional mRNA labeling (*in vivo*) and proteolysis processing in the liver. It is also possible that the CPT-II isoform acts as a master receptor regulator. The results of this work exhibited that treatment with prostaglandin inhibitors was able to reverse the effects of cachexia on hepatic CPT-II activities. The similarity between the potential obtained in mitochondria isolated from control cultured hepatocytes and those cultured with indomethacin predicts that PGE2 modulation of the CPT-II reduction potential is not the result of eicosanoid production by the hepatocytes themselves, but is likely due to the action of prostaglandins. In other wise, the *in vivo* situation is related to the large amounts of PGE2 production by the tumor and of course our data of this work needs more investigation regarding the role of CPT-II and the control of its translation for FAo mechanism in the pathophysiological positions. It is notable, liver is a central organ for lipid metabolism, therefor With the prevalence of NAFLD in Western countries and the significant risk of NAFLD patients to develop HCC, critical components in lipid metabolism could be potential targets for the treatment of NAFLD-induced HCC. PPARs and CPT proteins are among those potential targets. However, the exact role of PPAR-α in HCC has been controversial on whether PPAR-α promotes or suppresses tumor growth.

Conclusions

In conclusion, we demonstrated that by inhibiting CPT1 we can rescue CD4+ T cells and prevent HCC development. Our results provide useful information that CPT1 may be a potential target for NAFLD -promoted HCC therapy. Through this work, we have focused on the partial application of lipid metabolic pathways in disease pathogenesis and considered the modulating cases and clinical treatments of lipid systems in different tissues. Furthermore, through this work, we have characterized the pathophysiological function of both CPT-I and CPT-II systems in liver disease compared to several other chronic infections as well as cancer (Table 4).

Table 4. The role of CPT in inflammatory disease.

Goals	Associated patients	CPTs	Effects	References
Liver	injurey	CPT-I; CPT-II	Decreased in intrahepatic cholestasis model	[68, 69]
Liver	NAFLD	CPT-I	Improved the symptoms of the disease	[70]
Liver	HCC	CPT-I; CPT-II	Upregulated CPT elevated apoptosis of CD4+ T cells and promoted HCC formation in NAFLD	[71-73]
Cardiovascular	Cardiac dysfunction	CPT-I; CPT-II	Downregulated CPT induced cardiac injury during endotoxemia	[74]
Cardiovascular	Cardiac dysfunction	CPT-I	Downregulated CPT1 induced the injury	[75]
Pulmonary	Asthma	CPT2	Increased in asthmatic bronchial SMC	[76]
Pulmonary	Asthma	CPT-IB	Decreased CPT1B increased mortality; increased expression and decreased activity in aged ALI mice	[77]
Kidney	Kidney fibrosis	CPT-IA	Decreased during the disease	[78]
Kidney	Kidney fibrosis	CPT-IA	Overexpression of CPT1A showed protective effects	[79]
Colon	Colorectal cancer	CPT-IA	Exposure to adipocytes or FA upregulated CPT1A	[80]
Colon	Colorectal cancer	CPT-IA	Low expression in primary tumor tissues while high expression in CAFs	[81]
Colon	CAC	CPT-I	Suppressed CPT1 inhibited NLRP3 assembly in macrophages	[82]
Breast	Breast cance	CPT-IA	Upregulated in patients; a new biomarker for the diagnosis	[83]
Breast	Breast cance	CPT-IA	Increased in doxorubicin-treated tumours *in vivo*	[84]
Blood	AML	CPT-IA	Overexpression predicted poor clinical outcome	[85]
Pancreas	Pancreatic cancer	CPT-IA	Downregulated CPT1C inducted tumor senescence	[86]

(*Continued*)

Table 4. *Continuation.*

Goals	Associated patients	CPTs	Effects	References
Skin	Melanoma	CPT-IA	Inhibited CPT1A led to apoptosis in MAPKi-treated cells	[87]
Muscle	Muscle dysfunction	CPT-II	A conceptual overview on CPT2 deficiency	[88]
Gastric	Gastric Cancer	CPT-IC	Associated with poor prognosis; promoted proliferation of cancer cells	[89, 90]

Abbreviations

C: control rats; CH: cultured hepatocytes; CH-ind: hepatocytes cultured with indomethacin; C-ind: control rats submitted to indomethacin treatment; CPT: carnitine palmitoyltransferase; ETC: electron transport chain; FAMR: fatty acid metabolic regulation; FAO: fatty acid oxidation; FMP: fatty milk powder; IMM: inner mitochondrial membrane; LCFAs: long-chain fatty acids; NEFA: non esterification fatty acids; OMM: outer mitochondrial membrane; PGE2: prostaglandin E2; POPOP:

1,4-bis (5-phenyl-2-oxazolyl)-benzene; PPO: 2,5-diphenyloxazol; ROS: reactive oxygen species; TB: tumor-bearing rats; TB-ind: turnout-bearing rats given indomethacin; TNF: tumor necrosis factor;

Conflict of interest

All authors report that they do not have any conflicts of interest.

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Investigation and health risk assessment of heavy metals in cattle from slaughterhouses in Federal Capital Territory (FCT), Abuja, Nigeria

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Summary

Introduction: Heavy metals toxicity in cattle can cause health related issues in humans when they ingest cow meat polluted with heavy metals. The dearth of information regarding the level of heavy metals in cattle meat consumed in the Federal Capital Territory (FCT), Abuja, Nigeria, and its associated health risk to humans led to this study. **Aim:** to determine the level of the heavy metals- nickel, lead, cobalt, cadmium and chromium in cow muscle, intestine and liver from slaughterhouses in Federal Capital Territory (FCT), Abuja, Nigeria, as well as the health risk of consumption of the metals in the meat parts. **Methodology:** The heavy metals were assayed using Atomic Absorption Spectrophotometer Agilent 200 Series A. A. Health risk assessment of exposed adult men and women, adolescent and children to the heavy metals in the meat parts were evaluated via hazard quotient (HQ), hazard index (HI) and incremental lifetime cancer risk (ILCR) calculations. **Results:** The result of the study showed overall mean concentration of the metals in cow muscle,

intestine and liver samples ranging from 0.0417 ±0.0226 to 0.690 ±0.0570 mg/kg, fresh weight. HQ and HI values were less than 1, and ILCR values of the carcinogenic heavy metals ranged from 1.07×10^{-7} to 7.50×10^{-5}. **Conclusion:** Consumers of cow muscle, intestine and liver in FCT, Abuja, Nigeria are at low risk of developing chronic non-cancer health related issues, and cancer due to nickel, lead, cobalt, cadmium and chromium in cow meat parts from slaughterhouses in FCT, Abuja, Nigeria.

Keywords: Cattle, Hazard index, Hazard Quotient, Heavy metals and Incremental Lifetime Cancer Risk.

Resumen

Investigación y evaluación de riesgos para la salud de metales pesados en ganado vacuno de mataderos en el Territorio de la Capital Federal (FCT), Abuja, Nigeria

Introducción: La toxicidad por metales pesados en el ganado puede causar problemas de salud en los humanos cuando estos ingieren carne de vaca contaminada con estos metales pesados. La falta de información sobre el nivel de metales pesados en la carne de ganado consumida en el Territorio de la Capital Federal (FCT), Abuja, Nigeria, y el riesgo asociado para la salud de los seres humanos llevó a este estudio. **Objetivo:** determinar el nivel de metales pesados: níquel, plomo, cobalto, cadmio y cromo en el músculo, el intestino y el hígado de las vacas procedentes de mataderos en el Territorio de la Capital Federal (FCT), Abuja, Nigeria, así como el nivel de riesgo en la salud por el consumo de metales en las partes cárnicas. **Metodología:** Los metales pesados se analizaron utilizando un espectrofotómetro de absorción atómica Agilent 200 Serie A. A. La evaluación de riesgos para la salud de hombres y mujeres adultos, adolescentes y niños expuestos a los metales pesados en las diferentes partes de la carne se evaluó mediante el cociente de peligro (HQ), el índice de peligro (HI) y cálculos de riesgo incremental de cáncer de por vida (ILCR). **Resultados:** El estudio mostró una concentración media general de metales en muestras de músculo, intestino e hígado de vaca que oscilaba entre 0,0417 ±0,0226 y 0,690 ±0,0570 mg/kg de peso fresco. Los valores de HQ y HI fueron inferiores a 1, y los valores de ILCR de los metales pesados cancerígenos oscilaron entre $1{,}07 \times 10^{-7}$ y $7{,}50 \times 10^{-5}$. **Conclusión:** Los consumidores de músculo, intestino e hígado de vaca en FCT, Abuja, Nigeria, tienen un riesgo bajo de desarrollar problemas de salud crónicos no relacio-

nados con el cáncer, además de cáncer propiamente dicho, debido al níquel, plomo, cobalto, cadmio y cromo en las partes de carne de vaca de los mataderos en FCT, Abuya, Nigeria.

Palabras clave: Bovinos, índice de peligrosidad, cociente de peligrosidad, metales pesados y riesgo incremental de cáncer a lo largo de la vida.

Resumo

Investigação e avaliação do risco sanitário de metais pesados em bovinos provenientes de matadouros no Território da Capital Federal (FCT), Abuja, Nigéria

Introdução: A toxicidade de metais pesados em bovinos pode causar problemas de saúde em humanos quando estes ingerem carne de vaca poluída com metais pesados. A escassez de informações sobre o nível de metais pesados na carne bovina consumida no Território da Capital Federal (FCT), Abuja, Nigéria, e o risco associado à saúde humana levou a este estudo. **Objetivo:** determinar o nível de metais pesados - níquel, chumbo, cobalto, cádmio e cromo no músculo, intestino e fígado de vacas de matadouros no Território da Capital Federal (FCT), Abuja, Nigéria, bem como a saúde risco de consumo dos metais nas partes da carne. **Metodologia:** Os metais pesados foram dosados usando Espectrofotômetro de Absorção Atômica Agilent 200 Série A. A. A avaliação do risco à saúde de homens e mulheres adultos expostos, adolescentes e crianças aos metais pesados nas partes da carne foi avaliada através do quociente de perigo (HQ), índice de perigo (HI) e cálculos incrementais de risco de câncer ao longo da vida (ILCR). **Resultados:** O resultado do estudo mostrou concentração média global dos metais em amostras de músculo, intestino e fígado de vaca variando de 0,0417 ±0,0226 a 0,690 ±0,0570 mg/kg, peso fresco. Os valores de HQ e HI foram inferiores a 1, e os valores de ILCR dos metais pesados cancerígenos variaram de $1,07 \times 10^{-7}$ a $7,50 \times 10^{-5}$. **Conclusão:** Os consumidores de músculo, intestino e fígado de vaca na FCT, Abuja, Nigéria, apresentam baixo risco de desenvolver problemas crónicos não cancerígenos relacionados com a saúde e cancro devido ao níquel, chumbo, cobalto, cádmio e crómio em partes de carne de vaca provenientes de matadouros em FCT, Abuja, Nigéria.

Palavras-chave: Bovinos, Índice de Perigo, Quociente de Perigo, Metais Pesados e Risco Incremental de Câncer ao Longo da Vida.

Introduction

The muscle and edible offal of cow are the meat that are mainly consumed in Nigeria, because they are cheaper compared to that of goat, sheep and chicken. On the other hand, pig muscle and edible offal are avoided by some citizens of Nigeria due to religious and traditional beliefs. Cow muscle and edible offal provide cheap source of meat protein to the Nigerian people. Cattle rearing is highest among other domestic animals reared for meat consumption in Nigeria; cow meat account for 45% of the total meat eaten in Nigeria while meat from goat and sheep account for 35% [1]. Cow meat in Nigeria is mostly supplied by the Fulani tribe of Northern Nigeria whose occupation is cattle rearing. These Fulanis are nomads; moving from one region of the country to another in search of grazing for their cattle. As they move, wherever they find pasture and water (stream, ponds etc) they settle for their cattle to feed and or drink. Their movement from place to place may expose the cattle to heavy metal pollution.

Cows are herbivores; they feed on green plants (grasses). Green plants grow on soil. The problem that may arise from consumption of plants by cows reared in Nigeria is ingestion of heavy metal polluted plants, because some Nigeria soils are polluted with heavy metals [2-6], arising from dumping of scrap metals on soils [3], metal recycling activities [4], mining operations [6], power plant station emissions [7], vehicular exhaust emissions [8-10], oil spill [11-13], industrial effluent discharge [14-16], improper solid waste disposal [17, 18], auto mechanic and auto panel workshop activities [18, 19], application of fertilizers and pesticides etc. Some plants grown on heavy metal polluted soil have the ability to absorb heavy metal from soil through their root to other plant parts [16, 20], also, by atmospheric deposition heavy metals emitted into the atmosphere are deposited on plants and absorbed by plant parts [21, 22]. Apart from pollution of soil with heavy metals, heavy metals also pollute water.

Federal Capital Territory (FCT), Abuja, Nigeria is the capital city of Nigeria, with high population density, traffic and vehicular emission, which contributes to heavy metal pollution. Cattle consumed in the capital city are reared within and outside the capital city, majorly Zamfara, Sokoto and Niger states all within the Northern Nigeria. Zamfara, Sokoto and Niger states are rich in gold deposit. Among other metals, gold bearing ore contains minerals of cadmium, lead, nickel, and cobalt [23-26]. Artisanal gold mining in Zamfara, Sokoto and Niger states release heavy metals into the environment from gold mining tailings, Zamfara state reported lead poisoning in this state due to artisanal gold mining. Gold mine tailings are characterized by high levels of arsenic, cadmium, nickel, lead, copper, zinc, cobalt and mercury [27]. Studies have shown that soil, vegetables and livestock (chicken, goat, sheep and cattle) around some goldmine

areas in Zamfara state, Nigeria are polluted with heavy metals [28, 29]. When grazing animals such as cow eat and drink heavy metal polluted grasses and surface soil, and water, they can be exposed to heavy metal pollution [30].

Exposure of humans to heavy metals through ingestion of cow meat polluted with heavy metals can affect human health despite the nutritional benefits of meat. Heavy metals such as iron, zinc, copper, manganese, molybdenum, cobalt, chromium (Cr III), magnesium, selenium and nickel are essential mineral nutrients required for proper functioning of the human body [31]. However, excess amount of these heavy metal mineral nutrients causes adverse health effects in humans [32]. Non-essential heavy metals are not useful biologically in humans [20], they include cadmium (Cd), lead (Pb), mercury (Hg), arsenic (As), and chromium (Cr (VI)) [20]. These metals are toxic even at low concentrations [32]. Cadmium can cause osteoporosis, kidney, breast, lung, prostrate, nasopharynx and pancreas cancer [33]. Chromium VI is a human carcinogen [34]. Lead in the body can cause high blood pressure, neurological problems, kidney and brain damage as well as miscarriage, low birth weight, stillbirth, premature birth in pregnant women [35]. High concentrations of cobalt may cause cancer [36]. Nickel pollution is associated with lung fibrosis, cardiovascular disease, lung cancer, kidney diseases and nasal cancer [37]. Previous studies conducted in Nigeria on some meat samples from different regions in Nigeria showed pollution of the meat samples with heavy metals [38-41].

The objectives of this study are to evaluate levels of heavy metals – nickel (Ni), lead (Pb), cobalt (Co), cadmium (Cd) and chromium (Cr) in cow muscle, intestine and liver from slaughterhouses in FCT, Abuja, Nigeria and to determine the health risk of consumption of the heavy metals in cow meat parts by consumers in FCT, Abuja, Nigeria. The data generated will be a useful aid for policy formulation by relevant health and environmental regulatory agencies in FCT, Abuja and Nigeria in general.

Materials and methods

Study area and sampling

Federal capital territory (FCT), Abuja is the capital of Nigeria, it is found in longitudes 6° 46′ and 7° 37′ E and latitudes 8° 21′ and 9° 18′ N. It is situated in the center of Nigeria and located in the North Central geopolitical region of Nigeria. The vegetation in Abuja is a blend of savannah grassland and tropical rain forest. It has agricultural rich soil hence the local people of the area are farmers and livestock animal farmers. The weather conditions in the FCT are of two types; rainy season (similar to winter) and dry season (similar to summer). The raining season last for six months

beginning from April to October. Annual precipitation in the FCT range from 1100 mm to 1600 mm, while monthly mean temperature ranges from 25 °C to 30 °C. The savannah grassland, tropical rain forest and agricultural rich soil produce lots of plant and grasses for grazing animals such as cattle. There are four slaughterhouses in Federal Capital Territory (FCT), Abuja, Nigeria, namely Deidei, Gwagwalada, Karu and Kubwa slaughterhouses. Meat samples comprising of cow muscle, liver and intestine were randomly collected from 10 cows in each slaughterhouse located in FCT, making a total of 120 meat parts samples. The age of the cows ranged from three to four years. Each cow meat part of a cow was enclosed in a clean polyethylene bag, labelled and preserved in an ice box. The meat samples were taken to the laboratory and preserved in refrigerator preset at -18 °C, prepared and analyzed within five days.

Sample preparation and analysis

All glass wares and sample bottles used for sample preparation and storage were washed with detergent and rinsed with tap water, afterwards they were soaked overnight in 50% HNO_3 at room temperature, rinsed again in deionized water and air dried [42]. The meat part samples were washed with deionized water and cut into pieces with stainless-steel knife precleaned with detergent and rinsed with distilled water. 2 g each of meat sample was weighed into a beaker, 20 mL of aqua regia (3:1 ratio of HCl and HNO_3) prepared from 37% HCl and 69% HNO_3 from Merck, Darmstadt, Germany was added to the content in the beaker and heated on a hot plate in a fume cupboard for 50 minutes. The beaker was brought down from the hot plate and allowed to cool. The content in the beaker was filtered through a 110 mm filter paper into a 100 mL volumetric flask and made to the mark with deionized water and transferred into properly labelled sample bottle and assayed for heavy metal using atomic absorption spectrophotometer Agilent 200 Series A. A. Calibration curve of nickel was prepared with 3 mg/L, 6 mg/L, 9 mg/L and 12 mg/L standard solutions of nickel from 1000 mg/L stock solution of nickel, that of cadmium was prepared with 2 mg/L, 4 mg/L, 6 mg/L and 8 mg/L of standard solutions of cadmium from 1000 mg/L stock solution of cadmium, while that of lead was prepared with 1 mg/L, 1.5 mg/L, 2 mg/L and 2.5 mg/L of standard solutions of lead from 1000 mg/L stock solution of lead, calibration curve of cobalt was prepared with 2 mg/L, 4 mg/L, 6 mg/L and 8 mg/L of standard solutions of cobalt from 1000 mg/L stock solution of cobalt, that of chromium was prepared with 1 mg/L, 2 mg/L, 3 mg/L and 4 mg/L of chromium from 1000 mg/L stock solution of chromium. The heavy metals were determined at their wavelength 232.0 nm (nickel), 217.0 nm (lead), 240.7 (cobalt), 228 (cadmium) and 357.9 (chromium) 357.9. The limit of detection (LOD) of the metals was 0.00001 mg/kg, calculated as the concentration corresponding to three times the standard deviation (SD) of reagent blank, while the limit of quantification (LOQ) 0.000033 mg/kg was calculated as 10 times the standard deviation of reagent blank.

Quality control

Reagents used in the study were of analytical grade. Accuracy of the analysis was achieved by triplicate determination of the concentration of the heavy metals in the meat samples. Reagent blank was prepared and analyzed before each measurement.

Method and instrument validation

Recovery study of each heavy metal was determined so as to validate the digestion method and the spectrophotometer of the analysis. Recovery study for each metal was performed by weighing 2 g of muscle in two places, one of the 2 g was spiked with 1 mL standard solution of heavy metal (Ni (3 mg/L), Pb (0.5 mg/L), Co (2 mg/L), Cd (2 mg/L), Cr (1 mg/L), while the other 2 g was unspiked. Both spiked and unspiked muscle samples were digested with *aqua regia* and assayed for heavy metal.

The % recovery was calculated using equation:

$$\% \text{ recovery} = \frac{A_1 - A_2}{B} \quad (1)$$

A_1 = Concentration of spiked meat sample

A_2 = Concentration of unspiked meat sample

B = Concentration of standard solution added to the spiked sample.

Health risk assessment

Carcinogenic and non–carcinogenic health risk assessment of pollutant(s) or toxicant(s) can be evaluated by determining hazard quotient, hazard index and incremental lifetime cancer risk of the pollutant(s) or toxicant(s) [43-45]. To obtain hazard quotient, estimated daily intake (EDI) or average daily dose (ADD) of the pollutant(s) or toxicant(s) will have to be determined first. Hazard quotient and hazard index estimates non-carcinogenic chronic and adverse health effects, while, incremental lifetime cancer risk estimates carcinogenic risk due to exposure to carcinogenic pollutant(s) or toxicant(s) [43-45].

Estimation of Daily Intake (EDI) of nickel, lead, cobalt, cadmium and chromium in muscle and edible offal (intestine and liver) from slaughterhouses in FCT, Abuja, Nigeria.

This was calculated as shown in Eq. 2. [46-48]:

$$\text{EDI} = \frac{C \times IR}{BW} \quad (2)$$

where *C* is mean concentration (mg/kg) of heavy metals in muscle, intestine and liver of cow samples, *IR* is the daily ingestion rate (mg/day) of the meat parts, and *BW* is body weight (kg) of adult men, adult women, adolescent and children. There is no available data on daily consumption/ingestion rate of cow muscle and edible offal by Nigerians in food consumption database by international organization such as Food and Agricultural Organization of the United Nations (FAO) and World Health Organization (WHO), but, Ihedioha *et al.* (2014) [40] determined daily ingestion rate of cow muscle and edible offal by Nigeria adult men and women, adolescent and children. This was used to estimate the daily intake of the metals in the cow meat parts. Daily ingestion rates of cow muscle by adult men, adult women, adolescent and children are 25.54 g/day, 22.96 g/day, 26.27 g/day and 19.13 g/day respectively, while, ingestion rates of cow liver are 17.31 g/day, 21.47 g/day, 22.01 g/day, and 22.31 g/day respectively, and ingestion rates of cow intestine are 32.58 g/day, 23.50 g/day, 25.89 g/day and 21.67 g/day respectively. The ingestion rates were converted to mg/day to keep the unit consistent. Body weight of Nigeria adult men, adult women, adolescent and children, are 70, 63, 65 and 35 kg respectively [40].

Non–carcinogenic Risk Assessment

Hazard quotient is used to determine non-cancer risks due to exposure to a single pollutant or toxicant, while, hazard index evaluates non-cancer risks due to exposure of person(s) to more than one pollutant or toxicant that affect the same target organ or system and, or exposure of person(s) to same toxicant or pollutant via different exposure route [43, 45]. To obtain hazard quotient, the estimated daily intake of a chemical toxicant is compared to an acceptable level of exposure of the chemical toxicant. The expression used to calculate hazard quotient (HQ) is Eq. 3 [46, 48]:

$$HQ = \frac{EDI}{RfD} \tag{3}$$

Where RfD is oral reference dose (mg/kg/day). Oral reference dose is defined as the highest level at which no adverse health effects are expected on human population through daily exposure to a chemical toxicant. Value of HQ < 1 indicates that there is no significant health related illness envisaged from a toxicant, while HQ > 1 implies that possibility of non-carcinogenic health issues is likely. Hazard index (HI) is estimated from Eq. 4 [47, 49]:

$$HI = \Sigma HQ \tag{4}$$

HI < 1 implies non probability of chronic non-carcinogenic health issues or adverse risk and HI > 1 indicates possibility of chronic risk or adverse non-carcinogenic health effects [43-45].

Nickel, lead, cobalt, cadmium and chromium affect the same target organ (kidney and central nervous system); hence their HQ were summed up to derive HI.

Carcinogenic risks Assessment

The possibility of developing cancer by person(s) exposed from lifetime to carcinogenic heavy metals nickel, cadmium and chromium in cow muscle and edible offal samples from the study area was computed using incremental lifetime cancer risk (ILCR) Eq. 5 [46, 50]:

$$ILCR = CDI \times CSF \tag{5}$$

Where CDI is chronic daily intake of chemical carcinogen (mg/kg bw/day) calculated with Eq. (6) and CSF is the cancer slope factor.

$$CDI = \frac{EDI \times EF \times ED}{AT} \tag{6}$$

Where, EDI is the estimated daily intake (mg/kg bw/day), EF is the exposure frequency (days/year and 365 days/year), ED is the exposure duration (years) (in this study, exposure duration is 25 years for adult men and women, 16 years for adolescent, and 6 years for children), AT is the averaging time (days). AT is the period over which exposure is averaged, it is calculated as exposure frequency (days/years) multiplied by exposure duration (years). AT is of two types, namely, for carcinogens and non-carcinogens. For carcinogens AT is based on a lifetime exposure. The life expectancy of Nigerians is 53 years [51]. In this study, AT is 19345 (365 days/year multiplied by 53 years). ILCR between 1×10^{-6} and 1×10^{-4} is acceptable by United States Environment Protection Agency (EPA) [43].

Results and discussion

Table 1 shows the result of percentage recovery of Ni, Pb, Co, Cd and Cr in muscle of cow sample. The percentage recovery ranged from 83 to 101%.

Table 1. Percentage Recovery of Ni, Pb, Co, Cd and Cr from cow muscle.

Heavy metals	Added concentration (mg/L)	Concentration of spiked sample (mg/L)	Concentration of unspiked sample (mg/L)	% recovery
Nickel	3.00	2.917	0.042	96
Lead	0.50	0.446	0.030	83
Cobalt	2.00	2.115	0.098	101
Cadmium	2.00	2.013	0.002	101
Chromium	1.00	1.001	0.097	90

The mean concentrations (mg/kg, fresh weight) and range of Ni, Pb, Co, Cd and Cr in samples of muscle, intestine and liver of cattle from Deidei, Gwagwalada, Karu and Kubwa slaughterhouses in FCT, Abuja, Nigeria are presented in Table 2. Nickel in muscle, intestine and liver of cattle samples ranged from -0.63 ±0.005 to 0.614 ±0.004 mg/kg fresh weight, from 0.007 ±0.005 to 0.414 ±0.030 mg/kg fresh weight, and from -0.070 ±0.008 to 0.509 ±0.009 mg/kg fresh weight, respectively. Lead, cobalt, cadmium and chromium in the meat parts ranged as follows: from 0.01 ±0.072 to 0.34 ±0.006 mg/kg fresh weight (muscle), from 0.02 ±0.043 to 0.68 ±0.009 mg/kg fresh weight (intestine), from 0.010 ±0.007 to 0.280 ±0.004 mg/kg fresh weight (liver) for lead; from -0.225 ±0.003 to 0.673 ±0.008 mg/kg fresh weight (muscle), from -0.583 ±0.002 to 2.845 ±0.005 mg/kg fresh weight (intestine), and from -0.650 ±0.008 to 0.699 ±0.008 mg/kg fresh weight (liver) for cobalt; from 0.021 ±0.007 to 0.092 ±0.064 mg/kg fresh weight (muscle), from 0.018 ±0.004 to 0.239 ±0.042 mg/kg fresh weight (intestine), and from 0.018 ±0.002 to 0.120 ±0.061 mg/kg fresh weight (liver) for cadmium; from 0.545 ±0.005 to 0.789 ±0.006 mg/kg fresh weight (muscle), from 0.497 ±0.072 to 0.799 ±0.021 mg/kg fresh weight (intestine), and from 0.509 ±0.004 to 0.772 ±0.087 mg/kg fresh weight (liver) for chromium. Mean concentration of nickel in muscle is 0.194 ±0.158 mg/kg fresh weight, in intestine it is 0.167 ±0.125 mg/kg fresh weight and 0.169 ±0.139 mg/kg fresh weight in liver. The result shows that the highest concentration of Ni is in cow muscle. The order of concentration of Ni in the cow meat parts is: muscle > liver > intestine. Similar trend was observed in some previous studies, these studies showed that the concentration of Ni is higher in muscle than liver [40, 41, 52]. When ingested into humans and animals, the cell lining the small intestine absorb small quantity of ingested nickel [53]; the absorbed nickel is transported to other parts (tissue and organs), and accumulates majorly in the kidney [53, 54], but less in the liver [54]. The mean concentrations of Ni in all the meat parts were below the maximum limit of 0.5 mg/kg nickel in meat and meat products set by Russia [55]; Food and Agricultural Organization (FAO) of the United Nations and World Health Organization (WHO) does not have maximum limit for nickel in meat and meat products. Low levels of Ni in liver and kidney (from 0.04 to 0.3 mg/kg) of

cattle from Southern Nigeria [56], and in muscle, kidney, liver, intestine and tripe, (from 0.25 to 0.36 μg/g fresh weight) of cow consumed in an Urban Nigeria [40] was reported. Nickel is used in the industries to manufacture electrical appliances, coins, jewelry, alloys, as a component of stainless steel it is used to manufacture household utensils, it finds application in pigment, food processing and metallurgical industries, etc. [37]. Nigeria is not an industrialized nation, however, it has very few industries which include food processing and metallurgical industries. Release of nickel from industrial sources may be low because of few industries in the country. Release of nickel into the environment in Nigeria from other sources may be due to indiscriminate disposal of nickel containing waste (e.g., electronic waste) and combustion of fossil fuel (vehicular emission). The levels of nickel in the meat parts below recommended maximum limit suggest that they are not polluted with nickel and that the cows may have grazed on grasses, drank water and were exposed to air with low levels of nickel. Mean concentration of Pb in cow muscle is 0.101 ±0.084 mg/kg fresh weight, it is 0.127 ±0.134 mg/kg fresh weight in intestine and 0.118 ±0.09 mg/kg fresh weight in liver. The trend of concentration of Pb in the meat parts is: intestine > liver > muscle. Similar trend was observed in cow meat parts in some studies [52, 57], in these studies, the trend was kidney > liver > muscle (0.052 ±0.002 mg/kg in kidney, 0.036 ±0.018 mg/kg in liver and 0.013 ±0.010 mg/kg in muscle), and kidney > liver > muscle (109.42 μg/kg in kidney, 42.70 μg/kg in liver and 8.77 μg/kg in muscle) respectively. These studies and the present study showed that Pb accumulates in liver than muscle of cow**.** Maximum level of Pb permitted in cattle muscle and edible offal is 0.2 mg/kg [58], none of the concentrations of Pb in the meat parts exceeded the recommended maximum level of Pb in muscle and edible offal, meaning that the meat parts are not polluted with Pb, suggesting that the cows were exposed to low levels of Pb. The level of Pb in muscle and edible offal (from 0.013 ±0.010 to 0.052 ±0.002 mg/kg) of cow obtained from Benin City, Nigeria, from a previous studies, and (from 0.01 to 0.26 mg/kg) of Pb in meat parts from southern Nigeria were below FAO/WHO stipulated maximum level (57; 56), except in liver from Sapele, Nigeria (0.26 mg/kg) which was slightly above the maximum level, but, high levels of Pb was found in meat parts from Kaduna, Nigeria (41). Sources of Pb in the environment include smelting of Pb, battery manufacture and recycling, use of Pb in car repair, Pb based paints and pigments etc. [59]. In Nigeria, automobile mechanic makes up a large proportion of the informal sector; their activities include car body work (which involves panel beating and metal cutting), car spray painting (which uses paint), battery charging etc. The activities of the automobile mechanics release lead in the environment [60], other sources of lead in the environment in Nigeria are battery recycling activities, lead and gold mining activities and indiscriminate disposal of waste battery and other lead containing waste. Mean concentration of Cobalt in cow muscle, intestine and liver samples are: 0.256 ±0.255 mg/

kg fresh weight in muscle, 0.179 ±0.584 mg/kg fresh weight in intestine and 0.096 ±0.237 mg/kg fresh weight in liver. The trend of concentration of Co in muscle and edible offal of cow samples is muscle > intestine > liver, however, the work of Ogbomida *et al.* (2018) [57] showed a contrary trend of cobalt concentration in cow meat parts: liver > kidney > muscle. There is no maximum level for cobalt, but minimal risk level (MRL) [61], MRL is an estimate of the daily human exposure to a hazardous substance that is likely to be without appreciable risk of adverse non-cancer health effects over a specified duration of exposure [61]. The MRL oral exposure for cobalt is 0.03 mg/kg/day [61], Mean concentrations of Co in cow muscle, intestine and liver are higher than the recommended MRL oral exposure for Co. These concentrations indicates that the cow meat parts are polluted with Co and that the cows may have grazed on grasses, and or drank water and or were exposed to air with high levels of Co. Sources of Co include both natural and anthropogenic activities. Co is a natural component of soil, rock and air, and can be leached from soil and rock by rainwater and surface runoff into water bodies [62]. Anthropogenic activities such as mining and ore smelting activities releases Co from ore deposits and phosphate rocks into the environment [62]. Cobalt is among the heavy metals released into the environment during gold mining, High Co concentration ranging from 0.275 ±0.053 to 2.200 ±0.011 μg/g (mg/kg) in liver and muscle of goat, sheep and cattle reared in the gold-mining areas of Zamfara state in Nigeria was reported [29]. Significant levels of cobalt in soil, vegetables and livestock around some gold mine areas in Zamfara state, Nigeria was also reported [28]. Zamfara state is one of the states from which cattle is brought into FCT slaughterhouses. Other sources of cobalt include, airport and highway traffic, coal fired power plants, colored glass and ceramics and cobalt containing alloys [62]. In Nigeria, mining and ore smelting activities are among non-oil sector economic activities of the country and may have contributed to release and pollution of the environment with Co in addition to indiscriminate disposal of colored glass and ceramics and Co containing alloy substances and gold mining. FCT, Abuja being the capital city of Nigeria experience highway and airport traffic due to the population density and landing of local and international aircrafts, these too may have added Co to the environment. Co can cause neurological problem such as hearing and visual impairment, endocrine and cardiovascular health effects [63]. Mean concentrations of Cd in the meat parts samples are 0.040 ±0.0153 mg/kg fresh weight (muscle), 0.0475 ±0.041 mg/kg fresh weight (intestine) and 0.0417 ±0.0226 mg/kg fresh weight (liver). This result shows that the trend of the concentration of Cd in the cow meat parts is: intestine > liver > muscle. This result compares well with the result from previous studies which showed the trend, kidney > liver > muscle (0.890 ±1.035 mg/kg in kidney, 0.044 ±0.013 mg/kg in liver and 0.001 ±0.000 mg/kg in muscle) [57], and kidney > liver > muscle (62.56 μg/kg in kidney, 14.16 μg/kg in liver and 1.40 μg/kg in muscle) [52]. This suggest that, may be Cd concentrates more

in the liver of cow than in the muscle. According to Agency for Toxic Substances and Disease Registry [64], Cd concentration is highest in the kidney and liver. The maximum level of Cd in muscle and liver of cattle are 0.050 m/kg wet weight and 0.50 mg/kg wet weight respectively [65]. The levels of Cd in muscle and liver of the cow meat parts samples are lower compared to the approved maximum level of Cd in muscle and liver, also, the level of Cd in intestine is lower compared to recommended maximum level of Cd in both muscle and liver. This indicates that the meat parts are not polluted with Cd. Usman *et al.* (2022) [41] reported 0.05 μg/g (mg/kg) and 0.31 μg/g (mg/kg) of Cd in muscle and liver respectively and 0.16 μg/g (mg/kg) and 0.22 μg/g (mg/kg) of Cd in tripe and intestine of cow from Kaduna State, Nigeria. These levels of Cd in the meat parts are within the recommended maximum level of Cd in muscle and liver of cattle. Also, Cd levels in the work of Ogbomidia *et al.* (2018) [57] showed low level of cadmium in muscle (0.001 ±0.000mg/kg) and liver (0.044± 0.013 mg/kg) of cows from Benin, Nigeria. Cd can be released into the environment from mining and smelting, burning of fossil fuels, burning of plastics and nickel cadmium batteries [66], recycling of cadmium-containing steel scrap and electronic waste. The mean concentrations of Cr in cow muscle, intestine and liver samples are: 0.690 ±0.0570 mg/kg in fresh weight in muscle, 0.698 ±0.0680 mg/kg in fresh weight in intestine, and 0.687 ±0.062 mg/kg in fresh weight in liver; i.e. intestine > muscle > liver. Ogbomida *et al.* (2018) [57] reported more concentration of Cr in muscle (0.261 ± 0.216 mg/kg) of cattle than in liver (0.064 ±0.014 mg/kg) of cattle, as reported in the present study. Food and Agricultural Organization of the United Nations, World Health Organization and other international regulatory bodies have not recommended standards for Cr in meat and meat products in food, however, China set maximum limit of 1.0 mg/kg of Cr in meat and meat products [67]. The mean concentrations of Cr in the different meat parts are less than the recommended 1.0 mg/kg in meat and meat parts by China, this implies that the muscle and edible offal of the cattle samples are not polluted with Cr.

Table 2. Mean concentrations (mg/kg, fresh weight) of Ni, Pb, Co, Cd and Cr in muscle and edible offal of cattle from slaughterhouses in Federal Capital Territory (FCT), Abuja, Nigeria.

Cow meat parts	Statistics	Nickel	Lead	Cobalt	Cadmium	Chromium
Muscle	Min	-0.63	0.01	-0.225	0.021	0.545
	Max	0.614	0.34	0.673	0.092	0.789
	Median	0.157	0.08	0.284	0.039	0.696
	Range	0.677	0.33	0.898	0.071	0.244
	Mean ±SD	0.194 ±0.158	0.101 ±0.084	0.256 ±0.255	0.040 ±0.015	0.690 ±0.057

(*Continued*)

Table 2. *Continuation.*

Cow meat parts	Statistics	Nickel	Lead	Cobalt	Cadmium	Chromium
Intestine	Min	0.007	0.02	-0.583	0.018	0.497
	Max	0.414	0.680	2.845	0.239	0.799
	Median	0.119	0.08	0.172	0.039	0.709
	Range	0.407	0.66	3.428	0.221	0.302
	Mean ±SD	0.167 ±0.125	0.127 ±0.134	0.179 ±0.584	0.048 ±0.041	0.698 ±0.068
Liver	Min	-0.070	0.010	-0.650	0.018	0.509
	Max	0.509	0.280	0.699	0.120	0.772
	Median	0.144	0.09	0.106	0.034	0.705
	Range	0.579	0.27	1.349	0.102	0.263
	Mean ±SD	0.169 ±0.139	0.118 ±0.090	0.096 ±0.0237	0.042 ±0.023	0.687 ±0.062

The ingestion rate of muscle, intestine and liver, and the result of estimated daily intake (EDI, mg/kg bw/day) of Ni, Pb, Co, Cd and Cr in muscle, liver and intestine of cow meat samples are presented in Tables 3 and 4 respectively. The EDI of nickel in the meat parts was: i) for adult men: 7.00×10^{-5} mg/kg bw/day (muscle), 7.70×10^{-5} mg/kg bw/day (intestine) and 4.10×10^{-5} mg/kg bw/day (liver), ii) for adult women, it was 7.00×10^{-5} mg/kg bw/day (muscle), 6.20×10^{-5} mg/kg bw/day (intestine) and 5.70×10^{-5} mg/kg bw/day (liver), iii) for adolescent, it was, 7.80×10^{-5} mg/kg bw/day (muscle), 6.60×10^{-5} mg/kg bw/day (intestine) and 5.70×10^{-5} mg/kg bw/day (liver), and iv) for children, it was, 1.05×10^{-4} mg/kg bw/day (muscle), 1.03×10^{-4} mg/kg bw/day (intestine) and 1.07×10^{-5} mg/kg bw/day (liver). The tolerable daily intake (TDI) derived for nickel in food is 3 μg/kg bw/day [68], which is equivalent to 0.013 mg/kg bw/day. According to European Food Safety Authority (EFSA), TDI is an estimate of the amount of a substance in food or drinking water which is not added deliberately (e.g. contaminants) and which can be consumed over a lifetime without presenting an appreciable risk to health. EDI values of nickel in the meat parts obtained in this study for different groups (adult men and women, adolescent and children) are lower than the TDI set by EFSA. This indicates no risk to health over lifetime from consumption of the level of Ni in the meat parts. EDI of the other heavy metals in muscle and edible offal of the cow samples ranged from 2.90×10^{-5} to 7.80×10^{-5} mg/kg bw/day of Pb in muscle, intestine and liver for all the groups (adult men and women, adolescent and children). For Co, it ranged from 2.30×10^{-5} to 1.39×10^{-4} mg/kg bw/day of Co in muscle, intestine and liver for all the groups, while, for Cd, it ranged from 1.03×10^{-5} to 2.90×10^{-5} mg/kg bw/day of Cd in the meat parts for all the groups. For Cr, it ranged from 2.16×10^{-4} to 4.37×10^{-4} mg/kg bw/day of Cr in muscle and edible

offal for all the groups. There is no stipulated tolerable or acceptable daily intake for cobalt in muscle and edible offal or food substances by Food and Agricultural Organization of the United Nations (FAO), World Health Organization (WHO) and United States Protection Agency (USEPA) etc., However, French Food Safety Agency (Agence Francaise De Securite Sanitaire Des Aliments) set tolerable daily intake of cobalt in food between 1.6 and 8 μg/kg bw/day [69], equivalent to 0.0016 and 0.008 mg/kg bw/day. From the result of the study, EDI of cobalt in the muscle and edible offal of the cow samples are lower than the TDI of cobalt set by AFSSA. This suggest that there may be no risk to health over lifetime from consumption of the level of Co in the meat parts. FAO/WHO does not have TDI for lead but provisional tolerable weekly intake (PTWI) which is 0.025 mg/kg bw [70], conversion of this value to daily basis gives 0.0036 mg/kg bw, The EDI of Pb in the meat parts is lower than 0.0036 mg/kg bw, which implies that ingestion of the concentrations of Pb in the meat parts may not pose risk to health over lifetime from consumption of the meat parts. Acceptable daily intake (ADI) or TDI is not provided by relevant regulatory authorities for cadmium, however, Provisional Tolerable Monthly Intake (PTMI) of cadmium is 25 mg/kg/month [71], by converting the PTMI to daily intake, using 30 days in a month, gives the Provisional Tolerable Daily Intake (PTDI) 0.83 mg/kg/day [72], which when converted to mg/kg/day gives 0.00083 mg/kg/day. EDI of cadmium in muscle, intestine and liver of the cows by the groups are less than 0.00083 mg/kg/day. This suggests that there may be no risk to health to adult men and women, adolescent and children, due to consumption of the level of cadmium in the cow meat parts. There are no intake values for chromium (VI). The intake value available for chromium III is adequate intake (AI) [73]. Adequate Intake is the intake assumed to ensure nutritional adequacy; established when evidence is insufficient to develop a recommended dietary allowance (RDA) [73]. Adequate intake for children between the ages of 4 to 8 is 15 μg (male and female) (0.015 mg), 9 to 13 years are 25 μg (male) (0.025 mg) and 21 μg (female) (0.021 mg), 14 to 18 years are 35 μg (male) (0.035 mg) and 24 μg (female) (0.024 mg), 19 to 50 years 35 μg (male) (0.035 mg) and 25 μg (female) (0.025 mg), and 51 years and above 30 μg (male) (0.03 mg) and 20 μg (female) (0.02 mg) (73). The estimated daily intake of chromium in muscle, intestine and liver of the cow samples (from 2.16×10^{-4} to 4.37×10^{-4} mg/kg bw/day) by adult men and women, adolescent and children residing in FCT, Abuja, Nigeria, are lower than the recommended adequate intakes, indicating that health risk is unlikely to happen over lifetime from consumption of the level of Cr in the cow meat parts.

Table 3. Ingestion rate of Cow Muscle, Liver and Intestine in Nigeria

Group	Age (years)	Body weight (kg)	Daily meat consumption (g/day)		
			Muscle	Liver	Intestine
Adult men	25 – 55	70 ±10.4	25.54 ±2.20	17.31 ±2.86	32.58 ±6.10
Adult women	25 – 55	63 ±10.8	22.96 ±2.44	21.47 ±3.38	23.50 ±3.03
Adolescent	16 – 25	65 ±10.8	26.27 ±2.93	22.01 ±4.46	25.89 ±5.78
Children	6 – 15	35 ±5.9	19.13 ±1.83	22.31 ±3.76	21.67 ±4.16

Table 4. Estimated daily intake (mg/kg bw/day) of muscle and edible offal consumed by adult men, adult women, adolescent and children in FCT, Abuja, Nigeria.

Group	Cow meat part	Nickel	Lead	Cobalt	Cadmium	Chromium
Adult men	Muscle	7.00×10^{-5}	3.60×10^{-5}	9.30×10^{-5}	1.40×10^{-5}	2.51×10^{-4}
	Intestine	7.70×10^{-5}	5.90×10^{-5}	8.30×10^{-5}	2.20×10^{-5}	3.24×10^{-4}
	Liver	4.10×10^{-5}	2.90×10^{-5}	2.30×10^{-5}	1.03×10^{-5}	2.10×10^{-4}
Adult women	Muscle	7.00×10^{-5}	3.60×10^{-5}	9.30×10^{-5}	1.40×10^{-5}	2.51×10^{-4}
	Intestine	6.20×10^{-5}	4.70×10^{-5}	6.60×10^{-5}	1.76×10^{-5}	2.60×10^{-4}
	Liver	5.70×10^{-5}	4.00×10^{-5}	3.30×10^{-5}	1.42×10^{-5}	2.33×10^{-4}
Adolescent	Muscle	7.80×10^{-5}	4.00×10^{-5}	1.03×10^{-4}	1.60×10^{-5}	2.78×10^{-4}
	Intestine	6.60×10^{-5}	5.00×10^{-5}	7.10×10^{-5}	1.87×10^{-5}	2.78×10^{-4}
	Liver	5.70×10^{-5}	3.90×10^{-5}	3.20×10^{-5}	1.41×10^{-5}	2.32×10^{-4}
Children	Muscle	1.05×10^{-4}	5.50×10^{-5}	1.39×10^{-4}	2.17×10^{-5}	3.76×10^{-4}
	Intestine	1.03×10^{-4}	7.80×10^{-5}	1.10×10^{-4}	2.90×10^{-5}	2.32×10^{-4}
	Liver	1.07×10^{-4}	7.50×10^{-5}	6.10×10^{-5}	2.65×10^{-5}	4.37×10^{-4}

Table 5 shows the hazard quotient (HQ) and hazard index (HI) of nickel in the meat parts. Hazard quotient of nickel for adult men is 3.50×10^{-3} (muscle), 3.85×10^{-3} (intestine) and 2.05×10^{-3} (liver), for adult women it is, 3.50×10^{-3} (muscle), 3.10×10^{-3} (intestine) and 2.85×10^{-3} (liver), while for adolescent it is, 3.90×10^{-3} (muscle), 3.30×10^{-3} (intestine) and 2.85×10^{-3} (liver), and for children it is, 5.25×10^{-3} (muscle), 5.15×10^{-3} (intestine) and 5.35×10^{-3} (liver). HQs of nickel in the meat parts are less than 1. This suggest that the level of nickel in the meat parts do not pose potential health risk to its consumers. HQ of lead, cobalt, cadmium and chromium in muscle, intestine and liver of the cow meat parts consumed by all the groups range as follows: from 1.45×10^{-2} to 3.90×10^{-2} for lead, from 7.66×10^{-2} to 4.63×10^{-1} for cobalt, from 3.40×10^{-3} to 9.60×10^{-3} for cadmium, and 7.00×10^{-2} to 1.45×10^{-1} for chromium. Values of the HQs are less than 1, implying non likelihood of potential health risks to the groups due to consumption of the metals in the meat parts. Likewise, hazard index (HI) values of all the metals in muscle, intestine and liver consumed by the different groups are less than 1 (1.66×10^{-1} to 6.20×10^{-1}). This suggest that chronic non-cancer health risk is not likely to happen.

The result of incremental lifetime cancer risk (ILCR) is presented in Table 6. The range of the ILCR values of the carcinogenic metals (nickel, lead, cadmium and chromium) in the meat parts, for all the groups are as follows: from 1.07×10^{-7} to 3.23×10^{-5} for nickel, from 9.15×10^{-7} to 3.87×10^{-6} for cadmium, from 1.28×10^{-5} to 7.50×10^{-5} for chromium, and from 6.2×10^{-6} to 2.78×10^{-5} for lead. These values are below and within the recommended cancer risk values, namely from 1×10^{-6} to 1×10^{-4}, stipulated by United States Environmental Protection Agency [43]. This suggest that the possibility of adult men and women, adolescent and children who consume the carcinogenic metals in muscle, intestine and liver of cows' from FCT, Abuja, Nigeria, developing cancer is low.

Table 5. Hazard quotient and Hazard index of lead, cobalt, cadmium and chromium in muscle and edible offal consumed by adult men, adult women, adolescent and children in FCT, Abuja, Nigeria

Group	Cow meat parts	Nickel	Lead	Cobalt	Cadmium	Chromium	∑HI
Adult men	Muscle	3.50×10^{-3}	1.80×10^{-2}	3.10×10^{-1}	4.60×10^{-3}	8.30×10^{-2}	4.19×10^{-1}
	Intestine	3.85×10^{-3}	2.95×10^{-2}	2.70×10^{-1}	7.30×10^{-3}	1.08×10^{-1}	4.24×10^{-1}
	Liver	2.05×10^{-3}	1.45×10^{-2}	7.66×10^{-2}	3.40×10^{-3}	7.00×10^{-2}	1.66×10^{-1}
Adult women	Muscle	3.50×10^{-3}	1.80×10^{-2}	3.10×10^{-1}	4.60×10^{-3}	8.36×10^{-2}	4.19×10^{-1}
	Intestine	3.10×10^{-3}	2.35×10^{-2}	2.20×10^{-1}	5.80×10^{-3}	8.60×10^{-2}	3.38×10^{-1}
	Liver	2.85×10^{-3}	2.00×10^{-2}	1.10×10^{-1}	4.70×10^{-3}	7.70×10^{-2}	2.15×10^{-1}
Adolescent	Muscle	3.90×10^{-3}	2.00×10^{-2}	3.43×10^{-1}	5.30×10^{-3}	9.26×10^{-2}	4.64×10^{-1}
	Intestine	3.30×10^{-3}	2.50×10^{-2}	2.36×10^{-1}	6.10×10^{-3}	9.26×10^{-2}	3.63×10^{-1}
	Liver	2.85×10^{-3}	1.95×10^{-2}	1.06×10^{-1}	4.70×10^{-3}	7.70×10^{-2}	2.10×10^{-1}
Children	Muscle	5.25×10^{-3}	2.75×10^{-2}	4.63×10^{-1}	7.20×10^{-3}	1.25×10^{-1}	6.20×10^{-1}
	Intestine	5.15×10^{-3}	3.90×10^{-2}	3.66×10^{-1}	9.60×10^{-3}	7.73×10^{-2}	4.97×10^{-1}
	Liver	5.35×10^{-3}	3.75×10^{-2}	2.03×10^{-1}	8.80×10^{-3}	1.45×10^{-1}	3.99×10^{-1}

RfD: Ni = 0.02 mg/kg/day (USEPA), Pd = 0.002 mg/kg/day (USEPA), Co = 0.0003 mg/kg/day (USEPA), Cd = 0.003 mg/kg/day (USEPA), Cr = 0.003 mg/kg/day (USEPA)

Table 6. Incremental Lifetime Cancer Risk of nickel, cobalt, cadmium and chromium in muscle and edible offal consumed by adult men, adult women, adolescent and children in Abuja, Nigeria.

Group	Cow meat parts	Nickel	Cadmium	Chromium	Lead
Adult men	Muscle	2.94×10^{-5}	2.46×10^{-6}	5.81×10^{-5}	1.69×10^{-5}
	Intestine	3.23×10^{-5}	3.87×10^{-6}	7.50×10^{-5}	2.78×10^{-5}
	Liver	1.71×10^{-5}	1.81×10^{-6}	4.86×10^{-5}	1.36×10^{-5}

(*Continued*)

Table 6. *Continuation.*

Group	Cow meat parts	Nickel	Cadmium	Chromium	Lead
Adult women	Muscle	3.09×10^{-5}	2.46×10^{-6}	5.81×10^{-5}	1.69×10^{-5}
	Intestine	2.61×10^{-5}	3.09×10^{-6}	6.01×10^{-5}	2.22×10^{-5}
	Liver	2.39×10^{-5}	2.49×10^{-6}	5.39×10^{-5}	1.88×10^{-5}
Adolescent	Muscle	2.10×10^{-5}	1.80×10^{-6}	4.11×10^{-5}	1.21×10^{-5}
	Intestine	1.61×10^{-5}	2.10×10^{-6}	4.11×10^{-5}	1.53×10^{-5}
	Liver	1.52×10^{-5}	1.58×10^{-6}	3.43×10^{-5}	1.11×10^{-5}
Children	Muscle	1.05×10^{-5}	9.16×10^{-7}	2.08×10^{-5}	6.2×10^{-6}
	Intestine	1.03×10^{-5}	1.22×10^{-6}	1.28×10^{-5}	8.8×10^{-6}
	Liver	1.07×10^{-7}	1.11×10^{-6}	2.42×10^{-5}	8.9×10^{-6}

CSF: Ni = 0.91 mg/kg/day (EPD, Georgia), 2020), Cd = 0.38 mg/kg/day (USDOE), Cr = 0.5 mg/kg/day (USDOE), Pb = 0.0083 mg/kg/day.

Conclusion

Cow muscle, intestine and liver from slaughter houses in FCT, Abuja, Nigeria are not polluted with nickel, lead, cobalt, cadmium and chromium. Human population viz adult men and women, adolescent and children are at low risk of developing potential non-cancer health related issues, chronic non-cancer health related issues, and cancer due to consumption of nickel, lead, cobalt, cadmium and chromium in cow muscle, intestine and liver from at slaughter houses within FCT, Abuja, Nigeria.

Conflict of interest

All authors report that they do not have any conflicts of interest.

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Medication-related osteonecrosis of the jaw (MRONJ)

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Summary

Introduction: Medication-related osteonecrosis of the jaw, formerly known as bisphosphonate-associated osteonecrosis of the jaw, is a syndrome first described in 2003. Prescription drugs such as bisphosphonates, denosumab and anti-angiogenic agents, including monoclonal antibodies, have been implicated in the development of medication-related osteonecrosis of the jaw (MRONJ). **Purpose:** The aim of this research was to investigate drugs related to osteonecrosis of the jaw. **Method:** This were a quantitative, cross-sectional descriptive study, using a search of scientific databases to collect information on the drugs associated with the development of drug-related osteonecrosis of the jaw. **Result:** Bisphosphonates have been associated with the development of osteonecrosis of the jaw, as they inhibit osteoclastic activity by altering bone remodeling. Denosumab was introduced as a more effective alternative to bisphosphonates; however, it has also been linked to the production of osteonecrosis of the jaw, as this drug induces a sustained decrease in bone turnover. **Conclusions:** Osteonecrosis of the jaw has been linked to some drugs, which include bisphosphonates, especially zoledronic acid. Another drug that has been implicated in osteonecrosis of the jaw is denosumab, which is used for the treatment of osteoporosis and metastatic bone disease.

Keywords: Bisphosphonate–associated osteonecrosis of the jaw; osteonecrosis; maxilla; mandible; drug effects; pharmaceutical preparation.

Resumen

Osteonecrosis de la mandíbula relacionada con medicamentos (ONMRM)

Introducción: La osteonecrosis de la mandíbula relacionada con la medicación, anteriormente conocida como osteonecrosis de la mandíbula asociada a los bisfosfonatos, es un síndrome descrito por primera vez en 2003. Medicamentos recetados como los bisfosfonatos, denosumab y agentes antiangiogénicos, incluidos los anticuerpos monoclonales, han sido implicados en el desarrollo de la osteonecrosis de la mandíbula relacionada con la medicación (MRONJ, por sus siglas en inglés). **Propósito:** El objetivo de esta investigación fue investigar los medicamentos relacionados con la osteonecrosis de la mandíbula. **Método:** Este fue un estudio descriptivo cuantitativo, transversal, utilizando una búsqueda en bases de datos científicas para recopilar información sobre los medicamentos asociados con el desarrollo de la osteonecrosis de la mandíbula relacionada con la medicación. **Resultado:** Los bisfosfonatos han sido asociados con el desarrollo de osteonecrosis de la mandíbula, ya que inhiben la actividad osteoclástica al alterar la remodelación ósea. El denosumab fue introducido como una alternativa más efectiva a los bisfosfonatos; sin embargo, también ha sido relacionado con la producción de osteonecrosis de la mandíbula, ya que este medicamento induce una disminución sostenida en el recambio óseo. **Conclusiones:** La osteonecrosis de la mandíbula ha sido relacionada con algunos medicamentos, que incluyen los bisfosfonatos, especialmente el ácido zoledrónico. Otro medicamento que ha sido implicado en la osteonecrosis de la mandíbula es el denosumab, que se utiliza para el tratamiento de la osteoporosis y la enfermedad ósea metastásica.

Palabras clave: Osteonecrosis de la mandíbula asociada a los bifosfonatos; osteonecrosis; maxilar; mandíbula; efectos de medicamentos; preparación farmacéutica.

Resumo

Osteonecrose da mandíbula relacionada a medicamentos (ONMRM)

Introdução: A osteonecrose da mandíbula relacionada a medicamentos, anteriormente conhecida como osteonecrose da mandíbula associada a bifosfonatos, é uma síndrome descrita pela primeira vez em 2003. Medicamentos prescritos como bifos-

fonatos, denosumabe e agentes antiangiogênicos, incluindo anticorpos monoclonais, têm sido implicados na desenvolvimento de osteonecrose da mandíbula relacionada a medicamentos (MRONJ, na sigla em inglês). **Objetivo:** O objetivo desta pesquisa foi investigar medicamentos relacionados à osteonecrose da mandíbula. **Método:** Este foi um estudo quantitativo, transversal e descritivo, utilizando uma busca em bases de dados científicas para coletar informações sobre medicamentos associados ao desenvolvimento de osteonecrose da mandíbula relacionada a medicamentos. **Resultado:** Os bifosfonatos têm sido associados ao desenvolvimento de osteonecrose da mandíbula, pois inibem a atividade osteoclástica alterando a remodelação óssea. O denosumab foi introduzido como uma alternativa mais eficaz aos bifosfonatos; No entanto, também tem sido relacionado com a produção de osteonecrose da mandíbula, uma vez que este medicamento induz uma diminuição sustentada da remodelação óssea. **Conclusões:** A osteonecrose da mandíbula tem sido associada a alguns medicamentos, incluindo os bifosfonatos, especialmente o ácido zoledrônico. Outro medicamento implicado na osteonecrose da mandíbula é o denosumabe, usado para tratar a osteoporose e doenças ósseas metastáticas.

Palavras-chave: Osteonecrose da mandíbula associada a bifosfonatos; osteonecrose; maxilar; mandíbula; efeitos de medicamentos; preparação farmacêutica.

Introduction

Drug-related osteonecrosis of the jaw, formerly known as bisphosphonate-associated osteonecrosis of the jaw, is a syndrome first described in 2003 by Marx; it is characterized by the exposure of necrotic bone in the maxilla or mandible and is, triggered by the performance of an intraoral surgical procedure at the same time that the patient is under medical treatment with antiresorptive drugs such as bisphosphonates [1, 2].

Since the publication of its discovery, 19 years have passed, during which little scientific information has generated misinformation and certain fears among patients and doctors or dentists. Drug-related osteonecrosis of the jaw is undoubtedly a serious disease that can lead to a radical change in the daily habits of the patient [3].

The Food and Drug Administration (FDA) approves the use of bisphosphonates for administration in postmenopausal women with osteoporosis, men with osteoporosis, and patients with glucocorticoid-induced osteoporosis, hypercalcemia of malignancy, Pagt's disease, malignancy with bone metastasis, giant cell tumors or fibrous dysplasia. However, it should be reported that the same association (FDA) does not approve the

use of these drugs for the treatment of osteogenesis imperfecta in children and adults as a prevention of glucocorticoid-induced osteoporosis [4-6].

Bisphosphonates are antiresorptive drugs, that regulate the bone metabolism of calcium (Ca) and phosphorus (P) by binding to the hydroxyapatite of the mineralized matrix, remaining in the skeleton for a prolonged period, and exerting antiresorptive activity. These drugs, in turn, are classified into two categories: those containing nitrogen, such as alendronate, risedronate, ibandronate, pamidronate and zoledronic acid, and those without nitrogen, such as etidronate disodium, clodronate disodium and tiludronate disodium [5, 7-9].

The mechanism of action of bisphosphonates is to inhibit bone resorption by binding to hydroxyapatite binding sites in bones, particularly in areas of active resorption. Those drugs that contain nitrogen will inhibit farnesyl diphosphate, which is important for promoting osteoclast binding to bone and, as a result, the osteoclast will become detached from the bone surface, which inhibits bone resorption. Non-nitrogen-containing drugs result in osteoclast apoptosis, which, in turn, leads to an overall decrease in bone degradation [7, 8, 10].

The efficacy of bisphosphonates is based on improving the bone mineral density in postmenopausal women with osteoporosis. Thus, alendronate reduces the risk of vertebral fractures by 50% and hip fractures and other non-vertebral fractures by 30%; risedronate reduces vertebral and non-vertebral fractures by 40%; zoledronic acid reduces vertebral fractures by 70% and hip fractures and other non-vertebral fractures by 35%; and ibandronate reduces vertebral fractures by 50%. It is important to point out that alendronate, risedronate and ibandronate are administered orally, while zoledronic acid and pamidronate are administered intravenously. Denosumab acts as an antiresorptive agent by inhibiting osteoclast function and bone resorption because it does not bind to bone, and its effects on bone remodeling mostly diminish within six months after the discontinuation of treatment. This drug is effective at reducing metastatic bone disease from solid tumors when administered monthly subcutaneously, as it significantly reduces the risk of vertebral, non-vertebral and hip fractures in patients with osteoporosis [8, 11-13].

Antiresorptive drug administration may cause adverse gastrointestinal effects, such as gastrointestinal reflux, esophagitis, esophageal/gastric ulcers and gastritis; intravenous infusion reactions, as they are associated with an acute phase reaction characterized by flu-like symptoms, fever, myalgia, arthralgia and headache; hypocalcemia, which is more common from intravenously administered bisphosphonates and in patients with a vitamin D deficiency, a deficient calcium intake or hypoparathyroidism; arthralgia

and myalgia; ocular conditions, such as uveitis, conjunctivitis and scleritis; atypical femur fractures due to a rare effect that generally affects the diaphysis or the subtrochanteric region of the femur; and osteonecrosis, since most cases occur in patients with multiple myeloma or breast cancer that are treated with high doses of intravenous bisphosphonates [14, 15].

Invasive dental procedures, dental trauma, periodontal disease, exodontia, exostoses and dental implants are important risk factors for the development of osteonecrosis in patients with osteoporosis, with a percentage of 0-0.15% for bisphosphonate and 1% for denosumab. In patients with a history of cancer after exodontia, the percentage is 1.6% - 14.8% when consuming bisphosphonates. Osteonecrosis of the jaw occurs three times more frequently in the lower jaw (75%) than in the upper jaw (25%) due to anatomical factors, while patients with breast cancer, multiple myeloma or prostate cancer have a higher risk of developing osteonecrosis [11, 16, 17].

Patients receiving chemotherapy or corticosteroids, older adults, and Caucasians are at a greater risk of developing osteonecrosis; in addition, genetic factors (such as CYP2C8 gene polymorphism in patients with multiple myeloma who receive treatment with zoledronic acid and pamidronate) and smoking are risk factors for the development of osteonecrosis with bisphosphonate therapy [18].

There are several hypotheses that can explain the pathophysiology of drug-associated osteonecrosis. Among them is the inhibition of bone remodeling, since antiresorptive drugs, including bisphosphonates and denosumab (DMB), have direct effects on the formation, differentiation or function of osteoclasts; inflammation or infection, since, although extraction is the main triggering event for osteonecrosis, most teeth that are extracted have periodontal disease or an infection; the inhibition of angiogenesis, since bisphosphonates and denosumab inhibit the angiogenesis that is normally seen in post-extraction alveolar healing, and they also reduce the arterial area, venous area and general vascularization of periodontal tissues; dysfunction of the innate and acquired immune systems, as patients with comorbidities such as diabetes and rheumatoid arthritis or an immunocompromised state have a significantly increased risk of drug-associated osteonecrosis, as confirmed in animal studies where chemotherapy, steroids and antirheumatic drugs combined with antiangiogenic and antiresorptive drugs increased the prevalence of drug-associated osteonecrosis; and, finally, genetic factors, since there are some polymorphisms that are associated with the development of osteonecrosis, with most of these polymorphisms being located in genes associated with bone turnover and collagen formation [11, 19-21].

The American Association of Oral and Maxillofacial Surgeons states that patients are considered to have drug-related osteonecrosis of the jaw if they have certain specific characteristics, as can be seen in Table 1 [7, 8].

There is also drug-related, or spontaneous, mandibular necrosis without a predisposing event, which represents the second most common group responsible for triggering mandibular osteonecrosis. Spontaneous medication-related osteonecrosis of the jaw (MRONJ) is related to certain anatomical sites such as the torus, exostosis, and mylohyoid ridges. On the other hand, a bony protrusion contradicts the term spontaneous, as it represents a higher risk of trauma [11-13].

Drug-associated osteonecrosis of the jaw occurs and is classified according to the conditions present, which is why it can be classified into four stages, as can be seen in Table 2 [22-24].

Table 1. Characteristics required to consider a patient to have drug-associated osteonecrosis of the mandible.

Characteristics to be considered according to AAOMS	Current or previous treatment with antiresorptive or anti-angiogenic agents.
	Exposed bone or bone that can be probed by an extrabuccal or intrabuccal fistula, that is located in the maxillofacial region, and that has been maintained for more than eight weeks.
	No history of radiation to the jaws or metastases in the jaws.

Table 2. Stages of drug-associated osteonecrosis of the mandible.

Stage 0	Absence of necrotic bone, without bone exposure, but with specific clinical or radiographic findings. - SYMPTOMS Odontalgia. Pain in the mandibula that may radiate to the TMJ. Sinus pain. - CLINICAL FINDINGS Tooth mobility due to chronic periodontal disease. Intraoral or extraoral inflammation. - RADIOLOGICAL FINDINGS Bone loss not attributable to chronic periodontal disease. Absence of new bone in the alveoli post-extraction. Regions of osteonecrosis involving alveolar bone.

(*Continued*)

Table 2. *Continuation.*

Stage 1	Bone exposure and necrotic bone tissue or fistula where bone can be explored by probing; asymptomatic patients with the absence of an infection.
Stage 2	Infection, pain and/or erythema with necrotic bone exposure or fistula when probing bone.
Stage 3	Extension of necrotic bone and infection beyond the alveolar ridge, pathological fracture or oral-antral/oral-nasal communication, or an extraoral fistula.

The management of patients on bisphosphonate therapy prior to oral surgery should be performed under a protocol that includes asking all patients about their current or past bisphosphonate use and mode of administration, because intravenous bisphosphonates have a longer half-life and therefore pose a greater risk of the patient developing osteonecrosis than oral bisphosphonates. Patients who have not yet begun bisphosphonate therapy should first be examined to determine whether they require surgical dental procedures prior to therapy. A comprehensive treatment should be performed to minimize the need for future dental treatment. For patients who have already started therapy, any elective procedures should be avoided, if possible, to avoid the risk of bisphosphonate-induced osteonecrosis of the jaw. Root canal therapy should be performed in lieu of tooth extraction when possible. Patients should be educated about the importance of good oral hygiene, regular dental checkups and the symptoms of osteonecrosis of the jaw so that the patient can report early if symptoms develop. Patients in whom extractions are unavoidable should first consult with the treating physician to discontinue bisphosphonate therapy temporarily. The patient should be maintained on a chlorhexidine mouth rinse twice daily for two months and should be followed up postoperatively for 2 months [16, 25].

The most important therapeutic method is the prevention of drug-related osteonecrosis of the jaw by performing dental extractions or surgical interventions prior to the administration of antiresorptive and anti-angiogenic drugs. Prolonged perioperative antibiotic prophylaxis, adequate wound closure, and atraumatic procedures are important during dental treatment, as they minimize the risk of drug-related osteonecrosis of the jaw [26-31].

Asymptomatic lesions can be treated with antibacterial mouth rinses and prolonged antibiotic therapy. In cases where the patient presents with less severe symptoms, they can be treated by the removal of necrotic bone tissue, the smoothing of sharp bony edges, adequate wound closure and postoperative antibiotics. Those with symptomatic lesions are treated using a surgical approach, which may include a partial mandibulectomy

followed by bridging with reconstruction plates or a reconstruction with vascularized bone grafting in compromised patients [32-34].

The C-terminal telopeptide cross-linking (CTX) assay is used as a monitoring method in patients undergoing antiresorptive therapy, using a biological index (pg/ml) to measure bone remodeling and resorption as a parameter of osteoclastic activity. As bone resorption proceeds, osteoclastic enzymes digest the organic bone matrix and release type I collagen products, which include C-terminal telopeptide fragments of type I collagen called CTX and structural rings called pyridinoline crosslinks. During normal bone metabolism, mature type 1 collagen is degraded, and small fragments pass into the blood and are excreted through the kidneys. As a control, it has been suggested to measure CTX before starting bisphosphonate therapy, since the level will be reduced by 60% at 6 weeks when using the conventional dose. Whenever there is more rapid bone turnover, the CTX level will be higher, as in Paget's disease, but if the bone resorption rate is low, the CTX will also be low. It has been established that CTX values below 100 pg/ml represent a high risk of OMIB, values between 100 and 150pg/ml represent a moderate risk and values above 150 pg/ml represent minimal or no risk. CTX has been used in medicine to measure the bone turnover in diseases such as osteoporosis and bone metastases in response to bisphosphonates and is used as a parameter to assess the efficacy of oral bisphosphonate therapy [35].

However, since the 2014 American Association of Oral and Maxillofacial Surgery (AAOMS) paper, there has been a move away from bone turnover markers, so there are no validated biomarkers for clinical decision making and a test cannot be considered as a tool to estimate the ONJ risk [11].

For the performance of a surgical procedure on a patient that has been administered denosumab, a dentoalveolar surgery should be performed 3-4 months after the last dose, when the level of osteoclast inhibition is decreasing, and then the dose should be restored 6-8 weeks after surgery [36, 37].

For the management of patients who have already developed osteonecrosis due to medication, a panoramic radiograph is recommended to determine the extent of the necrosis and the position of the sequestration or osteomyelitis. Microbial cultures of soft tissue inflammation or the associated purulent discharge should be performed to identify any superinfection and the appropriate antimicrobial therapy; any dental trauma should be avoided, as it may further delay wound healing, and it should also be properly classified so that the appropriate treatment can be performed [16, 38, 39].

The treating physician may consider the non-invasive management of osteonecrosis of the jaw, initially with the administration of FDA-approved pentoxifylline for the treat-

ment of peripheral arterial disease, such as ischemic heart disease, since pentoxifylline has a mechanism of action that improves the peripheral blood flow by increasing vasodilatation, reducing blood viscosity, improving the arrival of erythrocytes, inducing effects against tumor necrosis factor alpha, inhibiting inflammation and decreasing fibrosis [40, 41].

This research focuses on describing the medications that produce mandibular osteonecrosis and the way in which these medications act on the bone tissue of each person, since it is very important for the dentist to know the side effects of these medications in order to look for alternatives when performing dental treatments.

Methods

A quantitative, cross-sectional, descriptive study was conducted by searching scientific databases for information on drugs associated with the development of drug-related osteonecrosis of the jaw. A literature search was carried out in databases and documentaries such as Pubmed, Scielo and Google Scholar; Boolean operators such as "AND", "OR" and "NOT" were applied with the keywords "bisphosphonate-associated osteonecrosis of the jaws", "osteonecrosis", "maxilla", "mandible", "drug effects" and "pharmaceutical preparation". In the bibliographic search, 127 articles were found, to which some inclusion criteria were applied, including the following: published between 2006 and 2023 and related to drugs that cause osteonecrosis of the jaw. Some bibliographies were discarded using exclusion criteria such as articles published earlier than 2014 and publications that did not contribute significant information to our search. This resulted in 103 articles of interest to us, which were classified by their year of publication (Figure 1).

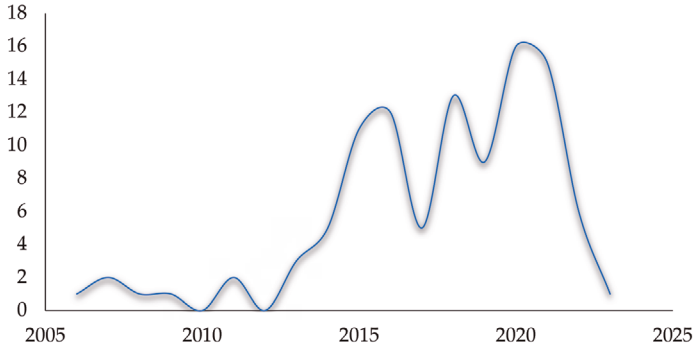

Figure 1. Analysis of study articles by year of publication.

Table 3. Other drugs associated with the production of osteonecrosis of the jaw other than the bisphosphonate family of drugs.

Group	Feature	Applications	Osteonecrosis	References
Denosumab	Introduced with the hope of decreasing the side effects of bisphosphonates; however, it has also been implicated in the production of osteonecrosis of the jaw. Suppresses bone resorption and osteoclast function. Alters bone turnover by directly altering osteoclasts. Its efficacy is similar to that of bisphosphonates. Strong blockade of osteoclastic activity may lead to maxillary and mandibular osteonecrosis. By blocking RANKL, it decreases the osteoblastic effect on osteoclasts and osteoclast progenitors and prevents their activation and differentiation, respectively. There is an increased risk of osteonecrosis of the jaw with denosumab compared to bisphosphonates. Decreases the resorption and destruction of bone tissue induced by tumor cells, thereby increasing the level of bone density and preventing future fractures. Enters the body subcutaneously or orally. Suppresses osteoclast formation by acting on preosteoclasts in the extracellular environment, as compared to bisphosphonates that act on active mature osteoclasts. Improves bone quality, strength, geometry and mineral density.	Used to combat osteoporosis and bone metastases. Prescribed in orthopedics, rheumatology and oncology. Prevents osteoclast-mediated bone resorption. Used for skeletal complications in metastatic bone lesions and to reduce vertebral and hip fractures in patients suffering from osteoporosis. Used for the treatment of multiple myeloma.	Yes	[42-73]

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Table 3. *Continuation.*

Group	Feature	Applications	Osteonecrosis	References
Bevacizumab	Decreases the action of vascular endothelial development component inhibitors and causes a reduction in angiogenesis.	Used to combat the metastasis of solid tumors, osteoporosis and multiple myeloma. It has been used in several types of cancer.		[74-76]
Pembrolizumab + denosumab	PEMBROLIZUMAB: A humanized monoclonal antibody targeting the programmed cell death protein. Bone tissue is the third most recurrent site of metastasis for a wide range of solid tumors, including prostate cancer, breast cancer, lung cancer and myeloma. DENOSUMAB: Reduces osteoclast function, formation and survival as an anti-osteoclastogenic agent.	The standard treatment in people with metastatic non-small cell lung cancer, as it increases progression-free preservation and resistance in these patients. Prevents skeletal-related events in advanced solid tumors with bone metastases.	Yes	[77]
Lenvatinib	Interacts with receptor tyrosine kinases (RTKs) involved in vascular endothelial growth (e.g., VEGFR1, VEGFR2 and VEGFR3), as well as other RTKs, including fibroblast development component receptor alpha, platelet-derived development component receptor alpha, RET and KIT. These RTKs are responsible for pathogenic angiogenesis, cancer progression and tumor growth.	Given to fight thyroid cancer that has returned or metastasized to other parts of the body.	Yes	[78]
Tyrosine kinase inhibitors (sunitinib, rafenib, pazopanib, axitinib, imatinib and regorafenib)	Linked to the development of osteonecrosis of the jaw. Limits the function of tyrosine kinase via various receptors, such as the vascular endothelial growth factor receptor.	Intervenes in the function of the cell surface receptors, which are known to be abnormally active in tumors.	Yes	[76, 79]

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Table 3. *Continuation.*

Group	Feature	Applications	Osteonecrosis	References
Monoclonal antibodies (rituximab, adalimumab, infliximab and romosozumab)	Reported to be possibly related to osteonecrosis of the jaw, along with denosumab and bevacizumab.	A rituximab injection (Rituxan) is used to fight pemphigus vulgaris. Adalimumab blocks the action of TNF (a substance in the body that causes inflammation). Infliximab is designed to bind to a chemical messenger in the body known as "tumor necrosis factor alpha". It is responsible for initiating the inflammatory process, occurring at increased levels in patients routinely treated with Remicade. Romosozumab increases bone formation and decreases bone breakdown.	Yes	[76]
mTOR inhibitors (everolimus and temsirolimus) and immunosuppressants (methotrexate and corticosteroids)	Linked to the development of osteonecrosis of the jaw; however, this event is extremely rare.	Given to combat rheumatoid arthritis, as a prophylaxis for transplant rejection and for the treatment of malignant diseases.	Yes	[76]
Imatinib	Works by blocking the action of the abnormal protein that tells cancer cells to multiply. This stops the spread of cancer cells.	Used to treat gastrointestinal stromal tumors, chronic myeloid leukemia and other cancers.	Yes	[76]

(*Continued*)

Table 3. *Continuation.*

Group	Feature	Applications	Osteonecrosis	References
Anti-angiogenic drugs	Anti-angiogenic drugs; i.e., they inhibit the formation of blood vessels.	Developed and supplied for various antineoplastic treatments (including treatments for ovarian cancer and glioblastoma, breast cancer, lung cancer, renal cancer and colorectal cancer).	Yes	[80]
Sunitinib	Blocks the growth of tumor cells. Intervenes in tumor cell division. Blocks the VEGF receptor, which is responsible for supplying blood to the tumor. Eliminates sources of nutrients to the tumor. Inhibits angiogenesis by preventing the formation of blood vessels.	Used for the treatment of gastrointestinal stromal cancer.	Yes	[71, 73, 77]
Everolimus	An immunosuppressive drug that inhibits the mTOR complex.	Used for advanced stages of kidney cancer, and also used to prevent rejection in transplants.	Yes	[72, 73]
Inhibitors of the mammalian target of rapamycin (mTOR)	A new therapy for the treatment of metastatic renal carcinoma.	Used for the treatment of metastatic renal carcinoma.	Yes	[80]
Aflibercept	Antiangiogenic drug with a different mechanism of action than bevacizumab, with the latter being a vascular endothelial growth factor receptor.	Used for the treatment of advanced colorectal cancer.	Yes	[73]
Thalidomide	This drug and its analogues (pomalidomide and lenalidomide) have an anti-angiogenic effect.	Used for treatment in patients with myeloma, who are largely receiving oral bisphosphonates and therefore at risk for MRONJ.	Yes	[73]

Table 4. Drugs related to osteonecrosis of the jaw according to the family to which they belong.

Group	Feature	Applications	Osteonecrosis	References
Bisphosphonates	Decreases bone turnover. Inhibits osteoclasts and disrupts normal bone healing. Strong inhibition of bone resorption may induce drug-associated osteonecrosis of the jaw. Induces apoptosis in osteoclasts. Increases bone density; inhibits osteoclastogenesis by interfering with prostaglandin and interleukin-6 secretion by osteoblasts. Reduces the risk of fractures and improves quality of life. Has been used as a tool to prevent and reduce bone complications caused by bone metastasis. Reduces bone resorption through intracellular effects. Believed to reduce osteolytic defects. Has a biological life of ten years. In osteoclastic resorption, bisphosphonates are internalized through pinocytosis in osteoclasts; the inhibition of the enzyme farnesyl-pyrophosphate synthase leads to reduced cellular activity, differentiation and apoptosis, depending on the dose of bisphosphonate applied. In the presence of these drugs, there is evidence of delayed healing after a surgical procedure. Drug-associated osteonecrosis of the jaw is the main side effect. Inhibits osteogenic cells, endothelial cells, human fibroblasts and macrophages, and decreases the viability of oral keratinocytes. Regulates the processing of phosphorus and calcium in bone tissue by binding to hydroxyapatite and reducing its resorption by osteoclasts.	Used to prevent osteoporosis and osteopenia and as a treatment for skeletal metastases (especially in lung cancer, breast cancer and prostate cancer), hypercalcemia associated with malignancies, osteogenesis imperfecta, Paget's disease and multiple myeloma. Used for the prevention of bone complications (pathological fractures or spinal cord compression). Helps prevent bone metastases from some types of carcinomas and multiple myeloma. As injectables, they are used to stabilize osteolysis of bone metastases from malignant tumors and improve malignancy-induced hypercalcemia, while oral bisphosphonates are mainly used to treat osteoporosis, and have also been reported to reduce the risk of fracture.	Yes	[44-49, 51, 52, 54-58, 60-64, 66, 68, 70-73, 76-78, 81-90]

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Table 4. *Continuation.*

Group	Feature	Applications	Osteonecrosis	References
	The half-life of bisphosphonates in circulation is considerably short, ranging from thirty minutes to two hours; however, once incorporated into bone, they can persist for more than 10 years.			
Pamidronate and zoledronate	Have demonstrated characteristics of inducing apoptosis and the death of osteoclasts.	Used to treat elevated blood calcium concentrations, which can be attributed to certain types of cancer. Used for the treatment of bone metastases.	Yes	[91]
Intravenous bisphosphonates	Impedes the physiological activity of osteoclasts. Limits bone resorption.	This drug is used for hypercalcemia related to malignant diseases, and it is also used in the treatment of osteolytic lesions and skeletal complications in patients with osteoporosis and osteopathic cancer.	Yes	[92]
Zoledronic acid	Has a good affinity for hydroxyapatite in bone tissue and reduces osteoclastic bone resorption. Has shown greater potency than other bisphosphonates. The poor vascularization caused by the impact of antiresorptive drugs in inhibiting physiological bone tissue remodeling appears to be essential for the development of necrotic bone tissue. Produces soft tissue toxicity and inhibits angiogenesis and immune dysfunction. The decrease in the creation of new vascularization networks impairs reconstruction after surgery. Has a long half-life. IV doses per year of zoledronic acid have a greater effect on bone remodeling compared to oral antiresorptive drugs. Improves bone mineral density and decreases the prevalence of hip fractures, non-vertebral fractures and vertebral fractures.	Used in the treatment of bone metastases. An important option in the treatment of osteoporosis and as a supportive therapy for bone metastatic malignancies by controlling bone resorption.	Yes	[47, 93-99]

Results

Once the information had been analyzed, in Table 3 we describe the mechanism of action of these drugs on the bone and the way in which they affect and produce osteonecrosis, and in turn, in Table 4 we proceeded to classify the drugs related to osteonecrosis of the jaw according to the family to which they belonged.

Osteonecrosis of the jaw has been linked to certain drugs, with bisphosphonates being the most commonly linked to the production of osteonecrosis of the jaw, as these drugs inhibit osteoclastic activity by altering bone remodeling. Denosumab was introduced as a more effective alternative to bisphosphonates. By introducing this drug, it was hoped that it would reduce the side effects; however, it has also been linked to the production of osteonecrosis of the jaw, as this drug induces a sustained decrease in bone turnover. With less scientific evidence, other drugs have been shown to be involved in the production of osteonecrosis of the jaw, including: Lenvatinib, tyrosine kinase inhibitors, monoclonal antibodies, mTOR inhibitors and immunosuppressants, imatinib, sunitinib, everolimus, temsirolimus, antiangiogenic drugs (bevacizumab, aflibercept), mammalian target of rapamycin limiters, aflibercept and thalidomide. These drugs have been used to treat certain malignancies by acting as inhibitors of vascular tissue construction and impeding the differentiation cascade in angiogenesis.

Discussion

Based on the data collected, drug-associated osteonecrosis of the jaw is increasing due to the frequent use of bisphosphonates, and the AAOMS recommends prior drug restriction for three months before dental procedures. Considering the half-life of these drugs, when treatments with bisphosphonates are prescribed for prolonged periods of time, it is essential to maintain constant vigilance to rule out adverse effects. Once the drug enters the body, it penetrates directly into the bone, accumulating in areas of increased resorption. When osteoclasts begin to act in areas with bisphosphonates, they enter and inhibit their activity, promoting their apoptosis and producing antiresorptive effects, which can be used in a variety of clinical situations: tumor hypercalcemia, Paget's disease, osteogenesis imperfecta, bone metastasis and osteoporosis. Long-term treatment with bisphosphonates inhibits bone remodeling, which is essential for repairing physiologically occurring micro-injuries, causing them to persist and resulting in hypo-dynamic and avascular bones.

Walton *et al.* [100] mentioned in 2019 that there are significant differences in the clinical appearance of drug-related osteonecrosis of the jaw depending on the underly-

ing primary disease condition in less advanced cancer patients, along with a tendency towards the dentate areas of the mandible. Radiographic findings often increase in parallel with the clinical staging, but not unequivocally, confirming that imaging provides additional information on the bone disease burden that the current clinical staging system does not provide. Klingelhöffer *et al.* [101] demonstrated that, in alveolar bone dynamics, there is a greater range of bone remodeling when compared to the different bone tissues of the appendicular or axial human body, which explains the incidence of necrosis in the maxillary bones.

Matsuda *et al.* [102] conducted a multicenter observational study on drug-induced osteonecrosis of the jaw in patients with advanced cancer and myeloma in northwest Italy in 2020 and stated that the primary diseases were breast cancer (46%), prostate cancer (21%), multiple myeloma (19%) and other types of carcinomas (14%). In the same study, they mentioned that dental treatment prior to the administration of intravenous bisphosphonates helps to reduce the prevalence of drug-associated osteonecrosis of the jaw. Ogawa *et al.* [103] stated in 2021 that dental extraction and local hard and soft tissue trauma are the main factors and triggers for the development of maxillary and mandibular osteonecrosis, and that these triggers, in addition to antiresorptive or antiangiogenic treatments, are responsible for producing bone necrosis of the jaws. However, in some cases, it has not been possible to identify a relationship to a specific triggering event. Blus *et al.* [104] stated in 2016 that bone remodeling is significantly delayed in the presence of bisphosphonates and antiresorptive agents, as they suppress normal bone turnover. Therefore, in the presence of certain drugs, a delay in healing is observed after a surgical procedure that alters tissue homeostasis and bone remodeling. Such procedures are considered a precipitating or triggering event for osteonecrosis of the jaw. Dental extractions have been identified as the triggering factor in 60-87% of cases. To further reiterate this, a study on ONJ patients with bone neoplasms reported that tooth extractions are the main trigger in 73% of cases. In addition to wound healing complications such as bleeding, postoperative infection and postoperative pain, certain medications, when administered, can alter the physiological phases of healing, e.g., anti-angiogenic and antiresorptive agents. Therefore, in the field of dentistry/oral surgery, dental extractions and dentoalveolar surgical procedures should be well planned, especially in patients receiving bisphosphonates and other antiresorptive drugs.

Hallmer *et al.* [49] mentioned in 2020 that the pathophysiology of drug-associated osteonecrosis of the jaw is still debated, with related causes including infection, inflammation, the impairment of bone tissue remodeling and resorption, and the inhibition of angiogenesis. Related factors for the development of drug-associated osteonecrosis of the jaw include the length of time the patient is on antiresorptive therapy. Local fac-

tors include tooth extractions and periodontal and dentoalveolar surgeries. An investigation in southern Sweden showed that the prevalence of mandibular and maxillary osteonecrosis associated with intravenous bisphosphonates was 2.8%. Over the course of time and with the implementation of antiresorptive drugs and the introduction of denosumab treatment, it has become necessary to study the predisposing factors for the development of drug-associated osteonecrosis of the jaw. On the other hand, Ikesue *et al.* [91] mentioned in 2021 that various factors have been suggested as predisposing factors for the development of osteonecrosis, including the factor of drugs; however, the drugs that have been most associated with osteonecrosis of the jaw are zoledronic acid and denosumab, although their mechanisms of action are different. Zoledronic acid has a greater affinity for the hydroxyapatite in bone tissue, which is why this drug is used to combat bone metastasis. In contrast, denosumab is considered a humanized monoclonal antibody with a high affinity for the RANKL ligand of nuclear factor B (NFkB). Another characteristic that differentiates them is the time of the effect on the bone, since zoledronic acid has a greater effect on the bone than denosumab, which has a temporary effect on the bone.

Dupic *et al.* [66] stated in 2015 that bisphosphonates are drugs used to treat malignant diseases, to prevent pathological fractures and spinal cord compression and to treat hypercalcemia, as well as to treat benign diseases such as Paget's disease and osteoporosis. Bisphosphonates have been implicated in the production of osteonecrosis of the jaw, with a prevalence of between 0 and 27.5% and an estimated 7% when administered via the venous route. In oncology patients, the prevalence increases, ranging from 1 to 15%, and in the case of administration via the venous route, it may increase even more, depending on the type of cancer. Denosumab has also been implicated in the production of osteonecrosis of the jaw. Similarly, Wasserzug *et al.* [61] stated in 2017 that bisphosphonates are antiresorptive drugs and that they prevent the resorption of trabeculated bone tissue by osteoclasts, which will lead to increased bone density. The most important effect of bisphosphonates is drug-associated osteonecrosis of the jaw. Two very important predisposing factors for osteonecrosis of the jaw are the route of drug administration and the cumulative dose of the drug. Studies have shown that there is a higher incidence of osteonecrosis of the jaw when the drug is administered intravenously rather than orally (0.88 to 1.15% versus 0.01 to 0.04%), and the time in which patients presented with osteonecrosis of the jaw was shorter in those treated with intravenous bisphosphonates.

Maluf *et al.* [63] mentioned in their 2016 research that denosumab has a different mechanism of action to bisphosphonates, mentioning that bisphosphonates inter-

vene in mature, active osteoclasts while denosumab acts on osteoclasts by inhibiting RANKL, thus preventing their function, differentiation and formation, and above all, intervening in bone resorption. Among the most important side effects of bisphosphonates is osteonecrosis of the jaw, which has a prevalence ranging from 0.9 to 5%; osteonecrosis of the jaw associated with denosumab is similar to that associated with bisphosphonates, ranging from 1 to 2%. Similarly, Voss *et al.* [62] noted in 2017 that the prevalence of drug-induced osteonecrosis of the jaw ranges from 0.001 to 1% in drugs prescribed for the treatment of osteoporosis and up to 20% in patients undergoing treatment for malignant pathologies.

Conclusion

The drugs that are related to adverse effects such as osteonecrosis are mainly bisphosphonates, especially zoledronic acid. Another drug that has been linked to osteonecrosis of the jaw is denosumab, which is used for the treatment of osteoporosis and metastatic bone disease. With less scientific evidence, other drugs that are also associated with osteonecrosis of the jaw have been described, including: Lenvatinib, tyrosine kinase inhibitors, monoclonal antibodies, mTOR inhibitors and immunosuppressants, imatinib, sunitinib, everolimus, temsirolimus, anti-angiogenic drugs (bevacizumab, aflibercept), mammalian target of rapamycin inhibitors, aflibercept and thalidomide. The severity of bone pathology will depend on the route of administration (oral or parenteral) and the dose accumulated in the body. As this is an extremely important topic in the field of dentistry, it is essential to keep up-to-date and to be aware of the drugs that affect physiological bone turnover in the jaw.

Compliance with ethical standards

Conflict of interest: The authors declare that they have no conflict of interest.

Ethical approval: This article does not contain any studies with human participants or animals performed by any of the authors.

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Quitosano derivado de quitina de langosta: actividad antioxidante y antimicrobiana

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Resumen

Introducción: El quitosano posee propiedades como antimicrobiana y antioxidante, que lo convierten en un compuesto interesante para diversas aplicaciones. **Objetivo**: Evaluar la actividad antioxidante y antimicrobina del quitosano, derivado de quitina de langosta (*Panulirus aurgus*), y el acetato de quitosano. **Métodos**: Se determinó la capacidad antioxidante de disoluciones de quitosano usando los métodos de capacidad reductora del hierro férrico (FRAP) y la capacidad secuestradora del radical (DPPH). Se evaluó la influencia de la concentración (0,5, 1,0 y 2,0 % (m/v)) del polímero y su sal de acetato en la actividad antimicrobiana. Las concentraciones

mínimas inhibitorias y microbicidas se determinaron a través del método de las diluciones seriadas y la actividad antimicrobiana por el de difusión en agar. Se utilizaron cepas atenuadas de microorganismos Gram positivos, Gram negativos, hongos y levaduras. **Resultados**: Las disoluciones de quitosano mostraron actividad antioxidante moderada, siendo mayor para el acetato de quitosano. La actividad antimicrobiana aumentó con el incremento de la concentración del biopolímero y su sal. Tanto el quitosano, como su sal de acetato, presentaron un efecto inhibitorio sobre los microorganismos Gram positivos y Gram negativos, así como los hongos. **Conclusiones:** Se demuestran las potencialidades de ambas materias primas para su empleo en productos farmacéuticos y cosméticos.

Palabras claves: acetato de quitosano, actividad antimicrobiana, actividad antioxidante, quitosano.

Summary

Chitosan derived from lobster chitin: antioxidant and antimicrobial activity

Introduction: Chitosan has antimicrobial and antioxidant properties, which make it an interesting compound for various applications. **Aim**: To evaluate the antioxidant and antimicrobial activity of chitosan, derived from lobster chitin (*Panulirus aurgus*), and chitosan acetate. **Methods**: The antioxidant capacity of chitosan solutions was determined using the ferric iron reducing capacity (FRAP) and radical scavenging capacity (DPPH) methods. The influence of the concentration (0.5, 1.0 and 2.0% (w/v)) of the polymer and its acetate salt on the antimicrobial activity was evaluated. The minimum inhibitory and microbicidal concentrations were determined through the serial dilution method and the antimicrobial activity by agar diffusion. Attenuated strains of Gram positive and Gram negative microorganisms, fungi and yeasts were used. **Results**: Chitosan solutions showed moderate antioxidant activity, being higher for chitosan acetate. The antimicrobial activity increased with increasing concentration of the biopolymer and its salt. Both chitosan and its acetate salt had an inhibitory effect on Gram positive and Gram negative microorganisms, as well as fungi. **Conclusions**: The potential of both raw materials for their use in pharmaceutical and cosmetic products is demonstrated.

Keywords: antimicrobial activity, antioxidant activity chitosan acetate, chitosan.

Resumo

Quitosana derivada de quitina de lagosta: atividade antioxidante e antimicrobiana

Introdução: A quitosana possui propriedades antimicrobianas e antioxidantes, o que a torna um composto interessante para diversas aplicações. **Objetivo:** Avaliar a atividade antioxidante e antimicrobiana da quitosana, derivada da quitina da lagosta (Panulirus aurgus), e do acetato de quitosana. **Métodos:** A capacidade antioxidante das soluções de quitosana foi determinada utilizando os métodos de capacidade redutora de ferro férrico (FRAP) e capacidade de eliminação de radicais (DPPH). Foi avaliada a influência da concentração (0,5, 1,0 e 2,0% (m/v)) do polímero e seu sal acetato na atividade antimicrobiana. As concentrações inibitórias e microbicidas mínimas foram determinadas através do método de diluição seriada e a atividade antimicrobiana por difusão em ágar. Foram utilizadas cepas atenuadas de microrganismos Gram positivos e Gram negativos, fungos e leveduras. **Resultados:** As soluções de quitosana apresentaram atividade antioxidante moderada, sendo maior para o acetato de quitosana. A atividade antimicrobiana aumentou com o aumento da concentração do biopolímero e seu sal. Tanto a quitosana quanto seu sal acetato tiveram efeito inibitório sobre microrganismos Gram positivos e Gram negativos, bem como fungos. **Conclusões:** Está demonstrado o potencial de ambas as matérias-primas para a sua utilização em produtos farmacêuticos e cosméticos.

Palavras-chave: acetato de quitosana, atividade antimicrobiana, atividade antioxidante, quitosana.

Introducción

El quitosano, obtenido por desacetilación de la quitina, es un polímero con estructura de hélice con grupos amino reactivos, lo que ofrece muchas posibilidades de modificación e interacciones iónicas. Se caracteriza por ser biodegradable, biocompatible y de baja toxicidad [1].

Posee diferentes propiedades bioactivas, como las antimicrobianas, antiinflamatorias, tensoactivas, antitumorales, mucoadhesivas, cicatrizantes y antioxidantes, que lo convierten en un compuesto interesante para aplicaciones en diferentes campos, como la industria alimentaria, farmacéutica, cosmética, la agricultura, la medicina, la industria textil y papelera, la química y el medio ambiente [1, 2].

El quitosano y sus oligómeros actúan como antioxidantes por el secuestro de radicales de oxígeno. Esta actividad depende tanto de su masa molecular (Mm) como de su grado de desacetilación (GD). Los oligómeros de baja Mm y alto GD tienen mayor actividad antioxidante que los de alta Mm. Los grupos hidroxilos y aminos activos en el quitosano pueden reaccionar con los radicales libres, pues en su estructura posee tres fuentes de hidrógeno libres en las posiciones C-2(NH_2), C-3(OH) y C-6(OH), respectivamente [1, 3].

El quitosano obtenido de la desacetilación de la quitina es un antioxidante aún más potente. Se ha descrito que la actividad antioxidante del quitosano aumentó en 2,5 veces cuando el tiempo de desacetilación se incrementó de 60 a 120 min. Esto demuestra que su actividad antioxidante está correlacionada con el GD. Las aminas suelen ser antioxidantes más potentes que las amidas [4].

Aunque los mecanismos de acción antioxidante de las moléculas de quitosano aún no están del todo claros, entre algunas razones, se justifica por su capacidad para formar complejos con muchos metales de transición. Las cadenas de quitosano son capaces de retrasar la oxidación lipídica mediante la quelación de iones de Fe^{2+} a través de sus grupos amino o hidroxilos, inhibiendo así la capacidad oxidante de este metal o su conversión en iones Fe^{3+} [4, 5].

La presencia de los grupos aminos (-NH_2) está intrínsecamente ligada a la actividad antioxidante del quitosano. La disponibilidad de un par de electrones libres en este grupo funcional da lugar a la captación de protones H^+ desde el medio, los cuales, posteriormente son cedidos a los radicales libres que forman parte de la cadena de reacciones que constituyen la degradación oxidativa, formando moléculas más estables y evitando la concreción de dicho fenómeno. En quitosanos de elevada Mm, la actividad antioxidante se relaciona, de manera más representativa, a un efecto quelante de metales pro-oxidantes. De este modo, puede considerarse que actúa como antioxidante tipo I y tipo II [4].

Este biopolímero ha demostrado ser antifúngico ante un amplio espectro de patógenos, provocando la inhibición total o parcial de estos según la especie fúngica, considerándose que existe una posible dependencia entre el grado de polimerización y de desacetilación del quitosano y el nivel de inhibición que provoca [1].

Además, posee carácter antimicrobiano frente a bacterias Gram positivas (*S. aureus*, *S. epidermidis*, *B. subtilis*) y Gram negativas (*P. aeruginosa*, *E. coli*, *Klebsiela Pneumoniae*, *Proteus vulgaris*) [6, 7].

La langosta es la fuente natural menos empleada internacionalmente, sin embargo, en Cuba existen plantas procesadoras de este crustáceo, empleándose como fuente de obtención de este biopolímero, y sus sales, lográndose procesos amigables con el medio ambiente [8, 9].

Existen evidencias de la actividad antioxidante de disoluciones de acetato y de lactato de quitosano, así como de su actividad antimicrobiana y antifúngica [10-13]. En sentido general, se sugiere su uso seguro por no presentar toxicidad (aguda y crónica), ser un producto no irritante para la piel y los ojos, no carcinógeno, no tóxico para la reproducción y con actividad antigenotóxica [14].

A pesar de las múltiples aplicaciones del quitosano y sus derivados en el campo biomédico y farmacéutico, la diversidad de fuentes y procesos de obtención, la caracterización incompleta y variabilidad de los quitosanos comerciales, aún no se encuentra aprobada por la *Food and Drug Administration* (*FDA,* por sus siglas en inglés).

Teniendo en cuenta la necesidad de profundizar en las propiedades del biopolímero derivado de quitina de langosta (*Panulirus aurgus*), que permitan el desarrollo de productos farmacéuticos y cosméticos seguros, en el presente trabajo se evaluó la actividad antioxidante y antimicrobiana del quitosano y el acetato de quitosano.

Materiales y métodos

Reactivos químicos

Como ingredientes activos se emplearon quitosano y acetato de quitosano, suministrados por la Planta de Producción de Productos Naturales y Sintéticos del Centro de Investigación y Desarrollo de Medicamentos (CIDEM, Cuba). Se emplearon tres lotes de cada uno, elaborados según los procedimientos descritos para el quitosano y el acetato de quitosano, los que cumplieron con los parámetros físicos y químicos establecidos [8, 9]. La masa molecular del quitosano fue de 310 000 g/mol y el grado de desacetilación fue de 77,63 % (Lote 10001), 79,63 % (Lote 11001) y para el lote 11002 de 77,86 %. El quitosano comercial empleado fue PRIMEX con un grado de desacetilación de 85,6 % (Primex Ingredients ASA, Noruega). El grado de desacetilación molar para los tres lotes de acetato de quitosano fue de 57,36 % (Lote 12001), 57,69 % (Lote 12002) y para el lote 12003 de 58,92 %.

Actividad antioxidante de disoluciones de quitosano y acetato de quitosano

Preparación de las disoluciones de quitosano

El quitosano fue disuelto en disoluciones de ácido acético al 1,0 % (v/v), con agitación magnética durante 2 h, obteniendo disoluciones de quitosano a las concentraciones de 5, 10, 15, 20, 25 y 30 μg/mL. Para el caso del acetato de quitosano también se emplearon disoluciones a las concentraciones de 5, 10, 15, 20, 25 y 30 μg/mL.

La actividad antioxidante *in vitro* del biopolímero y su sal se determinó mediante técnicas bioquímicas, a través de los ensayos de Capacidad Reductora del Hierro Férrico (FRAP) y la Capacidad Secuestradora del Radical (DPPH).

Capacidad Reductora del Hierro Férrico (FRAP)

El método se fundamenta en la reducción del complejo TPTZ – Fe^{3+} a la forma ferrosa por la acción de las sustancias antioxidantes presentes en las disoluciones de quitosano. La reacción fue monitoreada midiendo la absorbancia a 593 nm [15]. El ensayo FRAP consistió en añadir 900 μL del reactivo FRAP a un tubo de ensayo al que se le adicionaron 90 μL de las disoluciones de quitosano a las concentraciones de 5, 10, 15, 20, 25 y 30 μg/mL. Se empleó un lector de placas de 96 pocillos (SUMA, Cuba).

La reacción se llevó a cabo a temperatura ambiente, leyendo la absorbancia en un espectrofotómetro UV-Visible (SpectronicGenesys 2, LR 45227, EE. UU.), después de transcurridos 4 min. Como estándar se usó una disolución de ácido ascórbico (Sigma-Aldrich, Alemania) como control positivo. El FRAP se expresó como μM equivalentes de ácido ascórbico.

Capacidad secuestradora del radical (DPPH)

El método se basa en la reducción del radical estable 2,2-difenil-2-picrilhidrazilo (DPPH) por la acción de compuestos antioxidantes que contienen grupos -OH, que decoloran dicho reactivo [16]. En tubos de ensayo se mezclaron 1,5 mL de una disolución etanólica de DPPH (0,075 mg/mL) con 750 μL de las disoluciones de quitosano a diferentes concentraciones (5, 10, 15, 20, 25 y 30 μg/mL). La mezcla se agitó vigorosamente y se dejó reposar a temperatura ambiente durante 30 min, midiendo la absorbancia a 515 nm en un espectrofotómetro UV-visible (SpectronicGenesys 2, LR 45227, EE. UU.). Se empleó un lector de placas de 96 pocillos (SUMA, Cuba).

El valor de concentración de muestra requerida para disminuir el 50 % de la concentración del radical DPPH (CI_{50}) de las disoluciones de quitosano, se calculó usando la curva de dosis contra porcentaje de inhibición. Los resultados fueron expresados

como el porcentaje de decoloración de la disolución de DPPH (% DPPH), utilizando la siguiente ecuación:

$$\% \text{ DPPH} = (A_o - A_1) \times 100 / A_o \quad (1)$$

donde: A_o es la absorbancia del control y A_1 es la absorbancia de la muestra. Con fines comparativos, el Trolox 20 % (Sigma-Aldrich, Alemania) fue utilizado como antioxidante de referencia.

Para el análisis estadístico se empleó el programa GraphPad Prism 5.0 (GraphPad Software Inc., EE. UU.). Los datos se expresaron como Media/Desviación estándar (X/DE) de al menos tres ensayos independientes (n = 3). En todos los casos se consideraron diferencias significativas los valores de $p < 0,05$.

Actividad antimicrobiana del quitosano y el acetato de quitosano

Las cepas microbianas empleadas eran cultivos pertenecientes a las colecciones del Laboratorio de Microbiología del CIDEM y de la Facultad de Biología de la Universidad de La Habana: *Micrococcus luteus* ATCC 9341, *Micrococcus flavus* NCTC 7743, *Micrococcus flavus* ATCC 10240, *Staphylococcus aureus* ATCC 29737, *Staphylococcus epidermidis* ATCC 12228, *Sacharomyces cerevisiae* ATCC 9763, *Sacharomyces cerevisiae* ATCC 2601, *Escherichia coli* ATCC 10536, *Escherichia coli* ATCC 25922, *Bacillus subtillis AT*CC 6633, *Candida albicans* ATCC 10231.

Pruebas de pureza microbiológica

Las pruebas de límite microbiano para la evaluación de la pureza microbiológica de las muestras de quitosano y su sal de acetato, se realizaron según lo descrito para medicamentos no estériles [17].

Preparación del inóculo

Los microorganismos de prueba se cultivaron en tubos con agar inclinado Triptona Soya Agar (TSA) y Sabouraud Dextrosa Agar (SDA), según las características de los mismos. Concluido el periodo de incubación el crecimiento fue removido con 5 mL de disolución salina 0,9 % (estéril). La concentración del inóculo microbiano se ajustó al patrón 0,5 de la escala McFarland [18] correspondiente a 10^5 ufc/mL para bacterias y 10^4 ufc/mL para levaduras.

Determinación de la actividad antimicrobiana

Se empleó el método de difusión en placas de medio de cultivo Triptona Soya Agar para las bacterias y en medio Sabouraud Dextrosa Agar para las levaduras, previamente

inoculados con los microorganismos de prueba [19]. Se realizaron pozos de 6 mm de diámetro, utilizando un perforador estéril, colocando 100 µL de las muestras a ensayar, a las concentraciones de 0,5, 1,0 y 2,0 %, tanto para el quitosano como para el acetato de quitosano, respectivamente. Las placas se incubaron durante 24 h a 32-35 °C para las bacterias y 24-48 h a 30 °C para las levaduras.

La actividad antimicrobiana de las muestras se determinó midiendo el halo de inhibición alrededor de cada pozo, empleando para ello el lector de zona Fisherlily (EE. UU.). Se determinaron los valores promedios de los halos de inhibición, para el quitosano y su sal de acetato, para cada uno de los microorganismos evaluados. Con fines comparativos también se evaluó, bajo las mismas condiciones, el quitosano comercial PRIMEX.

Se consideró un resultado positivo cuando el halo de inhibición era igual o mayor a 8 mm de diámetro alrededor de cada pozo.

Concentración Mínima Inhibitoria y Concentración Mínima Microbicida

La concentración mínima inhibitoria (CMI) de cada muestra se determinó por el método de diluciones seriadas [18] en medio Triptona Soya Caldo y Sabouraud Dextrosa Caldo. Los tubos con las diluciones de quitosano se inocularon con los microorganismos de prueba y se incubaron a 37 °C para las bacterias y a 22 °C para las levaduras. Transcurrido el tiempo de incubación, se realizaron las lecturas correspondientes. Los resultados obtenidos se compararon con los tubos de medio de cultivo inoculados con los microorganismos de prueba, sin quitosano o sin acetato de quitosano, sometidas a iguales condiciones.

Transcurrido el período de incubación se hicieron resiembras de 0,1 mL, en placas Petri con medio agarizado selectivo, realizando el conteo de las colonias. La menor concentración de la muestra capaz de producir un daño sobre la célula microbiana fue definida como la Concentración Mínima Microbicida (CMM).

Se utilizaron como control diluciones estándar de Bacitracina, Gentamicina, Neomicina, Nistatina y Vancomicina, sustancias con actividad antimicrobiana reconocida frente a los microorganismos empleados en el estudio.

Resultados y discusión

Actividad antioxidante del quitosano y el acetato de quitosano

La evaluación de la capacidad antioxidante *in vitro* de las disoluciones de quitosano y su sal, por los dos métodos empleados, se presenta en la Tabla 1. Los resultados de

la capacidad antioxidante a Trolox, ensayo DPPH, muestran que, aunque con menor actividad secuestradora de este radical, respecto al patrón, en las condiciones experimentales empleadas y las concentraciones analizadas, el quitosano presentó un porcentaje de decoloración indicativo de capacidad antioxidante moderada, con un valor de CI_{50} de 28,09 ± 0,035 µL/mL, calculado por regresión lineal a partir de la ecuación: Y = 2,0852X - 7,5813, con un coeficiente de correlación (r^2) de 0,9932. Sin embargo, el acetato de quitosano alcanzó un bajo porcentaje de decoloración para las concentraciones ensayadas.

Tabla 1. Capacidad antioxidante de las disoluciones de quitosano y el acetato de quitosano

Concentración (µg/mL)	DPPH		FRAP
	Densidad óptica	% de inhibición	µM equivalentes de ácido ascórbico
Disoluciones de quitosano			
5	0,534/0,308	4,87	0,061/0,021
10	0,467/0,000	11,35	0,217/0,006
15	0,349/0,000	22,81	0,340/0,006
20	0,257/0,148	31,74	0,614/0,003
25	0,123/0,000	44,80	0,741/0,003
30	0,080 /0,001	55,05	0,905/0,002
Disoluciones de acetato de quitosano			
5	0,545/0,215	3,7	0,063/0,01
10	0,543/0,000	3,9	0,107/0,001
15	0,539/0,134	4,3	0,146/0,002
20	0,535/0,100	6,2	0,166/0,002
25	0,520/0,000	6,9	0,202/0,003
30	0,590/0,115	8,1	0,215/0,002

X/DE: Media/Desviación estándar: n=3

A partir del método FRAP, se corroboró que la actividad antioxidante de las disoluciones de quitosano aumentó con el incremento de su concentración, alcanzándose el valor máximo de 0,905 ± 0,002 µg equivalente de ácido ascórbico, a la concentración de 30 µL/mL. De igual forma, la sal mostró un incremento de la capacidad reductora del Fe^{3+}, con el aumento de la concentración de la disolución, pero menor respecto al quitosano, para igual concentración.

Estos resultados coincidieron, de forma general, con los reportados [11-13], donde los valores de la capacidad de secuestro de radicales libres de las disoluciones de quitosano

y su sal de acetato fueron superiores al 30 %, respecto al patrón empleado, incrementándose con el aumento de la concentración de quitosano y el tiempo de reacción de los ensayos. El acetato de quitosano no mostró una actividad antioxidante efectiva, según las condiciones evaluadas.

La capacidad antioxidante del quitosano puede explicarse por varios mecanismos. Uno de ellos es la capacidad de secuestro de radicales libres por acción del nitrógeno en el C-2. Esta capacidad de secuestro está relacionada con el hecho de que los radicales libres pueden reaccionar con los iones H^+ provenientes de los iones (NH_3^+) de las moléculas estables [11, 12].

Actividad antimicrobiana del quitosano y el acetato de quitosano

Al evaluar la calidad microbiológica de las muestras, empleando la técnica de límite microbiano, no se detectó la presencia de microorganismos contaminantes en ninguno de los lotes evaluados. En el proceso de obtención del quitosano se emplea una temperatura de mezcla de 80 °C y reacción de 100 °C /15 min dobles, se lava el polímero con agua purificada hasta neutralidad, y se seca por un tiempo de 4 h a 100 °C. El acetato de quitosano se obtiene por el método de secado por aspersión, a temperatura de entrada 160 ± 5 °C y salida 100 ± 5 °C [8, 9]. Por lo tanto, las condiciones del proceso de obtención, tanto del quitosano como del acetato de quitosano, favorecen la calidad del producto final y que se cumplan los límites establecidos para materias primas de origen natural y no estériles.

El efecto antimicrobiano del quitosano varía conforme al microorganismo en estudio, siendo diferente para hongos, bacterias Gram positivas y Gram negativas. Lo anterior podría estar fuertemente ligado a las estructuras externas características de cada microorganismo [20].

Con la prueba de difusión en agar se observó la variación de la inhibición a las diferentes concentraciones de quitosano utilizadas (Tabla 2). Nótese que para el quitosano la inhibición se hace visible a partir de la concentración de 0,5 %, no detectándose inhibición para las especies de *Micrococcus* empleadas en el estudio, a esa concentración. Sin embargo, cuando la concentración aumentó la actividad antimicrobiana se hizo visible para todos los microorganismos empleados.

Este comportamiento varió cuando se empleó la sal de acetato de quitosano (Tabla 3), donde con la concentración de 0,5 % ya hay valores de inhibición del crecimiento microbiano para todos los microorganismos, principalmente frente a *Candida albicans, Staphylococcus aureus, Sacharomyces cerevisiae* y *Staphylococcus epidermides*, comprobándose su actividad antimicrobiana frente a bacterias y hongos [6, 7].

Tabla 2. Actividad antimicrobiana del quitosano

Microorganismo	**Promedio de los halos de inhibición (mm)**		
	0,5 %	**1,0 %**	**2,0 %**
Micrococcus luteus ATCC 9341	ND	6,6	8,4
Micrococcus flavus ATCC 10240	ND	6,5	8,3
Micrococcus flavus NCTC 7743	ND	6,7	8,6
Staphylococcus aureus ATCC 29737	6,8	8,3	10,9
Staphylococcus epidermides ATCC 12228	6,2	8,0	10,3
Escherichia coli ATCC 10536	6,2	8,5	10,4
Escherichia coli ATCC 25922	7,5	8,2	11,8
Bacillus subtillis ATCC 6633	8,2	9,8	11,3
Sacharomyces cerevisiae ATCC 9763	9,9	11,0	12,9
Sacharomyces cerevisiae ATCC 2601	9,8	11,6	13,2
Candida albicans ATCC 10231	9,2	10,7	12,3

ND: no se detecta

Tabla 3. Actividad antimicrobiana del acetato de quitosano

Microorganismo	Promedio de los halos de inhibición (mm)		
	0,5 %	1,0 %	2,0 %
Micrococcus luteus ATCC 9341	7,6	9,8	12,5
Micrococcus flavus ATCC 10240	7,7	9,6	12,4
Micrococcus flavus NCTC 7743	7,9	9,8	12,8
Staphylococcus aureus ATCC 29737	10,9	11,8	13,3
Staphylococcus epidermides ATCC 12228	10,6	11,0	12,5
Escherichia coli ATCC 10536	11,2	11,9	13,8
Escherichia coli ATCC 25922	10,7	11,3	13,5
Bacillus subtillis ATCC 6633	11,4	12,2	13,3
Sacharomyces cerevisiae ATCC 9763	13,9	14,6	16,5
Sacharomyces cerevisiae ATCC 2601	13,9	14,4	16,9
Candida albicans ATCC 10231	13,1	13,9	15,8

La sal de acetato de quitosano fue obtenida a través del proceso de secado por aspersión, lo que favorece la disminución del tamaño de las partículas y, por ende, una mayor solubilidad, respecto al quitosano base, favoreciendo su actividad antimicrobiana.

Existen y se continúan investigando los mecanismos de acción a través del cual el quitosano ejerce su acción antimicrobiana. Se plantea que en las bacterias Gram positivas el polímero se une a los ácidos teicoicos y lipoteicoicos, ubicados en la pared celular, lo que altera la interacción entre ésta y la membrana celular, induciendo la salida de componentes esenciales y el desequilibrio osmótico de la célula [21]. En las bacterias Gram negativas interactúa, mediante atracciones electrostáticas, con lipopolisacáridos de la membrana externa de la bacteria desestabilizándola, lo cual altera su permeabilidad, tanto de la pared como de la membrana interna, favoreciendo la entrada y salida de componentes celulares esenciales [22]. En el caso de los hongos, los grupos amino de las unidades de glucosamina interactúan con componentes extracelulares de la pared y la membrana celular de los hongos, mediante atracciones electrostáticas, provocando la formación de poros y la salida del contenido intracelular, lo que ocasiona inestabilidad y muerte celular [21, 23].

En las Figuras 1, 2 y 3 se observa el comportamiento del quitosano, derivado de quitina de langosta (*Panulirus argus*) obtenido en Cuba y del quitosano comercial PRIMEX (Noruega). Se aprecia una diferencia en los valores de inhibición, siendo el quitosano comercial el que mejores resultados muestra, tanto para los microorganismos Gram positivos como los Gram negativos, los cuales aumentaron con el incremento de la concentración del biopolímero. Similar comportamiento muestra el quitosano nacional, pues sus valores de inhibición aumentaron al incrementarse su concentración, aunque inferiores a los del quitosano comercial. Esto pudiera atribuirse a las características propias de cada tipo de quitosano utilizado, el tamaño de las partículas, el GD, la solubilidad, entre otros.

La solubilidad del quitosano depende del GD y de la distribución de los grupos acetilos y aminos a lo largo de la cadena [8]. Para el caso que nos ocupa, al presentar un menor tamaño de partícula hay una mejor velocidad de disolución la cual varía entre los quitosanos evaluados (quitosano y quitosano comercial) favoreciendo su actividad antimicrobiana. Similar comportamiento sucede con la sal de acetato de quitosano, obtenida por secado por aspersión, donde además de obtenerse en forma de sal, el proceso de obtención de secado por aspersión, favorece la disminución del tamaño de las partículas y, por ende, el derivado de quitosano obtenido tiene una mayor velocidad de disolución.

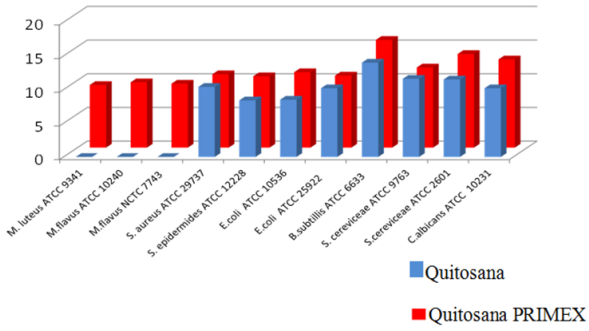

Figura 1. Actividad antimicrobiana del quitosano y el quitosano PRIMEX al 0,5 %.

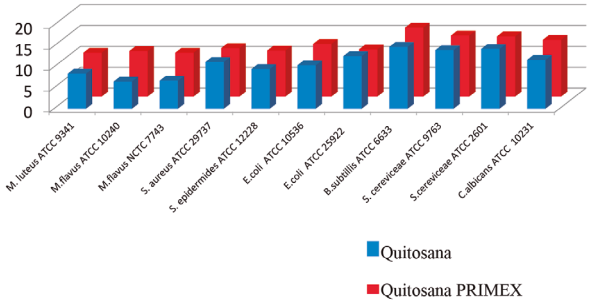

Figura 2. Actividad antimicrobiana del quitosano y el quitosano PRIMEX al 1,0 %.

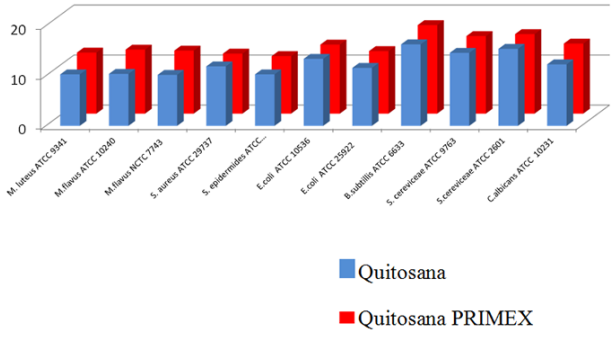

Figura 3. Actividad antimicrobiana del quitosano y el quitosano PRIMEX al 2,0 %.

En relación a las CMI y la CMM del quitosano y el acetato de quitosano (Tabla 4), se aprecia que en todos los casos se logró detener el crecimiento microbiano de todos los microorganismos evaluados. Este comportamiento es similar a lo reportado para las cepas de *Staphylococcus*, *E. coli* y las de levaduras [23]. La acción bactericida solo se evidenció en el acetato de quitosano frente a las cepas de microorganismos del género *Micrococcus.*

Tabla 4. Concentraciones mínimas inhibitorias (CMI) y mínimas microbicidas (CMM) del quitosano y el acetato de quitosano

Microorganismo	Quitosano		Acetato de quitosano	
	CMI (mg/mL)	**CMM (mg/mL)**	**CMI (mg/mL)**	**CMM (mg/mL)**
M. luteus ATCC 9341	1,5	-	0,34	1,5
M. flavus ATCC 10240	2,0	-	0,3	2,0
M. flavus NCTC 7743	2,0	-	0,3	1,5
S. aureus ATCC 29737	0,3	-	0,14	-
S. epidermides ATCC 12228	0,34	-	0,27	-
E. coli ATCC 10536	2,5	-	0,3	-
E. coli ATCC 25922	2,0	-	0,34	-
B. subtillis ATCC 6633	0,27	-	0,2	-
S. cerevisiae ATCC 9763	0,3	-	0,14	-
S. cerevisiae ATCC 2601	0,3	-	0,21	-
C. albicans ATCC 10231	0,27	-	0,2	-

La actividad antimicrobiana del quitosano depende de factores como el GD, Mm, temperatura, pH del medio, tipo de microorganismo, entre otros. Para el caso de las bacterias se plantea, que puede tener actividad intra y extracelular, según su Mm y las características del microorganismo. El quitosano de alta Mm es, generalmente, incapaz de penetrar la pared y la membrana celular, por lo que su potencial efecto antimicrobiano implica actuar como quelante de metales esenciales, impidiendo que los nutrientes sean absorbidos de las células extracelularmente, alterando la permeabilidad celular [22]. Sin embargo, la de baja Mm no sólo tiene actividad antimicrobiana extracelular, sino también intracelular, afectando así el ARN, la síntesis de proteínas y la función mitocondrial. Todo esto, unido a las características propias de cada grupo microbiano, hace que su acción antimicrobiana sea altamente dependiente del tipo de microorganismo [23].

En el caso de los hongos, sus propiedades antifúngicas están relacionadas, principalmente, con la interacción del quitosano con la pared celular o membrana celular. Sin embargo, la CMI del quitosano varía y está altamente asociada con la Mm, GD, pH y el tipo de hongo [24-26].

Conclusiones

Las disoluciones de quitosano mostraron mayor actividad antioxidante que la del acetato de quitosano. Tanto el quitosano, como su sal de acetato, presentaron un efecto inhibitorio sobre los microorganismos utilizados en el estudio y su actividad dependió de la concentración. La actividad antimicrobiana del acetato de quitosano fue superior a la del quitosano. La acción bactericida solo se evidenció frente a las cepas del género *Micrococcus*.

Conflicto de intereses

Los autores declaran no tener conflictos de intereses.

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Neuroprotective effect of *Mauritia flexuosa* on a unilateral 6-OHDA-induced Parkinson's disease in rats

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Summary

Introduction: Parkinson's disease (PD) is a neurodegenerative disorder characterized by motor and cognitive impairments, primarily due to the progressive loss of dopaminergic neurons in the substantia nigra. While there is currently no treatment to halt neuronal loss, evidence suggests that a diet rich in antioxidants may mitigate oxidative stress and disease progression. **Aims:** This study sought to investigate the neuroprotective effects resulting from the oral administration of ethanolic extract of *Maurita flexuosa* over a 21-day period, at doses of 1 mg/kg, 10 mg/kg, and 100 mg/kg. **Methods:** Initially, antioxidant activity assays revealed significant levels of various antioxidants, including β-carotene, gallic acid equivalent, and quercetin. A PD animal model was then induced via stereotaxic injection of 6-hydroxydopamine (6-OHDA) into the striatum, with apomorphine-induced rotation tests used for model validation. Motor behavior assessments were performed using open field tests and beam walking tests. **Results:** The open field test indicated improved motor behavior in the 1 mg/kg group compared to the 6-OHDA group. However, neurohistological analysis via Western blot testing revealed potential neurotoxic effects

associated with the 100 mg/kg treatment dose. **Conclusions:** Chronic administration of 1 mg/kg of ethanolic extract of *Maurita flexuosa* over 21 days demonstrated potential improvements in locomotion in a 6-OHDA-induced PD model. Nonetheless, a notable limitation of the study lies in the 6-OHDA model's failure to induce the expected level of damage in the striatum and substantia nigra, achieving only 29% damage, whereas a total PD model typically requires 70% damage for optimal replication.

Keywords: Mauritia flexuosa; antioxidant; neuroprotector; Parkinson Disease

Resumen

Efecto neuroprotector de *Mauritia flexuosa* en la enfermedad de Parkinson unilateral inducida por 6-OHDA en ratas

Introducción: La enfermedad de Parkinson (EP) es un trastorno neurodegenerativo caracterizado por deterioro motor y cognitivo, principalmente debido a la pérdida progresiva de neuronas dopaminérgicas en la sustancia negra. Si bien actualmente no existe un tratamiento para detener la pérdida neuronal, la evidencia sugiere que una dieta rica en antioxidantes puede mitigar el estrés oxidativo y la progresión de la enfermedad. **Objetivos:** Este estudio buscó investigar los efectos neuroprotectores resultantes de la administración oral de extracto etanólico de *Maurita flexuosa* durante un período de 21 días, en dosis de 1 mg/kg, 10 mg/kg y 100 mg/kg. **Métodos:** Inicialmente, los ensayos de actividad antioxidante revelaron niveles significativos de varios antioxidantes, incluidos β-caroteno, equivalente de ácido gálico y quercetina. A continuación, se indujo un modelo animal de EP mediante inyección estereotáxica de 6-hidroxidopamina (6-OHDA) en el cuerpo estriado, y se utilizaron pruebas de rotación inducidas con apomorfina para la validación del modelo. Se realizaron evaluaciones del comportamiento motor mediante pruebas de campo abierto y pruebas de marcha sobre vigas. **Resultados:** La prueba de campo abierto indicó una mejora del comportamiento motor en el grupo de 1 mg/kg en comparación con el grupo de 6-OHDA. Sin embargo, el análisis neurohistológico mediante pruebas de transferencia Western reveló posibles efectos neurotóxicos asociados con la dosis de tratamiento de 100 mg/kg. **Conclusiones:** La administración crónica de 1 mg/kg de extracto etanólico de *Maurita flexuosa* durante 21 días demostró posibles mejoras en la locomoción en un modelo de EP inducido por 6-OHDA. No obstante, una limitación notable del estudio radica en la incapacidad del modelo de 6-OHDA para inducir el nivel esperado de daño en el cuerpo estriado y la sustancia negra, logrando

solo un 29% de daño, mientras que un modelo de EP total normalmente requiere un 70% de daño para una replicación óptima.

Palabras clave: Mauritia flexuosa; antioxidante; neuroprotector; enfermedad de Parkinson

Resumo

Efeito neuroprotetor de *Mauritia flexuosa* em uma doença de Parkinson unilateral induzida por 6-OHDA em ratos

Introdução: A doença de Parkinson (DP) é uma doença neurodegenerativa caracterizada por deficiências motoras e cognitivas, principalmente devido à perda progressiva de neurônios dopaminérgicos na substância negra. Embora atualmente não haja tratamento para interromper a perda neuronal, as evidências sugerem que uma dieta rica em antioxidantes pode mitigar o estresse oxidativo e a progressão da doença. Objetivos: Este estudo buscou investigar os efeitos neuroprotetores resultantes da administração oral de extrato etanólico de *Maurita flexuosa* durante um período de 21 dias, em doses de 1 mg/kg, 10 mg/kg e 100 mg/kg. **Métodos:** Inicialmente, os ensaios de atividade antioxidante revelaram níveis significativos de vários antioxidantes, incluindo β-caroteno, equivalente de ácido gálico e quercetina. Um modelo animal de DP foi então induzido por injeção estereotáxica de 6-hidroxidopamina (6-OHDA) no estriado, com testes de rotação induzidos por apomorfina usados para validação do modelo. Avaliações do comportamento motor foram realizadas usando testes de campo aberto e testes de caminhada em viga. **Resultados:** O teste de campo aberto indicou comportamento motor melhorado no grupo de 1 mg/kg em comparação ao grupo de 6-OHDA. No entanto, a análise neuro-histológica por meio de testes de Western blot revelou potenciais efeitos neurotóxicos associados à dose de tratamento de 100 mg/kg. **Conclusões:** A administração crônica de 1 mg/kg de extrato etanólico de *Maurita flexuosa* ao longo de 21 dias demonstrou potenciais melhorias na locomoção em um modelo de DP induzido por 6-OHDA. No entanto, uma limitação notável do estudo está na falha do modelo de 6-OHDA em induzir o nível esperado de dano no estriado e na substância negra, atingindo apenas 29% de dano, enquanto um modelo de DP total normalmente requer 70% de dano para replicação ideal.

Palavras-chave: Mauritia flexuosa; antioxidante; neuroprotetor; Doença de Parkinson

Introduction

Parkinson's disease (PD) is a clinically heterogeneous neurodegenerative disease of adult onset [1]. PD is characterized by 4 main motor symptoms: resting tremor (with a frequency between 4 and 6 Hz), bradykinesia, rigidity and postural instability [1-3]. The prevalence of PD is currently high, and is predicted to double in size by 2040 [4], making it the fastest growing neurodegenerative disease [5]. From the neurohistopathological point of view, PD is characterized by a progressive loss of dopaminergic neurons in the pars compacta of the substantia nigra and its projection towards the striatum due to toxicity resulting from the accumulation and deposition of misfolded alpha synuclein at the intracellular level [6, 7]. Symptoms start on one side of the body when dopamine concentrations decline below 60 to 70% in the contralateral striatum [8]. One hypothesis for the etiology of PD is mitochondrial dysfunction and oxidative stress [7].

Antioxidants play a crucial role in mitigating oxidative damage caused by reactive oxygen species (ROS) and protecting against neurophysiological abnormalities [9, 10]. They act by decreasing the concentration of oxidants, binding to metal ions to prevent the formation of ROS, decreasing the reactivity of peroxides and decreasing the propagation and creation of free radicals [11].

Mauritia flexuosa, commonly known as buriti, moriche or aguaje, is a fruit with a high content of fats, proteins and vitamins (high content of B-carotene, vitamin A and tocopherol) [12-14]. In a study it was found a high level of antioxidants, especially in the pulp and peel extract with a concentration of 378.07 mg GAEq/100 g of phenols, 567.16 mg GAEq/100 g of flavonoids respectively, which is low in comparison to other fruits due to the high level of carotenoids; and in an extract of the pulp it was found 9.47 mg/100 g of total polyphenols and 23.36 mg/100 g of carotenoids [15, 16]. The main carotenoids found were β carotene at 60% and α carotene at 6% [16].

Therefore, the consumption of *Mauritia flexuosa* (aguaje) could act as a significant overprotective agent against the progression and as a preventive measure of PD, since it would act at the level of free radical depletion due to its high antioxidant content. The aim of the study was to evaluate the potential effect on locomotion and neuroprotection of 21 days oral administration (1 mg/kg, 10 mg/kg and 100 mg/kg) of ethanolic extract of *Mauritia flexuosa* of the morphotype "Color". *Mauritia flexuosa* administration is expected to improve motor performance and act as a neuroprotective agent at a substantia nigra level.

Material and methods

Extract preparation

The fruits of *Mauritia flexuosa* L.f. were of the "Color" morphotype. The preparation of the ethanolic extract and in vitro experiments were carried out at the Institute of Food Science and Nutrition of San Ignacio del Loyola University. The fruit was recollected from "Carretera Iquitos-Nauta" from Peruvian jungle.

β-carotene determination

The β-carotene content was carried out according to the methodology proposed by Zanqui *et al.* (2019) [17]. Approximately 100 mg of sample was placed in a conical tip centrifuge tube and then 5 mL of n-hexane was added. The extraction was conducted by vortexing at 1500 rpm for 30 min. Next, the extraction was continued in an ultrasound system at a frequency of 40 kHz, 30 °C and 30 min. The supernatant was obtained by centrifugation at 3500 rpm for 15 min. The absorbance value was made at 450 nm in a 1 cm optical path quartz cell using a Jasco V-770 spectrophotometer (Tokyo, Japan). To calculate the β-carotene content, the extinction coefficient of 2592 was considered. Values were expressed in mg β-carotene per gram.

Total polyphenols determination

Total polyphenol content was determined by the Folin-Ciocalteu method. An approximate amount of 500 mg of sample was extracted with 80% methanol. The polar fraction was extracted with an ultrasonic bath and subsequently centrifuged at 3500 rpm for 15 min. The supernatant was analyzed with Folin-Ciocalteu reagent and 7.5% sodium carbonate [18]. The blue color formed was read at 765 nm using a Jasco V-770 spectrophotometer. The results were expressed in mg of gallic acid equivalent per gram of sample.

Determination of antioxidant activity by TEAC and FRAC

The evaluation of antioxidant activity was carried out by TEAC and FRAP assay [19, 20]. For the TEAC assay, 20 μL of the extract and 980 μL of the ABTS radical were used. Readings were collected at 734 nm, after 5 min of reaction. The results were expressed as mmol of trolox equivalent/g sample.

For the FRAP assay, 120 μL of sample and 750 μL of FRAP reagent were used, which consisted of 25 mL of aqueous solution adjusted to pH 3.6, 2.5 mL of 10 mM ferric-2,4,6-tripyridyl-s-triazine complex (TPTZ) in 40 mM HCl and 2.5 mL of 20 mM

$FeCl_3$ in water. The absorbance reading was recorded at 593 nm after 5 min of reaction. The results were expressed as mmol $FeSO_4$/g.

Phenol profiling by HPLC-DAD

Phenol profiling was conducted by liquid chromatography with diode array detector (Hitachi Chromaster™, High-Technologies Corporation, Tokyo, Japan). Separation of the different analytes was performed on a LiChrospher® 100 RP-18 (5 μm) column (Merck KGaA, Germany). Mobile phase A consisted of a mixture of water/orthophosphoric acid and an equal parts methanol/acetonitrile mixture (B). The analytes were eluted according to the following gradient system (only the values of phase B are presented and the difference corresponds to phase A). The spectrum was recorded 280 nm and the injection volume was 20 μL. The results were obtained by comparison of the respective standards. The content of each phenol was expressed in g/kg of sample.

Maintenance and care of animals

21 Sprague-Dawley male rats, 2.5 months old and weighing 250 g – 300 g were used. The animals were kept in PET plastic cages with dimensions of 37.3 centimeters long, 23.4 centimeters wide and 14 centimeters high in groups of 4 rats. Cleaning was performed daily by renewing the cage shavings. The animals are from the automated Bioterio of the Universidad Andina del Cusco. The cages were kept in an automated environment with a 12-hour light/dark cycle with the light period starting at 6:00 am, temperature between 23 and 25 °C and relative humidity between 40 and 45%. Balanced pelleted feed and water were administered ad libitum. *Mauritia flexuosa* was administered by direct cannulation to the rat from day 1 to day 21. A 75-mm long cannula with a 2.3-mm diameter tip attached to a syringe was used and passed through the esophagus into the stomach, where the substance was expelled at a slow controlled rate avoiding regurgitation.

All procedures were performed under the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and with the approval of Cayetano Heredia University Institutional Animal Care and Use Committee (Protocol #2017-09) [21].

Injection with 6OHDA

The animal was anesthetized via intraperitoneal injection with Ketamine (70 mg/kg) and Xylazine (6 mg/kg). Prior to the procedure, the animal underwent a fasting period of 4 to 6 hours. Unilateral intracranial injection of 6-OHDA was performed on the right side using a concentration of 4 mg/mL and a volume of 4 mL in 0.02% ascorbic acid-saline [22]. In the negative control group, 5 mL of 0.02% ascorbic acid-saline was administered. The infusion rate was maintained at 1 mL/min, with the needle remain-

ing in place for 3 minutes before being slowly withdrawn. Injection coordinates were set at anteroposterior +1.0 mm, lateral +3.2 mm, and dorsal ventral -4.6 mm from the bregma [23]. Following the procedure, Dermicare was applied to prevent infections. Animals were closely monitored until they regained consciousness (45-90 minutes), after which they received individual care for two days before being grouped in sets of three rats.

Open field Test

The Open Field behavioral test was performed to measure levels of general locomotor activity and anxiety following the methodology of Shi *et al.* (2006) [24] with modifications. The test is performed on a 50 × 50 cm black acrylic square and a 50 cm wall around it. The square is divided into 4 sub-squares (2 × 2). Prior to the test the rats were habituated to the environment for 2 days for two hours and on the day of the test the rats were acclimatized for 60 minutes. The rat was placed in the center of the square and its behavior was recorded for 5 minutes with a Logitech BRIO webcam with 4K Ultra UH video with EthoVision XT 15 software.

Crossbar Test

This test consists of placing the rat in the middle of a horizontal bar and evaluating its ability to cross it to one of the two side platforms located at the ends of the bar. The bar has a diameter of 2.5 cm and a length of 60 cm. The platforms have a surface of 15 cm × 20 cm. The test has 6 trials per rat and a maximum duration of 60 seconds. The variables analyzed are: platform arrival latency, fall latency, number of falls, number of leg and tail errors and number of balance errors.

Apomorphine Test

The rotation test was performed to test the effects of injury based on the severity of motor behavior disorder by monitoring contralateral and ipsilateral reactions to unilateral injury with 6-OHDA. The methodology was collected from Wu *et al.* and animals will be given 0.25 mg/kg apomorphine in 0.5% ascorbic acid and saline subcutaneously. Rotations were measured at 5 and 10 minute intervals until minute 30.

Western Blot

Microdissection technique employing micropunch was utilized. Encephalons were extracted and immediately frozen in dry ice at -80 °C, followed by sectioning using a cryostat at 500 mm thickness. The frozen sections were then centrifuged along with Mammalian Lysis Buffer to release cellular content. Protein content quantification was carried out using a spectrophotometer (NanoDrop) with 1 mL of supernatant. For the Western Blot assay, polyacrylamide gels were prepared and waited 30 minutes for

gelation. Then the electrophoresis chamber was assembled and Running buffer 1X was added avoiding the formation of bubbles. 20 mL of pre-treated samples were placed and a constant voltage (100 V to 150 V) was administered.

Protein transfer from the gel to the nitrocellulose paper was achieved by immersing the materials in Towbin buffer, positioning them from the cathode (-) to the anode (+) at a constant amperage of 400 mA for 1 hour. In order to avoid unspecific binding of the antibody to the nitrocellulose paper, the protein-free spaces were blocked with molecular grade skimmed milk. Subsequently, the preparation of the polyclonal IgG anti-thyroxine hydroxylase antibody made in rabbits (Merck brand, code Ab152) was carried out and kept in incubation overnight at 4 °C. The next day washing was performed with 20 mL of PBS + Tween 20 0.1%. Biotinylated secondary antibody made in goat Anti-Rabbit (Invitrogen brand, code 656140) was incubated under agitation. Finally, it was incubated with ABC complex solution according to the instructions of the kit (Vectastain) and developed with the DAB kit. Subsequent measurement was performed using ImageJ software to quantify the optical density of protein expression.

Statistical Analysis

Since all variables were numerical, the means of the variable values were compared between groups using analysis of variance (ANOVA Tukey) if the data were normally distributed. If the data did not follow a normal distribution, the Kruskal-Wallis test was employed. All numerical data were analyzed using the STATA 15 program. Statistical significance was set at p-values less than 0.05.

Results

Antioxidant Activity

In order to identify the potential contributor(s) of antioxidant activity from the pulp of *Mauritia flexuosa*, β-carotene, total polyphenols, antioxidant activity by TEAC and FRAC Phenol profiling by HPLC-DAD were performed.

Table 1. Quantification of antioxidant activity

Antioxidant activity	Total content
mg β-caroten/g	0.68±0.02
mg equivalent gallic acid	4.11±0.20
TEAC (mmol TE/g)	21.85±0.84
FRAP (mmol $FeSO_4$/g)	36.28±0.70

(*Continued*)

Table 1. *Continuation.*

Antioxidant activity	Total content
3-4-dihidroxi benzoic acid (g/kg)	8.74±0.33
Chlorogenic acid (g/kg)	172.98±5.95
Caffeic acid (g/kg)	32.84±0.88
Syringic acid (g/kg)	0.68±0.02
Vainillin (g/kg)	3.57±0.03
Ferulic acid (g/kg)	17.30±0.15
Sinapic acid (g/kg)	1.88±0.03
Rutin (g/kg)	12.27±0.10
Quercetin (g/kg)	4.83±0.07

Motor behavior

Significant differences were observed in the Open Field Test variables, including speed, distance, immobility time, crossing between quadrants, and cumulative grooming at 14 days between the group administered with 1 mg/kg and the 6OHDA group, as determined by one-way ANOVA followed by Tukey's post hoc test ($p < 0.05$). At 21 days, significant differences were found only in speed and distance between the 6OHDA and 1 mg/kg groups, as well as grooming for the sham and 6OHDA group (Figures 1 and 2).

For Beam Walking Test, significant differences were found in latency to the platform for 14 days and fall latency for 21 days according to one-way ANOVA followed by used Dunn Test with Benjamini-Hochberg correction (Figures 3 and 4). To analyze the difference within groups it was found for 14 days in platform latency difference between sham and 6OHDA ($p = 0.02$). At 21 days there were found differences between the sham and 6OHDA groups ($p = 0.04$) in fall latency.

For the rotation test, no significant differences were found, but there was a greater number of gyrus in the 6OHDA group. The animals that presented ipsilateral rotation were eliminated from the study. Rats 7, 14, 18, B2, C2, D2, D3 and E5 were eliminated from the study.

Molecular activity

For the western blot assay, brains from rats 6, 7 and 9 were used for the 6OHDA group; 12, 13 and 14 for the 10 mg group; and 17, 18 and 19 for the 100 mg group. The analysis was performed in both the affected and contralateral hemispheres to obtain a control. A damage of 29% was obtained in the 6OHDA group, 27% in the 10 mg group and 53% in the 100 mg group (Figure 5 and 6).

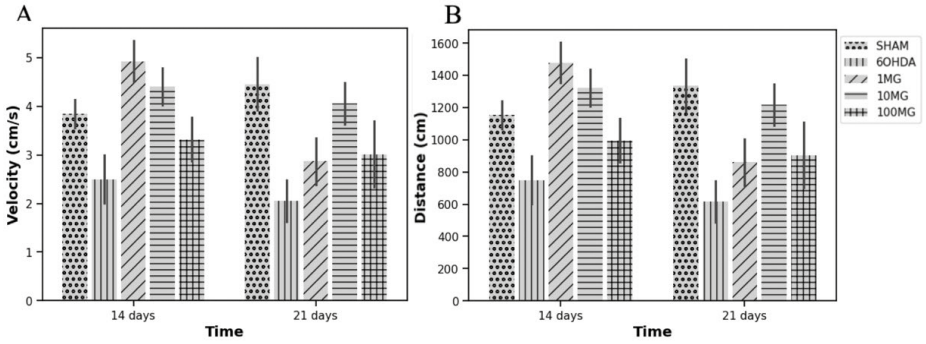

Figure 1. Determination of the effect of *Mauritia flexuosa* L.f. in velocity (A) and distance (B) in the open field test in animals pretreated with 6-OHDA. P < 0.01 (one-way ANOVA followed by Tukey's *post hoc* test). The figure shows an increased motor behavior in the experimental rats, being the 1 mg/kg group the one showing significance at 14 and 21 days.

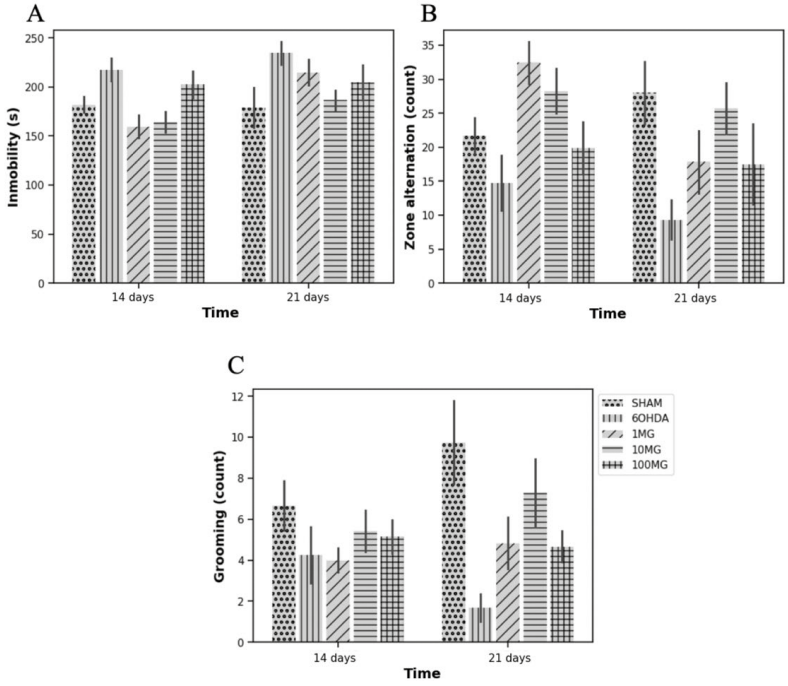

Figure 2. Determination of the effect of *Mauritia flexuosa* L.f. in immobility (A), zone alternation (B) and grooming (C) in the open field test in animals pretreated with 6-OHDA. P < 0.01 (one-way ANOVA followed by Tukey's *post hoc* test). The figure shows an increased motor behavior in the experimental rats, being the 1 mg/kg and sham group compared to the 6OHDA groups the ones showing significance for grooming at 21 days.

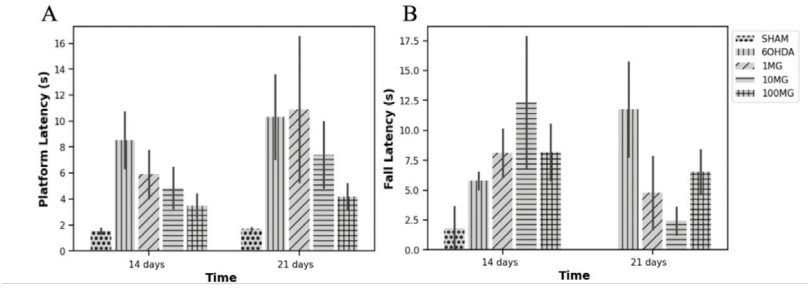

Figure 3. Determination of the effect of *Mauritia flexuosa* L.f. in platform latency (A) and fall latency (B) in the beam walking test in animals pretreated with 6-OHDA. $P < 0.01$ (one-way ANOVA followed by used Dunn Test with Benjamini-Hochberg correction).

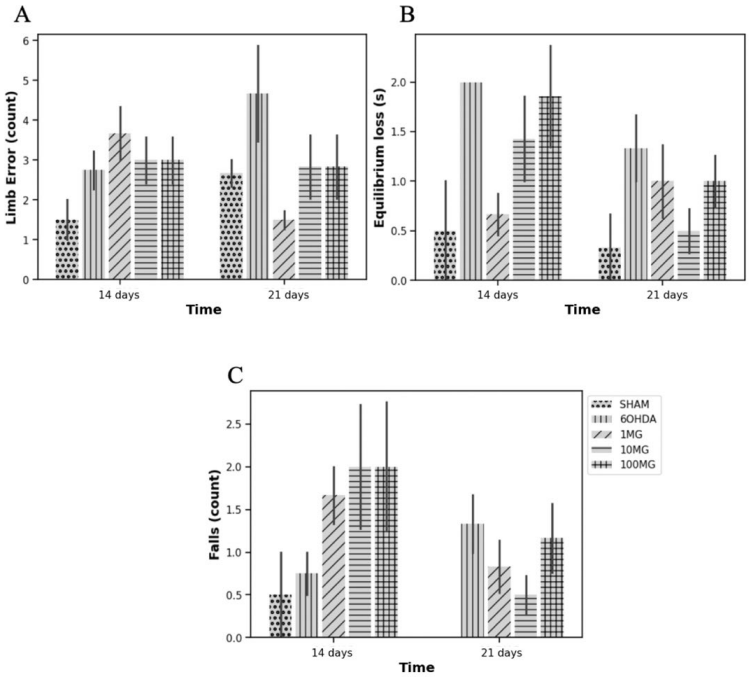

Figure 4. Determination of the effect of *Mauritia flexuosa* L.f. in errors: limb (A), equilibrium (B) and falls (C) in the beam walking test in animals pretreated with 6-OHDA. No significant differences found (one-way ANOVA followed by used Dunn Test with Benjamini-Hochberg correction).

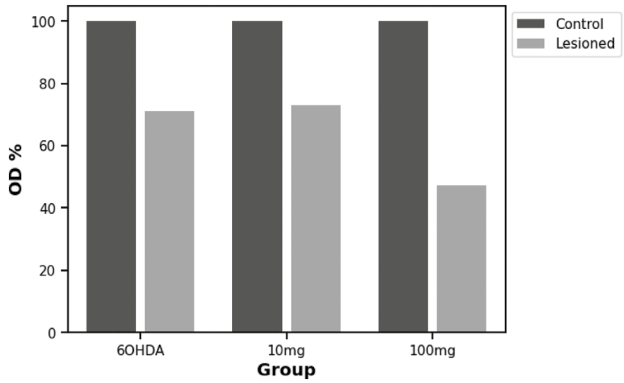

Figure 5. Western Blot percentages. Control is the side contralateral to the lesion.

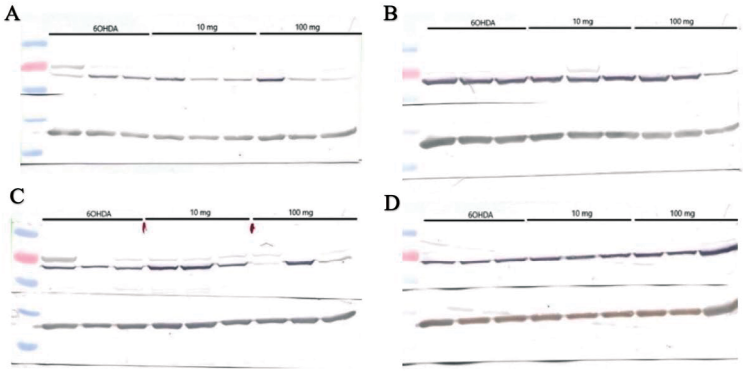

Figure 6. (A) Western Blot in 6OHDA substantia nigra. (B) Western Blot in control rats substantia nigra. (C) Western Blot in 6OHDA striatum. (D) Western Blot in control striatum.

Discussion

The study aimed to assess the potential impact of 21 days of oral administration (at doses of 1 mg/kg, 10 mg/kg, and 100 mg/kg) of ethanolic extract of *Mauritia flexuosa* (morphotype "Color") on locomotion and neuroprotection. The administration of *Mauritia flexuosa* was anticipated to enhance motor performance and act as a neuroprotective agent at the substantia nigra level. Antioxidant activity assessment revealed

significant levels of various components including β-carotene, gallic acid equivalent, 3-4-dihydroxy benzoic acid, Chlorogenic acid, Caffeic acid, Vanillin, Ferulic acid, Rutin, and Quercetin.

In the *in vivo* segment, the Open Field test unveiled notable differences in speed, distance, immobility time, crossing between quadrants, and cumulative grooming during the 14-day assessment. However, post-hoc analysis revealed differences between the 6OHDA group and the 1 mg/kg group. These results may indicate a potential overcompensation of the contralateral side to the lesion, resulting in higher-than-expected motor performance in the 6OHDA group. Additionally, the disparity between the 6OHDA and 1 mg/kg group could suggest neuroprotection offered by the administered dose to the injected rats.

In the same test conducted over 21 days, significant differences were found in the number of grooming and the post-hoc test showed differences between the Sham and 6OHDA groups. Grooming is the innate activity of the animal to maintain its hygiene, thermoregulation and communication and it has been found that its performance implies absence of damage to the striatum and dopaminergic inputs due to its necessary participation in the implementation of sequence and motor performance [25, 26]. Therefore, a lower amount of grooming in the 6OHDA group could imply a significant impairment in motor performance and in the present experiment these were presented by the rats of the 6OHDA group.

Regarding the second behavioral test performed, the cross bar test showed significant differences in latency to platform for 14 days and fall latency for 21 days. With respect to the 14-day test, the latency to platform is significantly higher in the 6OHDA group compared to the Sham group. These results are in accordance with expectations showing greater difficulty in the group injected with 6OHDA to reach the platform [27].

With respect to the latency to fall in 21 days the time is significantly longer for the 6OHDA group than the Sham group. Although the latency to fall is expected to be shorter for rats with 6OHDA [28], in the present experiment this group showed immobility when placed on the bar and immediate fall when attempting to move, whereas the Sham group presented rats that decided to launch themselves off the bar for possible stress avoidance or environmental recognition. Regarding the 6OHDA group in the same line, it was observed that the Sham rats presented grasping strategies involving the four limbs and the tail, while the 6OHDA rats did not.

Also, the rotation test not only serves to confirm the model, as treatment can reduce the number of rotations [29]. In the current experiment the mean for 6OHDA was higher than that of the Sham group. In the same vein it is important to mention that

studies show that an animal with less than 90% dopamine depletion in the striatum may not show remarkable differences in the number of gyri with apomorphine, so amphetamine is also used to generate gyri [23, 30].

Finally, western blot results indicate a 30% reduction for 6OHDA. According to studies it is possible to find no significant differences in HT between the sham and 6OHDA groups at 22 days [31], although HT reduction is expected in the 6OHDA group. A 2005 study investigated the effect of unilateral 6OHDA in mice and found high variability in the lesion with immunohistochemical results for TH reflecting dopaminergic loss between 4 and 100% [32]. On the other hand, the 100 mg/kg group showed a reduction of 53%, which could indicate a neurotoxic effect of the extract in that amount. It should be noted that the animals that received 100 mg/kg of *Mauritia flexuosa,* presented values closer to the 6OHDA group than the other treatment groups in the Open Field test, this being directly proportional to the results of the Western Blot, since they presented greater damage in the substantia nigra.

In summary, the findings suggest a potential neuroprotective effect associated with the administration of the 1 mg/kg dose, while indicating a possible neurotoxic effect linked to the 100 mg/kg dose. However, it is essential to acknowledge the main limitation of the study, which stems from the use of the 6OHDA model. This model fails to achieve the expected level of damage in the striatum and substantia nigra, reaching only 29% damage, whereas a total Parkinson's model typically involves damage reaching 70%. Therefore, it is advisable to replicate the research using a total Parkinson's model in rats induced with 6OHDA to validate and extend these findings.

Author statement

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed. We further confirm that the order of authors listed in the manuscript has been approved by all the members.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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Phytochemistry and antibacterial property of *Arisaema liemiana*

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Summary

Introduction: *Arisaema liemiana* was newly identified as a new species for the flora of Vietnam in 2020. It was discovered in Takou Nature Reserve, Binh Thuan province, Vietnam, characterized by its height of 20-50 centimeters, deciduous nature, light yellow spathe limb, white spathe tube, and spadix appendix covered with filiform neuter structures. **Aim:** This study aimed to investigate the chemical components and antibacterial activity of the acetone extracts derived from the aerial parts and tubers of *A. liemiana*. **Methodology:** *A. liemiana* specimens were collected from Takou Mountain within Takou Nature Reserve, Binh Thuan Province, and subjected to acetone extraction. The chemical components of these extracts were analyzed using GC-MS, while the antibacterial activity was assessed through the agar disc diffusion method. **Results:** A total of 57 compounds were identified in the aerial part and tuber extracts of which 32 components were found in the first extract

while the latter one contained 34 constituents. The antibacterial effectiveness of the tuber acetone extracts was assessed against 10 bacterial strains, revealing diverse inhibition zones. Notably, *S. aureus* ATCC 25923 exhibited the largest inhibition zone diameter, followed by *B. cereus*, *V. parahaemolyticus*, *S. flexneri*, *P. aeruginosa*, *E. coli*, *L. monocytogenes*, *S. saprophyticus*, *S. typhimurium*, and *S. enteritidis*. **Conclusion:** The chemical composition and antibacterial properties of acetone extracts derived from *Arisaema liemiana* was investigated for the first time. The observed notable antibacterial efficacy suggests potential pharmacological implications of *A. liemiana*. Additionally, this research unveils broader applications of *A. liemiana* in herbal medicine.

Keywords: Aracae, *Arisaema liemiana*, GC-MS, antibacterial activity.

Resumen

Fitoquímica y propiedad antibacteriana de *Arisaema liemiana*

Introducción: *Arisaema liemiana* fue identificada recientemente como una nueva especie para la flora de Vietnam en 2020. Fue descubierta en la Reserva Natural de Takou, provincia de Binh Thuan, Vietnam, caracterizada por su altura de 20-50 centímetros, naturaleza caducifolia, extremidad de espata de color amarillo claro, tubo de espata blanco y apéndice del espádice cubierto de estructuras neutras filiformes. **Objetivo:** Este estudio tuvo como objetivo investigar los componentes químicos y la actividad antibacteriana de los extractos de acetona derivados de las partes aéreas y tubérculos de *A. liemiana*. **Metodología:** Los especímenes de *A. liemiana* se recolectaron de la montaña Takou dentro de la Reserva Natural de Takou, provincia de Binh Thuan, y se sometieron a extracción con acetona. Los componentes químicos de estos extractos se analizaron mediante GC-MS, mientras que la actividad antibacteriana se evaluó mediante el método de difusión en disco de agar. **Resultados:** Se identificaron 57 compuestos en los extractos de la parte aérea y del tubérculo, de los cuales 32 componentes se encontraron en el primer extracto, mientras que el último contenía 34 constituyentes. Se evaluó la eficacia antibacteriana de los extractos de acetona del tubérculo frente a 10 cepas bacterianas, lo que reveló diversas zonas de inhibición. Cabe destacar que *S. aureus* ATCC 25923 exhibió el mayor diámetro de zona de inhibición, seguido de *B. cereus*, *V. parahaemolyticus*, *S. flexneri*, *P. aeruginosa*, *E. coli*, *L. monocytogenes*, *S. saprophyticus*, *S. typhimurium* y *S. enteritidis*. **Conclusión:** Se investigó por primera vez la composición química y las propiedades

antibacterianas de los extractos de acetona derivados de *Arisaema liemiana*. La notable eficacia antibacteriana observada sugiere posibles implicaciones farmacológicas de *A. liemiana*. Además, esta investigación revela aplicaciones más amplias de *A. liemiana* en la medicina herbal.

Palabras clave: Aracae, *Arisaema liemiana*, GC-MS, actividad antibacteriana.

Resumo

Fitoquímica e propriedade antibacteriana de *Arisaema liemiana*

Introdução: *Arisaema liemiana* foi recentemente identificada como uma nova espécie para a flora do Vietnã em 2020. Foi descoberta na Reserva Natural de Takou, província de Binh Thuan, Vietnã, caracterizada por sua altura de 20-50 centímetros, natureza caducifólia, membro de espata amarelo claro, tubo de espata branco e apêndice de espádice coberto com estruturas neutras filiformes. **Objetivo:** Este estudo teve como objetivo investigar os componentes químicos e a atividade antibacteriana dos extratos de acetona derivados das partes aéreas e tubérculos de *A. liemiana*. **Metodologia:** Espécimes de *A. liemiana* foram coletados da Montanha Takou dentro da Reserva Natural de Takou, Província de Binh Thuan, e submetidos à extração de acetona. Os componentes químicos desses extratos foram analisados usando GC-MS, enquanto a atividade antibacteriana foi avaliada através do método de difusão em disco de ágar. **Resultados:** Um total de 57 compostos foram identificados na parte aérea e extratos de tubérculos, dos quais 32 componentes foram encontrados no primeiro extrato, enquanto o último continha 34 constituintes. A eficácia antibacteriana dos extratos de acetona de tubérculos foi avaliada contra 10 cepas bacterianas, revelando diversas zonas de inibição. Notavelmente, *S. aureus* ATCC 25923 exibiu o maior diâmetro de zona de inibição, seguido por *B. cereus*, *V. parahaemolyticus*, *S. flexneri*, *P. aeruginosa*, *E. coli*, *L. monocytogenes*, *S. saprophyticus*, *S. typhimurium* e *S. enteritidis*. **Conclusão:** A composição química e as propriedades antibacterianas dos extratos de acetona derivados de *Arisaema liemiana* foram investigadas pela primeira vez. A notável eficácia antibacteriana observada sugere potenciais implicações farmacológicas de *A. liemiana*. Além disso, esta pesquisa revela aplicações mais amplas de *A. liemiana* na medicina herbal.

Palavras-chave: Aracae, *Arisaema liemiana*, GC-MS, atividade antibacteriana.

Introduction

Arisaema Martius is one of a large genus belonging to Araceae family which includes 15 sections [1] and about 201 species [2] widely found in the tropical Asia, Himalayas, Australia and New Guinea [3-7]. About 25 species belonging to 6 sections have been recorded for the flora of Vietnam [2, 3, 7-11]. So many *Arisaema* members have been known as the medicinal plants. For example, *A. rhizomatum* is known for its traditional medicine to treat contusions, injuries from falls, strains. It is also used to dispel wind, quicken blood, free the flow of network vessels, etc. [11]. Apart from that, the tuber extracts of *A. amurense*, *A. asperatum*, *A. calcareum*, *A. serratum*, and *A. heterophyllum* were used as antitumor, analgesic, and pesticide agents [12]. In addition, studies provided the chemical compositions and pharmaceutical properties of the extracts obtained from various *Arisaema* plants [13-18].

Arisaema liemiana Luu, H.T.Van, H.C.Nguyen & V.D.Nguyen was first described as a new species for the flora of Vietnam in 2020 which the type specimens collected from Takou Nature Reserve, Binh Thuan province, Vietnam. *A. liemiana* is a deciduous herbs and the morphology of this species are characterized by having: 20-50 high deciduous, the light yellow spathe limb, white spathe tube, spadix appendix covered with filiform neuters [2]. To date, *A. liemiana* is a rare species and it is only found in the location where its type specimens were collected. Thus, the present study firstly investigated the chemical components and antibacterial activity of the acetone extracts from the aerial parts and tubers of *A. liemiana*.

Materials and methods

Plant

Arisaema liemiana was collected from Takou Mountain, Takou Nature Reserve, Binh Thuan province (Figure 1) where the type specimens of this species were discovered in the year 2020 [2].

Figure 1. *Arisaema liemiana* in its habitat

Bacterial strains

In this study, ten tested bacteria were used to identify the antibacterial activities of the acetone extracts of the studied species, including four *Gram-positive* bacteria (*Bacillus cereus* ATCC 11774, *Listeria monocytogenes* ATCC 19111, *Staphylococcus aureus* ATCC 25923, *Staphylococcus saprophyticus* BAA750), and six *Gram-negative* bacteria (*Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 13311, *Salmonella enteritidis* ATCC 13976, *Pseudomonas aeruginosa* ATCC 27853, *Shigella flexneri* ATCC 9199, *Vibrio parahaemolyticus* ATCC 17802).

Extract preparation

The fresh aerial and tuber of *A. liemiana* were dried at 50°C and then ground into powder. Five hundred milliliters of 99% acetone solution (Thermo Fisher Scientific, USA) were used to soak 100 grams of the sample for for 48 hours at room temperature. The extract was obtained by filtration using Whatman paper and its residue was re-extracted two more times. The final filter was evaporated under vacuum condition at 45°C to remove acetone solution.

Gas Chromatography-Mass Spectrometry (GC-MS)

This method was performed using a TRACE™ 1310 Gas Chromatograph (Thermo Fisher Scientific Inc., Waltham, MA, USA) coupled with an ISQ 7000 Single Quadrupole Mass Spectrometer. A DB-5MS column (30 m × 0.25 mm × 0.25 μm) was utilized as the stationary phase, and Helium at a flow rate of 1.2 mL/min was used as

the carrier gas to determine the chemical composition present in the acetone extract. The sample was injected into the GC system using a sample splitting method, adopting a 30:1 split ratio, a non-split flow duration of 1 minute, at 250°C with a flow rate of 36 mL/min. The injection temperature was consistently held at 250 °C. The thermal program of the oven was designed to start at 80 °C, sustained for 5 minutes, followed by an increment of 20 °C/min until a terminal temperature of 280 °C was reached, which was then maintained for 10 minutes. Electron impact ionization was set at 70 eV and the source filament temperature at 250 °C. The mass scan range of the MS was 29-650 m/z with a scan rate of 2 scans per second.

Spectral analysis was conducted using Thermo Scientific Xcalibur software, which facilitated data generation. Chemical component identification within the extract was predicated on retention times, mass spectra, and the peak areas of the compounds, which were compared against the known spectra in the library (NIST library 2017). The percentage composition of the compounds was quantified based on the peak area of each compound relative to the total peak area of all compounds, multiplied by 100.

Antimicrobial methods

The agar disc diffusion method was utilized to assess the antibacterial activity of the samples [19]. Bacterial strains are cultivated in Mueller-Hinton Broth (MHB) until a turbidity of 0.5 McFarland standard was attained. A 0.1 mL sample of the bacterial suspension was inoculated onto a Petri dish containing Mueller-Hinton Agar (MHA) and spread uniformly. Paper discs were then placed onto the agar surface, and 10 µL of the sample solution was applied onto these discs. Gentamycin antibiotic discs (Nam Khoa, Vietnam) were used as positive controls, while a 10% acetone solution serves as the negative control. The Petri dishes are stored at 4 °C for 2 hours to allow the sample solution to permeate into the agar. The cultures were then incubated at 37 °C for 16-18 hours to evaluate the resistance of the extract against the bacterial strains. The diameter of the inhibition zone was measured after 16-18 hours. The antibacterial activity of the sample solutions against the bacterial strains was determined based on the diameter of the inhibition zones, deducting the diameter of the paper disc (6 mm), which will be the zone of bacterial inhibition of the research sample.

To evaluate the differences between the means of the experimental treatments, the analysis of variance (ANOVA) method, following the LSD test, was employed. Statgraphic Centurion software version 15.2 and Excel 2010 were used to calculate the mean and standard deviation among the measurements.

Results and discussion

Chemical compositions of acetone extracts from *A. liemiana* tuber and aerial part

The chemical constituents of the acetone extracts of the *A. liemiana* tuber and aerial part were shown in the Figure 2 and Table 1. The aerial part extract was mainly composed of 2-pentanone, 4-hydroxy-4-methyl- (12.62%), 2,3-butanediol (11.61%), n-hexadecanoic acid (8.09%), neophytadiene (8.87%), phytol (6.64%), 9(E),11(E)-conjugated linoleic acid (6.49%), linolenic acid (6.38%), 2,6-cresotaldehyde (4.93%), γ-sitosterol (4.42%), linolein, 2-mono-(4.14%), stigmasterol (3.95%). The major compounds in the tuber extract were consisted of 2-pentanone, 4-hydroxy-4-methyl- (21.95%), γ-sitosterol (13.91%), stigmasterol (12.08%), campesterol (10.72%), ascorbic acid 2,6-dihexadecanoate (7.67%), 9(E),11(E)-conjugated linoleic acid (6.89%), cis-vaccenic acid (4.28%).

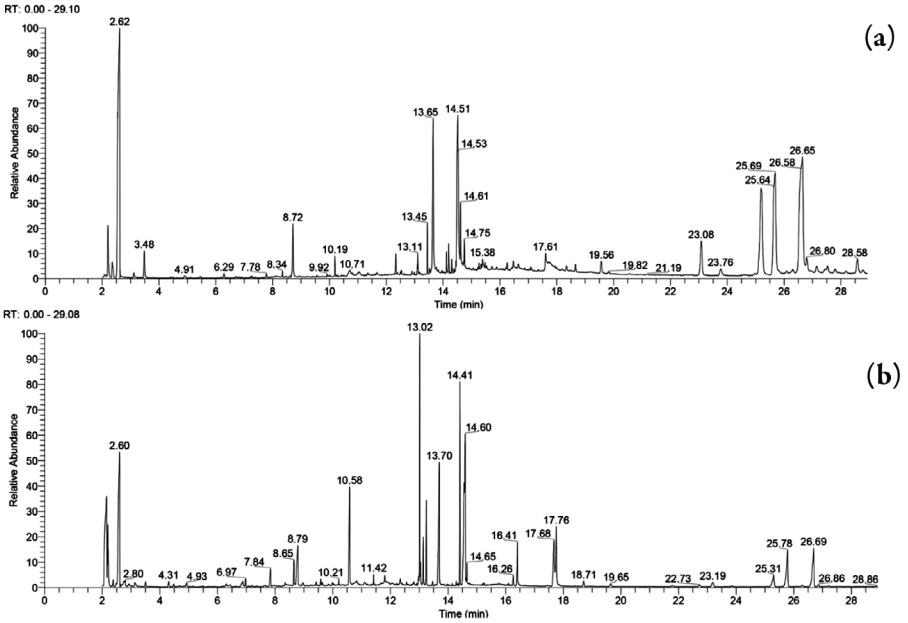

Figure 2. The GC chromatogram of acetone extracts from *A. liemiana* tuber (a) and aerial part (b)

The chemical compositions of the essential oils or the various solvent extracts obtained from the *Arisaema* plants using GC-MS technique were also reported by prior studies. For instance, the chemical compounds of the essential oils isolated from four organs like fruits, leaves, tubers and petioles of *A. amuremse* were also reported. Accordingly,

the fruit essential oil was found to be rich in hexadecanoic acid methyl ester (53.45%), 13-octadecenoate (7.82%), and (Z, Z)-9,12-octadecadienoic acid methyl ester (5.4%). The petiole oil contained 7,9-di-tert-butyl-1-oxaspiro (4,5) deca-6,9-diene-2,8-dione (7.9%); 14-methylpentadecanoic acid methyl ester (7.7%); 1-hexadecanol acetate (4.05%) as the major compounds. The tuber essential oil was characterized by the prominence of 3-cyclohexyl-1-phenyl propane (14.86%), 2-pentadecanone (8.9%), perhydrofarnesyl acetone (6,10,14-trimethyl-2-pentadecanone (7.15%) whereas 6,10,14-trimethyl-2-pentadecanone (46.27%); hexadecanoic acid methyl ester (38.62%) and (Z) 9-octadecenoic acid methyl ester (12.27%) were the main components in the leaf oil [13].

In the case of the methanolic extract of *A. tortuosum* leaf, Garg *et al.* demonstrated the identification of a total of fifty-three phytochemicals. The initial compound eluted was nonane, which exhibited a retention time of 4.064 minutes within the leaf extract. Predominant peak areas were observed for 6,10,14-trimethyl-2-pentadecanone at 54.55% and phytol at 18.86% within the leaf extract. Other notable phytochemicals identified included 2-pentadecanone, 6,10,14-trimethyl-; phytol; benzene, 1,3-dimethyl-, etc. [20]. Moreover, the chemical components of the ethanolic extract, and its fractions like n-hexane and chloroform obtained from *A. tortuosum* leaf were also investigated. Accordingly, the ethanolic extract included 9,12,15-octadecatrienoic acid, methyl ester, (Z,Z,Z)- (37.53%); hexadecanoic acid, methyl ester (20.57%) and 9,12-octadecadienoic acid, methyl ester (20.14%) as the major compounds. n-hexane fraction was found to be rich in phytol (16.90%); ethyl (9z,12z)-9,12-octadecadienoate (14.97%); hexadecanoic acid, ethyl ester (14.60%); dibutyl phthalate (19.33%); 1,2-benzenedicarboxylic acid, Bis(2- methylpropyl) ester (12.11%) and 1,2-benzenedicarboxylic acid (8.53%) [21].

Table 1. The chemical compounds of acetone extracts of *A. liemiana* aerial part and tuber

RT	Compounds	Relative peak area (%)	
		Aerial part	Tuber
2.15	2,3-Butanediol	11.61	-
2.21	3-Penten-2-one, 4-methyl-	-	2.00
2.36	2-Pentanone, 4-hydroxy-	-	0.79
2.60	2-Pentanone, 4-hydroxy-4-methyl-	12.62	21.95
2.80	L-Lactic acid	0.45	-
3.12	2-Ethyl-trans-2-butenal	-	0.24
3.48	2-Heptanol, acetate	-	1.16
4.31	2,5-Hexanedione, 3,4-dihydroxy-3,4-dimethyl-	0.40	-

(Continued)

Table 1. *Continuation.*

RT	Compounds	Relative peak area (%)	
		Aerial part	Tuber
4.91	1,3-Dioxane, 2-methyl-	-	0.26
4.93	2-Acetoxyisobutyryl chloride	0.36	-
5.45	Tetraethylene glycol, diacetate	-	0.12
6.29	Benzeneacetaldehyde	-	0.21
6.70	D-Alanine, N-propargyloxycarbonyl-, isohexyl ester	-	0.15
6.97	Pyrazine, tetramethyl-	0.41	-
7.84	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl-	1.07	0.23
8.34	2-Pentanol, 2,4-dimethyl-	-	0.19
8.65	Coumaran	1.03	-
8.79	5-Hydroxymethylfurfural	3.75	2.28
9.58	2-Methoxy-4-vinylphenol	0.41	
10.19	1,6-Octadiene, 2,6-dimethyl-	-	0.60
10.58	2,6-Cresotaldehyde	4.93	-
11.42	Phenol, 4-ethenyl-2,6-dimethoxy	0.45	-
12.33	13-Methyltetradecanal	-	0.70
12.53	Tetradecanoic acid	-	0.24
13.02	Neophytadiene	8.87	-
13.04	3,7,11,15-Tetramethylhexadec-2-ene	0.56	-
13.09	Pentadecanoic acid	-	0.27
13.11	(E)-Hexadec-2-enal	-	0.49
13.24	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	3.71	-
13.47	Hexadecanoic acid, methyl ester	0.26	-
13.65	Ascorbic acid 2,6-dihexadecanoate	-	7.67
13.70	n-Hexadecanoic acid	8.09	-
14.12	Heptadecanoic acid	-	0.67
14.20	Verticiol	-	0.79
14.30	8,11-Octadecadienoic acid, methyl ester	-	0.49
14.41	Phytol	6.44	-
14.53	cis-Vaccenic acid	-	4.28
14.56	9(E),11(E)-Conjugated linoleic acid	6.49	6.89
14.60	Linolenic acid	6.38	-
14.65	Octadecanoic acid	0.71	1.94
15.38	2-cis-9-Octadecenyloxyethanol	-	0.45

(Continued)

Table 1. *Continuation.*

RT	Compounds	Relative peak area (%)	
		Aerial part	Tuber
16.26	Heptacos-1-ene	0.58	-
17.61	7-Methyl-Z-tetradecen-1-ol acetate	-	0.66
17.68	Linolein, 2-mono-	4.14	-
17.76	Butyl 9,12,15-octadecatrienoate	3.80	-
18.34	E,E,Z-1,3,12-Nonadecatriene-5,14-dio	-	0.22
18.71	Squalene	0.51	-
19.56	Octadecane, 3-ethyl-5-(2-ethylbutyl)-	-	0.62
19.65	17-Pentatriacontene	0.41	-
21.79	γ-Tocopherol	0.31	-
22.73	17-Pentatriacontene	0.37	-
23.19	Vitamin E	0.81	2.74
25.31	Campesterol	1.58	10.72
25.78	Stigmasterol	3.95	12.08
26.69	γ-Sitosterol	4.42	13.91
26.80	Stigmasta-5,24(28)-dien-3-ol, (3β,24Z)-	-	0.76
28.58	α-Tocopheryl acetate	-	0.89
	Total	**99.88**	**97.66**

Antibacterial activity of acetone extracts from *A. liemiana* tuber and aerial part

Overall, the acetone extract from *A. liemiana* tuber displayed activity against all the tested bacteria whereas these strains were not sensitive to the aerial part extract (Table 2). Accordingly, the tuber extract showed the strongest antibacterial effects against *S. aureus* ATCC 25923 with the inhibition zone diameter of 7.7 mm, followed by *B. cereus* (6.2 mm), *V. parahaemolyticus* (5.3 mm), *S. flexneri* (4.8 mm), *P. aeruginosa* (4.7 mm), *E. coli* (4.7 mm), *L. monocytogenes* (4.3 mm), *S. saprophyticus* (4.2 mm), *S. typhimurium* (2.3 mm), *S. enteritidis* (1.8 mm).

Prior reports demonstrated that members of the genus *Arisaema* grown in some Asian countries were used in the ethnopharmacology as an antimicrobial agent. For instance, in traditional medicine in India and China, *A. consanguineum* was extensively used as the antifungal and antibacteral medications [21]. Furthermore, *A. jacquemontii* was traditionally used as a folk remedy to treat microbial illness In Bhutan, India, Nepal, and Pakistan [22]. Similarly, In Chinese and Indian traditional medicine, *A. flavum* was also used as bacterial efficacy [23]. In addition, studies also provided the antibacterial activities of different solvent extracts obtained from the *Arisaema* species. For instance,

the ethanolic extract of the *Arisaema tortuosum* leaf had an inhibitory effect on *B. subtilis* and *S. typhimurium* whereas two its fractions, n-hexane and chloroform was active against *S. aureus* and *E. coli*, respectively [24]. Furthermore, the different extracts from *A. jacquemontii*, acetone, chloroform, distilled water, and methanol, was found to be effective against so many bacterial strains, including *Salmonella typhi*, *Pseudomonas aeruginosa*, and *Shigella dysenteriae*, *Lysteria monocytogenes* and *Staphylococcus aureus* [25]. Similarly, the ethanol and methanol extracts from *A. jacquemontii* displayed activity against some bacterial and fungal strains, including *Micrococcus luteus* and *Aspergillus flavus* [17]. The methanolic extract of *A. jacquemontii* root was also reported to have an inhibitory effect on many microorganisms, such as *Proteus mirabilis*, *Streptococcus faecalis*, *Escherichia coli*, *Salmonella enteritidis*, *Micrococcus luteus*, *Enterobacter cloacae*, *Bacillus subtilis*, *Staphylococcus aureus*, and *Pasteurella multocida* [26].

Table 2. The antibacterial activity of acetone extracts from *A. liemiana* tuber

Bacterial strains	Inhibition zone diameter (mm)		
	Tuber	Gentamicine	Negative control
Bacillus cereus ATCC 11774	6.2 ± 0.6[a]	18.7 ± 0.8[b]	-
Escherichia coli ATCC 25922	4.7 ± 0.3[a]	14.8 ± 0.3[b]	-
Listeria monocytogenes ATCC 19111	4.3 ± 0.3[a]	6.3 ± 0.6[b]	-
Pseudomonas aeruginosa ATCC 27853	4.7 ± 1.2[a]	24.7 ± 0.6[b]	-
Staphylococcus aureus ATCC 25923	7.7 ± 0.6[a]	25.2 ± 1.0[b]	-
Staphylococcus saprophyticus BAA750	4.2 ± 0.3[a]	23.7 ± 0.6[b]	-
Salmonella enteritidis ATCC 13976	1.8 ± 1.3[a]	12.3 ± 0.3[b]	-
Salmonella typhimurium ATCC 13311	2.3 ± 0.6[a]	14.8 ± 0.8[b]	-
Shigella flexneri ATCC 9199	4.8 ± 0.8[a]	18.2 ± 1.0[b]	-
Vibrio parahaemolyticus ATCC 17802	5.3 ± 0.6[a]	12.3 ± 0.3[b]	-

Different lowercase letters within a row indicate significant differences in the inhibition zone diameter (± SD) (mm) between acetone extracts of Tuber and Gentamicin (LSD test: $P < 0.05$)

Conclusion

This research provides insights into the recently identified plant species *Arisaema liemiana* found in the flora of Vietnam in Takou Nature Reserve, Binh Thuan province. By examining acetone extracts from both aerial parts and tubers, the study investigated the chemical composition and antibacterial effects of *A. liemiana*. GC-MS analysis facilitated the identification of the chemical constituents, while evaluation against 10 bacterial strains revealed significant antibacterial activity of the tuber acetone extract. These findings enhance the understanding the pharmacological potential of *A. liemiana* and underscore its importance in medicinal and ecological research. Further

investigations are warranted to elucidate the mechanisms underlying its antibacterial properties and explore its broader pharmacological applications.

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Compliance with ethical standards

Conflict of interest: The authors have reported no conflicts of interest

Ethics approval: Not applicable.

Author contributions: All authors contributed to the development, analysis, and drafting of this article

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An analytical of an experimental method for evaluation and validation from measuring dissolution of Metformin and Empagliflozin for generic tablets: Confirmation the results by docking method

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Summary

Aim: Validation for assay and dissolution of metformin and Empagliflozin in tablet 500:5 mg for evaluating the efficacy and safety of linagliptin vs. placebo in patients with type 2 diabetes. **Materials and methods:** The HPLC system used was Shimadzu LC-2010 *HT* with UV detector and Empagliflozin standard stock 1: 28 mg of Empagliflozin was accurately prepared. Separately 10 µL of standard solution and sample solution was injected into the chromatographic system. Patients with inadequate glycaemia control despite stable-dose metformin received open-label Empagliflozin as add-on therapy for several weeks. All patients continued treatment with metformin and Empagliflozin or metformin and Empagliflozin. The primary endpoint was change from baseline. **Results:** The experiments were significantly

exhibited a reducing the problem in patients with type 2 diabetes. In addition, results of docking method confirmed also the effectiveness of Metformin/ Empagliflozin tablets to better controlling of diabete-type-2 disease. **Conclusions**: Empagliflozin in Metformin/Empagliflozin for 24 weeks improved glycaemia control and was well tolerated.

Keywords: Metformin; Empagliflozin; type 2 diabetes; HPLC; glycaemia control; docking simulation; molecular dynamic; drug design.

Resumen

Análisis de un método experimental para la evaluación y validación de la medición de la disolución de metformina y empagliflozina en comprimidos genéricos: confirmación de los resultados mediante el método de acoplamiento

Objetivo: Validación del ensayo y disolución de metformina y empagliflozina en comprimidos de 500:5 mg para evaluar la eficacia y seguridad de la linagliptina frente a placebo en pacientes con diabetes tipo 2. **Materiales y métodos:** El sistema HPLC utilizado fue Shimadzu LC-2010 HT con detector UV y para la solución madre estándar de empagliflozina 1: se prepararon con precisión 28 mg de empagliflozina. Se inyectaron por separado 10 µL de solución estándar y la solución de muestra en el sistema cromatográfico. Los pacientes con un control inadecuado de la glucemia a pesar de la administración de metformina en dosis estables recibieron empagliflozina de etiqueta abierta como terapia complementaria durante varias semanas. Todos los pacientes continuaron el tratamiento con metformina y empagliflozina o metformina y empagliflozina. El criterio de valoración principal fue el cambio con respecto al valor inicial. **Resultados:** Los experimentos mostraron una reducción significativa del problema en pacientes con diabetes tipo 2. Además, los resultados del método de acoplamiento confirmaron también la eficacia de los comprimidos de metformina/empagliflozina para controlar mejor la enfermedad diabética tipo 2. **Conclusiones:** La empagliflozina en combinación con metformina/empagliflozina durante 24 semanas mejoró el control de la glucemia y fue bien tolerada.

Palabras clave: metformina; empagliflozina; diabetes tipo 2; HPLC; control de la glucemia; simulación de acoplamiento; dinámica molecular; diseño de fármacos.

Resumo

Uma análise de um método experimental para avaliação e validação da medição da dissolução de Metformina e Empagliflozina para comprimidos genéricos: Confirmação dos resultados pelo método de encaixe

Objetivo: Validação para ensaio e dissolução de metformina e Empagliflozina em comprimido 500:5 mg para avaliar a eficácia e segurança da linagliptina vs. placebo em pacientes com diabetes tipo 2. **Materiais e métodos:** O sistema HPLC usado foi Shimadzu LC-2010 HT com detector UV e estoque padrão de Empagliflozina 1: 28 mg de Empagliflozina foram preparados com precisão. Separadamente, 10 µL de solução padrão e solução de amostra foram injetados no sistema cromatográfico. Pacientes com controle glicêmico inadequado, apesar da metformina em dose estável, receberam Empagliflozina de rótulo aberto como terapia complementar por várias semanas. Todos os pacientes continuaram o tratamento com metformina e Empagliflozina ou metformina e Empagliflozina. O desfecho primário foi a mudança da linha de base. **Resultados:** Os experimentos exibiram significativamente uma redução do problema em pacientes com diabetes tipo 2. Além disso, os resultados do método de encaixe confirmaram também a eficácia dos comprimidos de Metformina/Empagliflozina para melhor controle da doença diabética tipo 2. **Conclusões:** A empagliflozina em Metformina/Empagliflozina por 24 semanas melhorou o controle da glicemia e foi bem tolerada.

Palavras-chave: Metformina; Empagliflozina; diabetes tipo 2; HPLC; controle da glicemia; simulação de encaixe; dinâmica molecular; design de fármacos.

Introduction

Analytical Method

Analytical chemistry offers with the look at of the separation, quantification, and chemical identity pharmaceutical analysis could be very crucial within the dedication of prescription drugs formula and bulk drugs regarding quality manage and high-quality guarantee. The quick increase in pharmaceutical industries of herbal and non-natural substances composed with one or more compounds or element. It is categorized into two main types, a qualitative analysis that is concerned with the identification of chemical compounds present in the sample whereas quantitative the analysis determines the

number of certain elements or compounds in the sample and the producing of drugs in and around the sector cells searching for novel and advance analytical techniques in prescribed drugs. As a result, analytical method development has become a popular activity of analysis. Development in scientific and advanced analytical techniques is due to the advancement of analytical instruments [1-3]. The aim of the present work was to develop an accurate and sensitive HPLC analytical method validation for assay and dissolution of metformin and Empagliflozin in metformin/Empagliflozin tablet 500:5 mg that might be improved glycaemia controlling. Here we also described the optimization of the instrumental parameters as well as the extraction procedure for human type 2 diabetes disease through dissolution of Metformin and Empagliflozin in Metformin/Empagliflozin. The method had been validated by evaluating the precision, accuracy and other validation parameters [3-7].

Metformin mono-therapy

Metformin mono-therapy, the recommended first-line pharmacotherapy for patients with type 2 diabetes (T2D), while initially effective, often fails to maintain glycaemia control as T2D progresses.1, 2 Therefore, additional effective and well tolerated therapies are required when metformin mono-therapy fails to maintain glycaemia control. There are no uniform recommendations regarding the best agent to combine with metformin [8]. Although metformin mono-therapy, for patients with type 2 diabetes (T2D) [8] initially was effective, but sometimes does not act to control the diabetes progresses properly [8, 9]. Therefore, additional drugs are needed when metformin mono-therapy fails to maintain glycaemia control [8, 10]. Empagliflozin, a potent and selective inhibitor of the sodium glucose co-transporter [9] (SGLT2), is one of the most recommended items for T2D [10] due to that SGLT2 inhibitors reduce renal glucose reabsorption, thereby increasing urinary glucose excretion and reducing hyperglycaemia [11]. In a unique testing in patients with T2D, Empagliflozin 10 mg and 25 mg given as add-on to metformin for 6 months provided clinically relevant reductions in HbA1c, fasting plasma glucose (FPG), weight and systolic blood pressure (SBP) vs. placebo effects, exactly [12]. If using dual mixtures of anti-diabetes agents did not work properly, triple combinations are required [8, 10]. Di-peptidyl peptidase-4 (DPP-4) inhibitors, which recommended as second- or third-line treatment option for patients with T2D is a potent and selective DPP-4 inhibitor [13, 14]. Since DPP-4 destroyed incretin hormones, in contrast linagliptin increases the concentrations of active incretin hormones, stimulating the release of insulin in a glucose-dependent manner and decreasing the levels of glucagon in the circulation, resulting in better regulation of glucose homeostasis [15]. For patients without controlling diabetes disease with metformin and a sulphonyl-urea, or with metformin and pioglitazone, linagliptin improved glycaemia control, with a low risk of hypo-glycaemia and a neutral effect on weight

[16-23]. The combination of SGLT2 inhibitors and DPP inhibitors with metformin is a recommended third-line treatment option for patients with T2D.

Docking Simulation

Although metformin is a compound that belongs to the class of drugs known as biguanidine and is the first therapeutic choice for Type 2 Diabetes Mellitus, unfortunately it has been used for more than four decades with several side effects. Therefore a novel drug with no side effects should be designed and replace. Since metformin inhibits hepatic gluconeogenesis and prevents hyper-glycaemia without impairing insulin secretion, hypoglycemia, or weight gain, it is an essential step to simulate the molecules, including 1-Carbamimidoyl-1,2-dimethyl-guanidine hydrochloride, Metformin hydrochloride, *N*1,*N*1-Dimethyl-*N*5-methylbiguanide hydrochloride, and *N*1,*N*1,*N*5,*N*5-Tetrakis (methyl-bi-guanine hydrochloride], via molecular docking simulation for analyzing the properties of these four analogues of metformin, as well as through a combination with Empagliflozin.

Materials and methods

HPLC system

The HPLC system (228 nm, 1.0 mL·min^{-1}) was used by Shimadzu LC-2010 *HT* with UV detector, including 2.8 g monobasic potassium phosphate at pH=4.5 with 10 μL acetonitrile as buffer. Using 'LC Solution software Version 1.21' data processing and chromatographic integration was carried out. A reciprocating shaker used for liquid–liquid extraction was procured from Trishul Equipment. The nitrogen evaporator used to evaporate the samples was procured from Takahe Analytical Instruments. Hitachi centrifuge machine was used for the sample preparation. Deep freezers used for storage of plasma samples were procured from Sanyo (Japan). Dissolution Condition: Medium: 900 mL, 6.08 gr monobasic potassium phosphate in 1000 mL water. PH=6.8 Apparatus: (2) paddle, Time: 30 min, RPM: 50

Preparation of standard solution

Empagliflozin standard stock 1: 28 mg of Empagliflozin WS was accurately weighed and taken in 50 mL clean and dry volumetric flask then adding containing 20 mL of Acetonitrile by Sonicated for 2 min and then the solution was made up to the mark by medium solution. Empagliflozin standard stock 2: 2 mL of stock solution were transferred as 1 to 20 mL volumetric flask and made up to mark by medium solution. Standard solution: 28 mg of Metformin HCl WS was accurately weighed and taken in a 50 mL clean and dry volumetric flask then adding to containing 20 mL of medium solu-

tion by sonication for 2 min and then adding 5 mL of Empagliflozin standard stock 2 to the flask. Thus, the solution was made up to the mark by medium solution (In assay preparation use Diluent instead of medium solution) (Metformin/ HCl: Empagliflozin standard; 0.56:0.0056 mg/mL).

Preparation of Test Solution (TS)

Assay: Not less than 10 tablets were taken, powdered and test stock solution of Metformin HCl: Empagliflozin was prepared by transferring weight equivalent to 500 mg of Metformin HCl and 5 mg of Empagliflozin to 70 mL of Diluent which is sonication and shake intermittently for 10 min and later made up to 100 mL with diluent. Transfer 1 mL of this solution to 10 volumetric flasks and fill to volume with diluent. This solution was filtered using a 0.22-micron syringe filter. (Metformin HCl, Empagliflozin standard, 0.50:0.005 mg/mL). Dissolution: Metformin HCl/ Empagliflozin tablet was dissolved in 900 mL medium for dissolution test (Metformin HCl: Empagliflozin standard; 0.56:0.0056 mg/mL).

Preparing stock solution

Not less than 10 tablets were taken, powdered and test stock solution was prepared by transferring weight equivalent to 500 mg of Metformin HCl and 5 mg of Empagliflozin to 80 mL of Diluent which is sonicated and shacked intermittently for 10 min and later made up to 100 mL with diluent.

Hydrolytic degradation under acidic condition

1 mL stock solution of Metformin HCl: Empagliflozin was prepared and taken in a 10 mL volumetric flask; 0.75 mL of 0.1 N HCl was added. Then the volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N NaOH and the volume was made up to the mark with the Diluent. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Hydrolytic degradation under alkaline condition

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 0.75 mL of 0.1N NaOH was added. Then the volumetric flask was kept at normal condition for 90 minutes and then neutralized with 0.1 N HCl and the volume was made up to the mark with the Buffer. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Thermal induced degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 1.5 mL of the Diluent was added. Then the volumetric flask was kept at reflex condition for 60 minutes and further the volume was made up to the mark with the Buffer. The resultant solution was filtered with 0.45 microns' syringe filters and placed in the vials.

Oxidative degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and taken in a 10 mL volumetric flask; 0.5 mL of 3 % w/v of hydrogen peroxide solution was added and the volume was made up to the mark with Diluent. Further the volumetric flask was kept at room temperature for 15 min. The resultant solution was filtered with 0.45-micron syringe filters and placed in the vials.

Procedure

Separately inject 10 µL of standard solution and sample solution into the chromatographic system. Record the chromatograms and measure the peak responses. Calculate the assay and dissolution results of samples by the following formula: Result% = $(A_T/A_S)\times(C_S/C_T)\times100$, where: A_T = sample area, A_S = standard area, W_s = Concentration of standard, W_T = Concentration of sample.

Method precision

Method precision was determined by performing assay and dissolution of the sample under the tests of (i) repeatability (Intraday precision) and (ii) Intermediate precision (Inter day precision or ruggedness) performed during 2 consecutive days by two different analysts, at working concentration.

Method validation

Validation of the analytical method is the process that is established by laboratory studies in which the performance characteristics of the method meet the requirements for the intended analytical application. HPLC method developed was validated according to International Conference on Harmonization (ICH) guidelines for validation of analytical procedures. The method was validated for the parameters like linearity, accuracy, system precision, intra-day precision, inter-day precision/ intermediate precision/ruggedness.

Results

Selectivity of the analytical method is defined as the degree to which a method can quantify the analyte in the presence of interferes. Specificity study of the chromatographic method is performed by the separation of the analyte from the other potential components such as impurities, degradants or excipients etc. In addition, forced degradation studies are carried out to challenge the method. The forced degradation studies are of particular importance when the impurities are not available. During forced degradation studies, the sample is subjected to the stressed conditions of light, heat, humidity, acid/base hydrolysis and oxidation.

Linearity

The linearity of the analytical procedure is its ability (within a given range) to obtain the test results which are directly proportional to the concentration (amount) of analyte in the sample. Standard solutions of Metformin HCl: Empagliflozin at different concentrations level (50-150%) were prepared. Calibration curve (Fig. 1) was constructed by plotting the concentration level of drug versus peak area. The results show an excellent correlation between peak area and concentration level of Metformin HCl: Empagliflozin within the concentration range (tables 1 and 2). The correlation coefficient was greater than 0.999, which meets the method's validation acceptance criteria and hence the method is said to be linear in the range of 0.28-0.84 mg/mL for Metformin HCl and 0.0028-0.0084 mg/mL for Empagliflozin (figures 1 and 2).

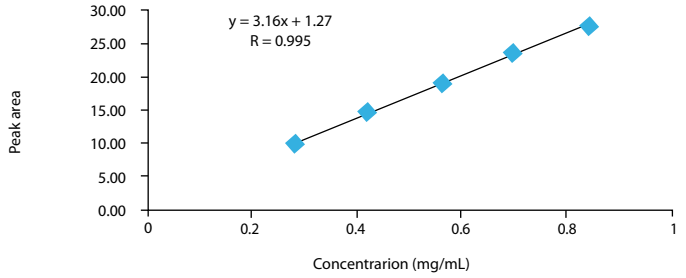

Figure 1. The Linearity plot of Metformin HCl

Table 1. Linearity results (part a)

Standards	1 (50%)	2 (75%)	3 (100%)	4 (125%)	5 (150%)
Metformin HCl Concentration (mg/mL)	0.28	0.42	0.56	0.7	0.84
Peak Area × 10^6	9.9	14.8	19	23.6	27.7

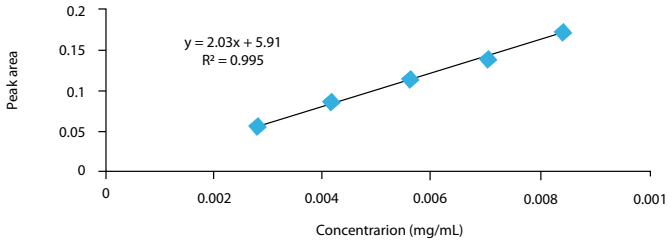

Figure 2. The Linearity plot of Metformin HCl

Table 2. Linearity results (part b).

Standards	1 (50%)	2 (75%)	3 (100%)	4 (125%)	5 (150%)
Empagliflozin Concentration (mg/mL)	0.0028	0.0042	0.0056	0.007	0.0084
Peak Area × 10^4	5.74	8.62	11.36	14.34	17.10

System precision

The precision of an analytical procedure expresses the closeness of measurements obtained from multiple sampling of the same homogenous sample under the prescribed conditions implied five replications of the standard solution at the target concentrations were injected. Results showed Relative Standard Deviation (RSD) was less than 2%, which indicates acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in tables 3-a,b)

Table 3. System precision

Y=3.16X+1.27, lim of RSD<2% (a)		
Area number	**Cal. conc.(mg/mL)**	**Assay %**
Area 1	0.5657	100.8
Area 2	0.5653	100.7
Area 3	0.5617	100.1
Area 4	0.5657	100.8
Area 5	0.5686	101.3
medium	1.9189	100.75
STDEV	7.86	0.41
RSD	0.44	0.41

(Continued)

Table 3. *Continuation*.

Y=2.0X+5.91, lim of RSD<2% (b)		
Area 1	0.00554	99.5
Area 2	0.00558	100.2
Area 3	0.00554	99.6
Area 4	0.00559	100.7
Area 5	0.00556	99.9
medium	0.00557	100
STDEV	530	0.47
RSD	0.47	0.47

Repeatability (Intraday precision)

Three replications of the sample solution at the three concentrations (50%, 100% and 150%) were injected. Results showed Relative Standard Deviation (RSD) was less than 2%, which indicates acceptable reproducibility and thereby the precision of the system. System precision results are tabulated in (tables 4-a, b, c, d, e, f).

Table 4. Intraday precision of Empagliflozin/metformin·HCl

Y=3.16X+1.27, lim of RSD<2% a) Repeatability of 50%.			**Y=2.0X+5.91, lim of RSD<2% d) Repeatability of 50%.**		
Area number	Assay	Cal. conc. (mg/mL)	Assay %	Cal. conc. (mg/mL)	Assay %
Area 1	51.9	0.2721	100.8	0.00280	50.5
Area 2	52.1	0.2728	100.7	0.00280	50.6
Area 3	52.1	0.2729	100.1	0.00281	50.8
medium	52	0.273	100.75	0.00280	50.7
STDEV	0.08	16084	0.41	7.86	0.12
RSD	0.16	0.16	0.41	0.44	0.24
Y=3.16X+1.27, lim of RSD<2% b) Repeatability of 100%.			**Y=2.0X+5.91, lim of RSD<2% e) Repeatability of 100%.**		
Area 1	100.6	0.5644	99.5	0.00561	100.8
Area 2	100.5	0.5611	100.2	0.00559	100.3
Area 3	100	0.5640	99.6	0.00559	100.7
medium	100.4	0.5631	100	0.00560	100.6
STDEV	0.30	0.00	0.47	0.00	0.26
RSD	0.30	0.32	0.47	0.26	0.26

(Continued)

Table 4. *Continuation.*

Y=3.16X+1.27, lim of RSD<2% c) Repeatability of 150%.			Y=2.0X+5.91, lim of RSD<2% f) Repeatability of 150%.		
Area 1	147.3	0.8446	99.5	0.00843	151.3
Area 2	146.6	0.8407	100.2	0.00848	152.2
Area 3	148.1	0.8499	99.6	0.00851	152.6
medium	147.3	0.8452	100	0.00557	152
STDEV	0.77	0.00	0.47	0.00	0.67
RSD	0.52	0.55	0.47	0.44	0.44

Intermediate precision (Inter day precision/Ruggedness)

Precision between two consecutive days performed by different analysts of the sample showed % RSD less than 2, which indicate the method developed is inter day precise/ rugged (tables 5-a, b, c, d, e, f).

Table 5. Intermediate precision of Empagliflozin/metformin·HCl

Y=3.16X+1.27, lim of RSD<2% a) Repeatability of 50%			Y=2.0X+5.91, lim of RSD<2% d) Repeatability of 50%		
Area number	Assay	Cal. conc. (mg/mL)	Assay %	Cal. conc. (mg/mL)	Assay %
Area 1	51.9	0.2721	100.8	0.00280	50.5
Area 2	52.1	0.2728	100.7	0.00280	50.6
Area 3	52.1	0.2729	100.1	0.00281	50.8
medium	52	0.273	100.75	0.00280	50.7
STDEV	0.08	16084	0.41	7.86	0.12
RSD	0.16	0.16	0.41	0.44	0.24
Y=3.16X+1.27, lim of RSD<2% b) Repeatability of 100%			Y=2.0X+5.91, lim of RSD<2% e) Repeatability of 100%		
Area 1	100.6	0.5644	99.5	0.00561	100.8
Area 2	100.5	0.5611	100.2	0.00559	100.3
Area 3	100	0.5640	99.6	0.00559	100.7
medium	100.4	0.5631	100	0.00560	100.6
STDEV	0.30	0.00	0.47	0.00	0.26
RSD	0.30	0.32	0.47	0.26	0.26
Y=3.16X+1.27, lim of RSD<2% c) Repeatability of 150%			Y=2.0X+5.91, lim of RSD<2% f) Repeatability of 150%		
Area 1	147.3	0.8446	99.5	0.00843	151.3
Area 2	146.6	0.8407	100.2	0.00848	152.2
Area 3	148.1	0.8499	99.6	0.00851	152.6
medium	147.3	0.8452	100	0.00557	152
STDEV	0.77	0.00	0.47	0.00	0.67
RSD	0.52	0.55	0.47	0.44	0.44

Photolytic degradation

1 mL Metformin HCl: Empagliflozin stock solution was prepared and exposed to near ultraviolet lamp in photo stability chamber providing illumination for 1 hr. Then sample was dissolved in Diluent and the volume was made up to mark [10 mL] (Table 6).

Table 6. Specificity of Metformin HCl: Empagliflozin in tablet

No	Degradation studies	Metformin HCl Result%	Empagliflozin Result%
1	Hydrolytic degradation alkaline	101.9	99.5
2	Hydrolytic degradation acidic	101.3	99.5
3	Thermal degradation	101.5	100.0
4	Oxidative degradation	100.7	101.1
5	Photolytic degradation	100.0	99.8

Evaluating the accuracy of methods

The accuracy of an analytical method expresses the closeness of agreement between the value accepted either as a conventional true value or an accepted reference value and the value found. Accuracy was determined by means of recovery experiments, by the determination of % mean recovery of sample by standard addition method at three different levels. At each level, three determinations were performed. Percent mean recovery was calculated as shown in table 7. The accepted limits of recovery are 98%-102% and all observed data are within the required range, which indicates good recovery values and hence the accuracy of the method developed.

Table 7. Evaluating the accuracy of methods in Metformin/Empagliflozin tablet

Control solution of 50%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (a)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	1.016	1.011	1.005	1.013	55624	0.550
Observed conc. (mg/mL)	0.280	0.270	0.280	0.270	0.0	0.0
Calculated conc. (mg/mL)	0.280	0.278	0.277	0.278	1.37	0.27
Relative error	-0.25	0.36	1.00	0.37		
Accuracy	100.26	99.64	99.00	99.63		

(Continued)

Table 7. *Continuation*.

Control solution of 100%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (b)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	1.89	1.90	1.91	1.90	56430	0.290
Observed conc. (mg/mL)	0.560	0.560	0.560	0.560	0.0	0.0
Calculated conc. (mg/mL)	0.559	0.561	0.562	0.561	1.38	0.27
Relative error	0.14	-0.28	-0.48	-0.21		
Accuracy	99.86	100.28	100.48	100.21		
Control solution of 150%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=3.16X+1.27 (c)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^7	2.83	2.83	2.83	2.83	25153	0.089
Observed conc. (mg/mL)	0.8400	0.8400	0.8400	0.8400	0.0	0.0
Calculated conc. (mg/mL)	0.85597	0.85478	0.85446	0.85507	0.77	0.27
Relative error	-1.90	-1.76	-1.72	-1.79		
Accuracy	101.90	101.76	101.72	101.79		
Control solution of 50%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (d)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^4	5.74	5.77	5.73	5.75	183	0.32
Observed conc. (mg/mL) × 10^{-3}	2.80	2.80	2.80	2.80	0.0	0.0
Calculated conc. (mg/mL)	0.003	0.003	0.003	0.003	0.273	0.27
Relative error	-0.05	-0.44	0.2	-0.1		
Accuracy	100.05	100.44	99.8	100.1		
Control solution of 100%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (e)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^5	1.13	1.13	1.14	1.13	594.6	0.52
Observed conc. (mg/mL) × 10^{-3}	5.6	5.6	5.6	5.6	0.0	0.0
Calculated conc. (mg/mL)	0.006	0.006	0.006	0.006	0.28	0.27
Relative error	0.61	1.04	0.0	0.55		
Accuracy	99.39	98.96	100	99.45		

(Continued)

Table 7. *Continuation*.

Control solution of 150%/Accuracy of determination for Empagliflozin/metformin. HCl					**Y=2.0X+5.91 (f)**	
Control Soul.	1	2	3	Medium	STDEV	RSD.
Area × 10^5	1.71	1.71	1.71	1.71	430	0.25
Observed conc. (mg/mL) × 10^{-3}	8.4	8.4	8.4	8.4	0.0	0.0
Calculated conc. (mg/mL)	0.008	0.008	0.008	0.008	0.28	0.27
Relative error	-0.28	0.19	-0.20	-0.10		
Accuracy	100.28	99.81	100.20	100.10		

Limit of detection (LOD) and Limit of quantitation (LOQ)

There are several terms that have been used to define LOD and LOQ. In general, the LOD is taken as the lowest concentration of an analyte in a sample that can be detected, but not necessarily quantified, under the stated conditions of the test. The LOQ is the lowest concentration of an analyte in a sample that can be determined with acceptable precision and accuracy under the stated conditions of the test. For a linear calibration curve, it is assumed that the instrument response y is linearly related to the standard concentration x for a limited range of concentration. It can be expressed in a model such as y=a+bx. This model is used to compute the sensitivity b and the LOD and LOQ. Therefore, the LOD and LOQ can be expressed as: LOD = 3 × Sa / b, LOQ = 10 × Sa / b, where Sa is the standard deviation of the response and b is the slope of the calibration curve. The standard deviation of the response can be estimated by the standard deviation of either y-residuals, or y-intercepts, of regression lines. This method can be applied in all cases, and it is most applicable when the analysis method does not involve background noise. It uses a range of low values close to zero for calibration curve, and with a more homogeneous distribution will result in a more relevant assessment (Figure 3).

Ranges of testing: Range of analytical method can be obtained from Linearity, precision and accuracy data. Report the range in % with respect to sample concentration. According to the result it can be concluded that the range of analytical method for the determination of assay and dissolution of Metformin HCl/Empagliflozin tablet 500/5 mg is 50-150% of target concentration. The null hypotheses of no treatment effect for the primary and key secondary endpoint were tested hierarchically to control diabetes type 2. For each endpoint, the superiority of linagliptin 5 mg was tested. The trial was powered separately for linagliptin 5 mg vs. placebo as add-on to Empagliflozin 10 mg and metformin and linagliptin 5 mg vs. placebo as add-on to Empagliflozin 25 mg and metformin (Figure 4).

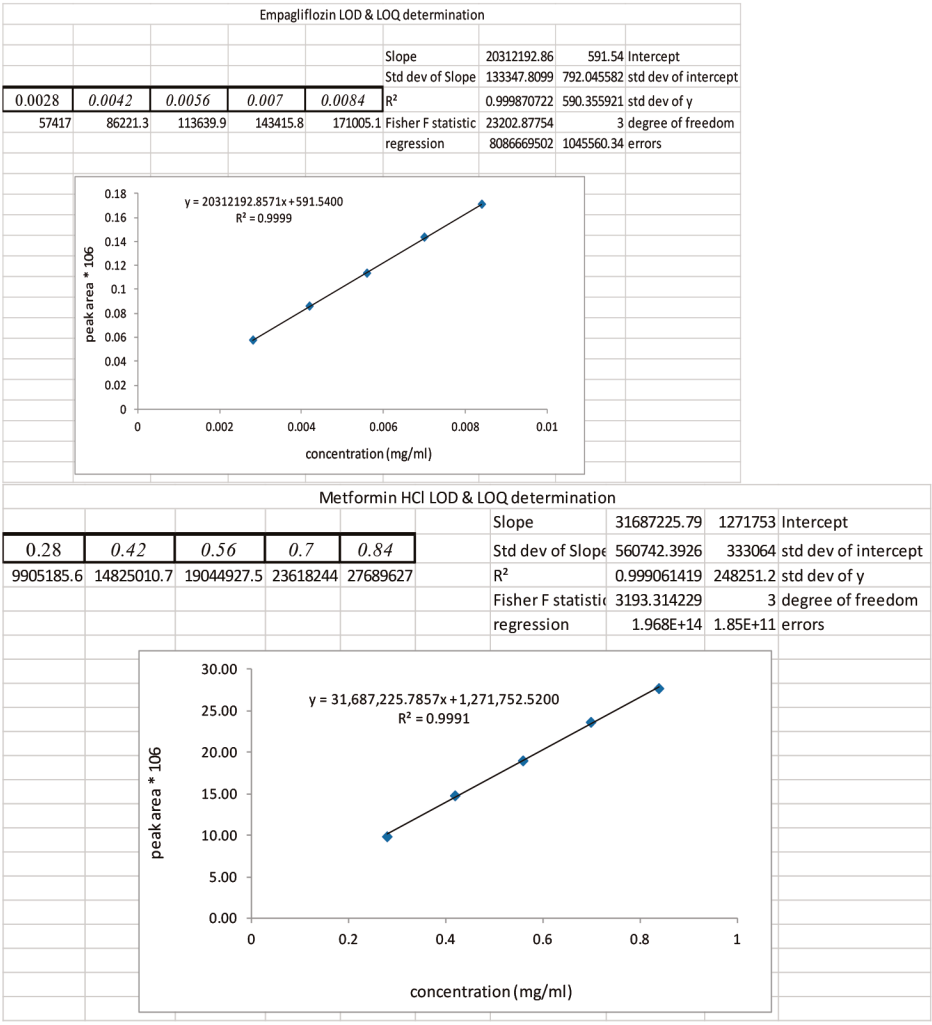

Figure 3. Peak versus concentration

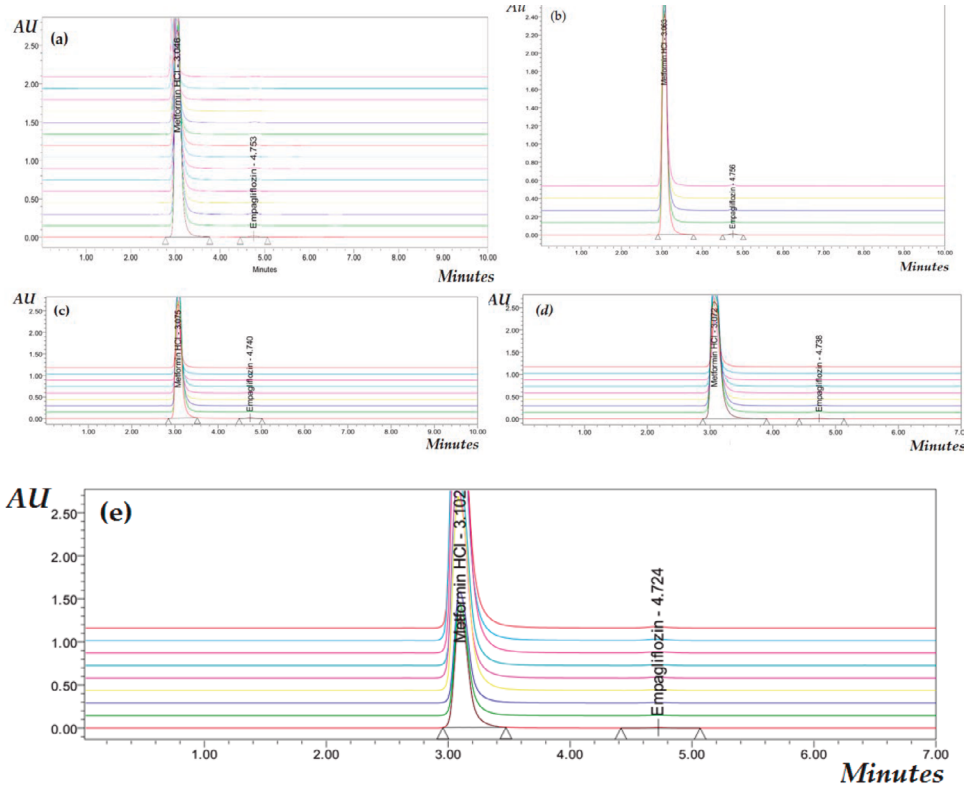

Figure 4. Several sample peaks

Results of Docking Simulation

The Metformin and its five analogues were downloaded from Pub-Chem database (Table 8), and then were converted into PDB format. In addition, XRD structure of 7VSI.pdb, that is structure of human SGLT2-MAP17 complex bound with Empagliflozin, and 8JTS.pdb a crystal structure in complex with metformin in outward open conformation, were gotten from Brookhaven Protein Data Bank (Figure 5). Finally all the analogues were converted into PDBQT file format using Auto Dock Tool (ADT) for further analysis and also docking was performed with iGemdock algorithms. At the first step the interactive maps of the docked molecules (five molecules in table 1) were done by two mentioned PDB files, and the docked minimum energies were then analyzed for finding the suitable places, which any types of peptides could attached with higher affinity to the both SGLT2-MAP17 and human organic cation transporter. NMR data of Empagliflozin and Metformin cation have plotted in figure 6.

Table 8. The Metformin and its five analogues were downloaded from Pub-Chem database

No.	Compounds analogs
1	Metformin cation
2	Metformin-d6 hydrochloride
3	Metformin Hydrochloride
4	Empagliflozin
5	*N*1,*N*1-Dimethyl-*N*5-methylbiguanide hydrochloride

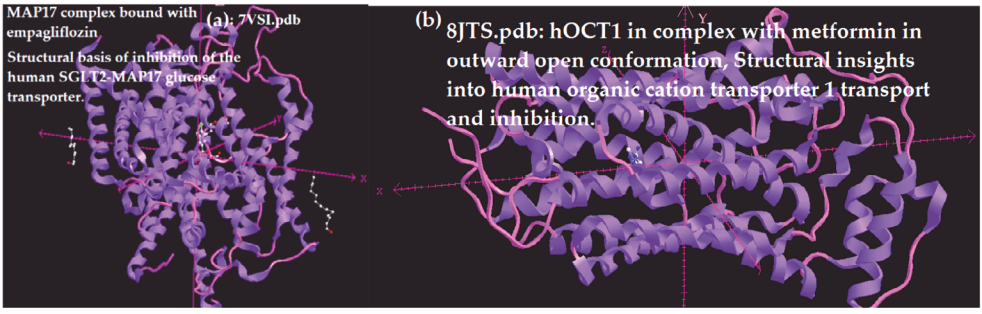

Figure 5. Brookhaven Protein Data Bank of (a): human SGLT2-MAP17 complex bound with Empagliflozin, and (b): Structural insights into human organic cation transporter 1 (HOCT).

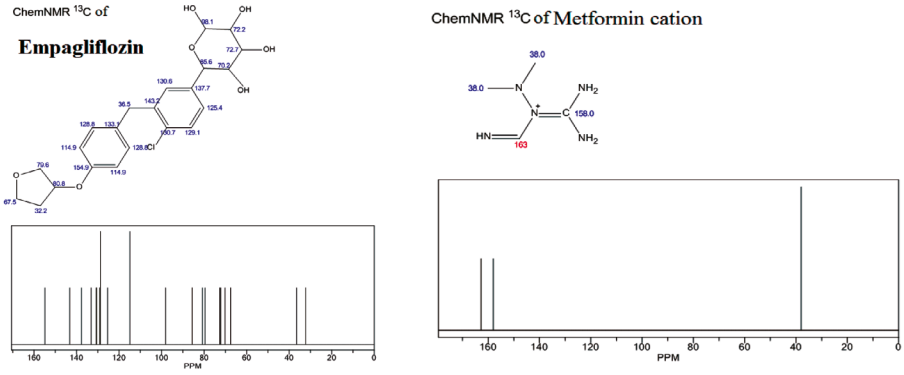

Figure 6. NMR data of Empagliflozin and Metformin cation

The potential binding sites were calculated and plotted using Acsite software. Docking was performed with iGemdock algorithms. At the first step the interactive maps of the docked molecules were done by LIGPLOT, and the docked minimum energies were then analyzed for finding the suitable places, which any types of each molecules from

Table 8 could attached with higher affinity to the SGLT2-MAP17 complex .Three of these molecules including, Metformin cation, Metformin-Hydrochloride, and Empagliflozin docked, exhibited suitable binding energies, and docking were repeated by increased stringency using a reduced cavity size as a receptor. This resulted in one matching peptide again modeled with SGLT2-MAP17 Data Bank, and HOCT a template using MODELLER software. The scoring function, X-score, was used to compare the binding energies Table 9 and Figure 7.

Table 9. Docking energies for three complexes

Molecules No	Name	Complex	Ka ($(10_4$ M	VDW (kcal/mol)	H-bond (kcal/mol)	Docking Fitness (kcal/mol)
(1)	Metformin cation	Metformin cation- SGLT2	3.15	-46.74	-16.52	-63.24
(3)	Metformin Hydrochloride	Metformin Hydrochloride-SGLT2	1.45	-29.12	-14.18	-43.28
(4)	Empagliflozin	Empagliflozin--SGLT2	2.45	-57.61	-13.59	-71.194

Compound	Energy	H-M CYS 536	H-M LEU 539	H-S THR 543	V-M LEU 539	V-S LEU 539	V-M LEU 540	V-S LEU 540
cav7vsi_PLM-Metformin -1.pdb	-43.3	-6.9	-3.5	-3.5	-4.7	-4.5	-5.4	-6.7

Compound	Energy	H-M VAL 532	H-M PHE 535	H-M LEU 539	V-M LEU 533	V-S PHE 535	V-M SER 537	V-M GLY 538
cav7vsi_PLM-Empagliflozin-0.pdb	-71.2	-3.5	-6.6	-3.5	-7.5	-8.2	-6.8	-14.1

Compound	Energy	H-M GLY 491	H-M GLY 492	H-M CYS 536	V-M GLY 491	V-M CYS 536	V-S LEU 540
cav7vsi_PLM-Empagliflozin-0.pdb	-63.3	-2.5	-3.5	-5	-6.9	-4.6	-4.8

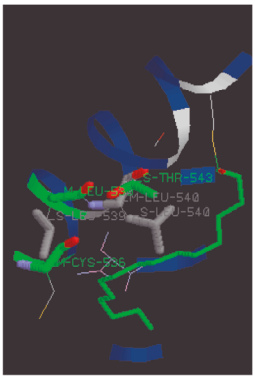

Figure 7. Docking position of amino acids for binding of three complexes

Discussion

In these two-Phase III studies, designed to evaluate the efficacy and safety of linagliptin 5 mg vs placebo as add-on to Empagliflozin 10 mg or 25 mg and metformin for 24 weeks

in patients with T2D, use of linagliptin resulted in statistically significant reductions in HbA1c and FPG vs placebo. Importantly, the proportion of patients with baseline HbA1c ≥7% who reached HbA1c <7% after 24 weeks with linagliptin was more than twice that with placebo as add-on to Empagliflozin 10 or 25 mg and metformin. These improvements in glycaemic control were observed despite a smaller proportion of patients receiving rescue therapy in the linagliptin vs the placebo as add-on to Empagliflozin and metformin groups. At week 24, the reduction from baseline in HbA1c observed with linagliptin as add-on to Empagliflozin 10 mg or 25 mg and metformin was >0.5%. The impact on weight is an important consideration when choosing a treatment for T2D. Metformin, the recommended first-line treatment for patients with T2D, is considered weight neutral. However, some second- and third-line therapies such as sulphonylureas, glinides, thiazolidinediones and insulin, are associated with weight gain. Weight loss or avoidance of weight gain is desirable for patients with T2D, weight gain being associated with decreased treatment satisfaction and health-related quality of life [24]. In line with the mechanism of action of DPP-4 inhibitors [25-28] and data from linagliptin Phase III trials [29], linagliptin did not markedly change weight during the double-blind treatment periods, while Empagliflozin has been shown to reduce weight [30].

Docking technique needs selecting the three-dimensional coordinate space of the binding site in the target and measuring the binding interactions of the molecule's resultant orientation within the binding site, forming the complex. The importance and sensitivity of binding affinity values are calculated by the maximum magnitude negative number (highest binding affinity or lowest binding energy), which represents the most advantageous conformation of the complex produced when the ligand involved binds efficiently to the target's active pockets. Additionally, docking simulations were used to confirm the anti-diabetic potency of Metformin cation- SGLT2, Metformin Hydrochloride- SGLT2, and Empagliflozin-SGLT2, by examining the binding affinity and orientation of ligands inside the SGLT2 receptor pocket. The docking poses were rated as per their score values, and Table 9 summarized the binding affinities of the best pose for each of the four analogues with the SGLT2 target (Figure 7). Additionally, the molecules in this analysis have a higher binding affinity, comparable to that of a regular drug (metformin).The four compounds could dock into the active site of SGLT2 successfully. The tight binding can be explained in terms of hydrogen bonding with target protein. All the three complexes were involved in the hydrogen bonding with a residue. Metformin hydrochloride interacted with SGLT2 forming H-bonds at active site region involving residues CYS, LEU, and THR (7). Analysis of these interaction results confirmed that the selected four analogues showed the efficient binding with

diabetic target protein SGLT2 like the standard drug metformin. So it was confirmed that, these analogues might be a potential lead compounds for experimentally validation for diabetes management. Our docking results confirmed the experimental data significantly.

Conclusion

The present study was carried out to develop a sensitive, precise and accurate HPLC method for the analysis of Metformin HCl and Empagliflozin in Bulk as well as in Metformin HCl /Empagliflozin tablets. In order to method development under gradient conditions, was tested as mobile phase on a (L10 (250×4.6 mm); column. The retention times obtained for Metformin HCl/Empagliflozin was around 3.09 and 4.7 min respectively. In conclusion, linagliptin improved glycaemia control compared with placebo as add-on to Empagliflozin 10 or 25 mg and metformin and was well tolerated. Therefore, linagliptin could provide a valuable treatment option for patients with T2D who are inadequately controlled with Empagliflozin and metformin, improving glycaemia control with a low risk of hypo glycaemia and no increase in weight.

Author contributions

Motahareh Dehghandar, Parisa Latifi, and Iman Eskandary designed, measured, and drawn the experimental data, images, graph, and experimental activities and methods. Sara Shahriari and Fatemeh Mollaamin checked the molecular structures and chemical abbreviation in viewpoint of chemistry and biochemistry . Majid Monajjemi accomplished the molecular docking simulation. Majid Monajjemi and Fatemeh Mollaamin revised the English, grammar and dictation of the manuscript.

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Conflict of interest

All authors report that they do not have any conflicts of interest.

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Resúmenes del V Congreso Latinoamericano de Métodos Alternativos al Uso de Animales de Experimentación (V COLAMA), Bogotá D.C., Colombia, septiembre 16 al 20 de 2024

Organizado por:

Universidad Nacional de Colombia, Departamento de Farmacia, Facultad de Ciencias

Asociación Colombiana para la Ciencia y Bienestar del Animal de Laboratorio (ACCBAL)

Pontificia Universidad Javeriana, Unidad de Biología Comparativa

Universidad de los Andes, AnimalCore

Universidad de La Salle, Observatorio Colombiano de Salud y Bienestar Animal (OCSBA)

Universidad CES, Unidad de Toxicidad In Vitro (UTi)

Federación de Sociedades Sudamericana de Ciencia en Animales de Laboratorio, (FESSACAL)

Centro Brasilero para la Validación de Métodos Alternativos (BraCVAM)

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Diseño de programas de atención veterinaria siguiendo el modelo de AAALAC

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Resumen

El Programa de Atención Veterinaria según AAALAC International: La atención veterinaria es uno de los puntos fundamentales dentro del programa de cuidado y uso de los animales de una institución. Esta actividad se debe enmarcar dentro de un programa cohesionado de atención veterinaria, con la participación de diversos actores e incluyendo actividades concretas. El programa de atención veterinaria es una responsabilidad colectiva de la(s) persona(s) con autoridad en la institución, el/la responsable veterinario/a (responsable veterinario), y el Comité Institucional para el Cuidado y Uso de los Animales de Laboratorio (CICUAL). El responsable veterinario debe tener la autoridad y los recursos, otorgados por la dirección de la institución, para establecer un programa eficiente. Para lograrlo, también debe interactuar con los investigadores para conocer las necesidades y riesgos específicos derivados de los proyectos experimentales. La participación del responsable veterinario en el CICUAL es la manera más eficiente de asegurar la comunicación con el resto de actores implicados, que también incluye a los técnicos del bioterio. Por otra parte, la especialización en experimentación animal del responsable veterinario, y más concretamente en las especies utilizadas, es también necesaria para el éxito del programa. El programa de atención veterinaria no solamente consiste en atender animales enfermos, sino que incluye otras actividades como la opinión sobre aspectos de los proyectos experimentales que puedan afectar el bienestar y la salud animal; las instrucciones sobre pautas anestésicas y analgésicas, así como sobre manipulaciones y condiciones de estabulación de los animales; los cuidados perioperatorios; o el establecimiento de las condiciones sobre la potencial reutilización o adopción de animales ya utilizados. Esta presentación analizará todos estos aspectos de responsabilidad institucional, comunicación, y actividades necesarias para el establecimiento de un programa de atención veterinaria, incluyendo algunas preguntas que nos podemos hacer para evaluar si el resultado es satisfactorio (performance standards).

Derechos de los animales de investigación

Arthur Regis

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Resumen

Un nuevo paradigma se ha presentado ante la sociedad global: el reconocimiento científico y legal de los animales como seres sensibles, sintientes o conscientes. Dando como resultado, incluso, una nueva área de conocimiento multidisciplinar: el Derecho Animal (basado en el reconocimiento de la dignidad intrínseca de los animales). Incluso cuando la población no puede percibirlos claramente, los problemas animales están estrechamente relacionados con la vida social cotidiana: elección de la dieta, vestimenta, actividades de entretenimiento, investigación científica etc. En este contexto, la nueva percepción de los animales también afectará a las ciencias de los animales de laboratorio, ya que el animal utilizado en la investigación y la enseñanza no puede verse como un insumo, sino como un ser vivo digno que se utiliza en contra de su voluntad para servir a los intereses humanos, y deben, como mínimo, ser tratados con respeto y debe garantizarse su bienestar. De esta percepción surgen varias obligaciones hacia estos seres vivos: necesidad de enriquecimiento ambiental, imposición de técnicas de eutanasia, establecimiento de un punto final humanitario etc. Es claro que los principios de sustitución, reducción y refinamiento (Russel & Burch, 1959), aunque válidos, ya no son suficientes para abordar la cuestión, requiriendo la aplicación de nuevos conceptos que se ajusten al paradigma de la dignidad animal. Los reflejos del reconocimiento de la dignidad animal se dan en las normativas de los países, como, por ejemplo, en la prohibición de probar productos cosméticos en animales: Ley 2.047/2020 de Colombia; Resolución Normativa CONCEA 58/2023 de Brasil; Ley 21.646/2024 de Chile. Otro elemento importante es el desarrollo de métodos alternativos, preservando animales que no han elegido vivir toda su vida en una jaula de laboratorio. Es necesario construir un futuro sostenible y más justo para todas las formas de vida.

Optimización ética en investigación: estrategias para integrar las 3Rs en experimentación animal

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Resumen

El término 3Rs, acuñado por Russell y Burch en 1959, describe un enfoque integral para la optimización ética de la investigación que implica el Reemplazo, Reducción y Refinamiento de la experimentación con animales. Esto se orienta hacia la sustitución de los animales por métodos que no los empleen, la disminución del número de animales utilizados y la mejora de las técnicas experimentales para minimizar el sufrimiento de los mismos. Actualmente, la mayoría de las legislaciones incluyen disposiciones para la implementación de las 3Rs en investigación animal. Antes de llevar a cabo experimentos con animales, los investigadores deben buscar y considerar los métodos de reemplazo disponibles. Si no se pueden encontrar alternativas de reemplazo adecuadas para sustituir completamente o en parte el uso de animales, se debe priorizar la reducción y el refinamiento de los procedimientos experimentales. Sin embargo, la falta de conocimiento sobre las alternativas disponibles o la resistencia a su adopción pueden obstaculizar la aplicación de estas metodologías de reemplazo. Por lo tanto, es crucial realizar búsquedas sistemáticas y estar al tanto de diversas fuentes de información y estrategias de búsqueda. Los comités de ética de experimentación animal desempeñan un papel fundamental en la supervisión y garantía de la aplicación adecuada de las 3Rs por parte de los investigadores. Esta conferencia proporcionará una revisión de las bases de datos más adecuadas, así como de las estrategias recomendadas para identificar los métodos de reemplazo, reducción y refinamiento más apropiados para su implementación en la experimentación.

Criterios de cuidado y bienestar en la investigación con organismos acuáticos

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Resumen

La investigación con organismos acuáticos juega un papel crucial en diversos campos científicos, desde la biología marina hasta la ecotoxicología. Sin embargo, es fundamental abordar esta práctica con un enfoque ético y responsable que garantice el bienestar de los animales involucrados. En este contexto, la bioética, el bienestar animal y el refinamiento experimental se erigen como pilares fundamentales. La bioética proporciona un marco conceptual que permite evaluar los aspectos morales de la investigación con animales. Los principios bioéticos de beneficencia, no maleficencia, autonomía y justicia deben ser considerados al diseñar y llevar a cabo experimentos con organismos acuáticos. Esto implica buscar un equilibrio entre los beneficios potenciales de la investigación y el posible daño causado a los animales. El bienestar animal, por su parte, se centra en el estado físico y psicológico de los animales. En el caso de los organismos acuáticos, el bienestar se ve influenciado por factores como la calidad del agua, la disponibilidad de alimento, la densidad de población y la presencia de estímulos estresantes. Los investigadores deben tomar medidas para proporcionar un entorno lo más natural posible y evitando procedimientos dolorosos o innecesarios. El refinamiento experimental es una estrategia clave para mejorar el bienestar de los animales de laboratorio. Consiste en modificar los procedimientos experimentales para reducir al mínimo el estrés de los animales. Algunas técnicas de refinamiento incluyen el uso de anestésicos, analgésicos y sedantes, así como el desarrollo de métodos alternativos que no requieran el uso de animales. La cultura del cuidado es un aspecto esencial para promover el bienestar animal en la investigación. Implica la adopción de una actitud respetuosa hacia los animales y la sensibilización de los investigadores y técnicos sobre la importancia del bienestar animal. Además, es fundamental contar con protocolos y guías de buenas prácticas que establezcan los estándares mínimos para el cuidado de los organismos acuáticos en investigación. En conclusión, los criterios de cuidado y bienestar en la investigación con organismos acuáticos son fundamentales para garantizar la ética y la calidad de la investigación. La bioética, el bienestar animal, el refinamiento experimental y la cultura del cuidado son elementos interrelacionados que deben ser considerados de manera integral para promover el uso responsable de los animales en la ciencia.

Author Guide for Addressing Animal Methods Bias in Publishing

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Summary

One of the biases that can affect the peer review of scientific articles is called animal method bias, which is the reviewer's preference for animal-based methods where they are not necessary or where non-animal methods may already be suitable. This preference, or the lack of expertise to properly evaluate non-animal methods, results in the unfair assessment of non-animal research and can lead to the request of additional (animal) experiments to "validate" non-animal data, which affects the likelihood and/or timeliness of acceptance for publication. A cross-sectional survey has recently shown that researchers have performed animal experiments for the sole purpose of anticipating reviewers' requests, and that the request to add animal experimental data is quite prevalent. A similar survey is being launched during COLAMA to assess the prevalence of animal methods bias in Latin-America. Aiming to help avoiding and overcoming this bias, the Coalition to Illuminate and Address Animal Method Bias (COLAAB) have developed a guide containing measures, resources, and tools for authors, including how to find and select appropriate non-animal methods and pre-register the research plan; tips for manuscript preparation and submission, including how to discuss methods, choose journals and reviewers; and suggestions for the peer review process, including language and literature to help authors in responding to biased reviews. The author's guide for addressing animal method bias in publishing is a living resource, available at animalmethodbias.org. Animal methods bias not only has ethical, time, and cost implications, but may also contribute to the lack of translatability of findings from animal studies to human health improvements. Its prevention requires systemic solutions, including the implementation of guidelines and bias training for researchers, funding agencies, editors and reviewers.

Modelos alternativos para entrenamientos quirúrgicos

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Resumen

El uso de animales de laboratorio ha sido el *gold standart* para la capacitación de disciplinas como la cirugía, siendo un tema polémico que no se reduce al ámbito científico, teniendo implicancias legales, de objeción de conciencia e impacto en medios de comunicación y la opinión pública. El objetivo es demostrar cómo fueron modificándose los métodos de entrenamiento y capacitación a través de los años en un Hospital de Pediatría Público para alcanzar niveles de aprendizaje aceptables mediante alternativas como: uso de material inanimado, simulación, software, reutilización de cadáveres, conservación de tejidos e impresión 3D se desarrolló un programa de entrenamiento permanente comparando resultados de aprendizaje generados por los método alternativos se obtuvieron beneficios adicionales como ahorro de tiempo y costos, potencial mejorado para la parametrización y la repetición del ejercicio de aprendizaje, incremento de la confianza y satisfacción del cirujano, mayor cumplimiento con la legislación sobre el uso de animales, eliminación de las objeciones del uso de animales sacrificados con objetivos específicos y la integración de la perspectiva y ética clínica en las primeras etapas de instrucción de los cirujanos. La evidencia demuestra que los educadores pueden servir mejor a sus alumnos, y a la vez minimizar las cargas financieras y de tiempo, con la introducción de métodos de entrenamiento bien diseñados que no dependan del uso dañino de animales. La implementación en forma gradual de modelos de bajo costo y otros de alta tecnología hacen una combinación perfecta en las herramientas necesarias para llevar a cabo dicha tarea de entrenamiento y capacitación. Nos encontramos, por tanto, en un punto de inflexión. La Ciencia y la Sociedad está avanzando respecto a hace unos años, pero aún quedan muchos puntos por cubrir y muchos obstáculos por superar. Por ello, es importante que no cesen los esfuerzos por parte de la comunidad científica para que apoyen los métodos alternativos, para que se siga invirtiendo en su desarrollo e implementación en todos los ámbitos, ya que de este modo todos nos beneficiamos: los humanos porque desarrollaremos modelos más fiables) y los animales no humanos, porque evitaremos su sufrimiento innecesario.

Biomateriales y su impacto en la docencia universitaria

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Resumen

Los biomateriales están transformando la educación superior, particularmente en la ingeniería biomédica, al integrarse de manera efectiva en la formación académica y la investigación aplicada. A través de estrategias pedagógicas innovadoras como el aprendizaje basado en proyectos y la enseñanza invertida, los estudiantes desarrollan habilidades para resolver problemas complejos mediante la creación de soluciones prácticas, como nanovehículos para la administración dirigida de medicamentos y bioinks 3D para la regeneración de tejidos. Estas iniciativas no solo mejoran la comprensión teórica, sino que también fomentan el desarrollo de tecnologías alternativas que minimizan la dependencia de modelos animales, abordando cuestiones éticas y científicas contemporáneas. La implementación de simulaciones multifísicas y herramientas avanzadas como COMSOL ha potenciado la capacidad de los estudiantes para modelar y predecir el comportamiento de estos biomateriales en aplicaciones biomédicas, facilitando la transición del conocimiento teórico a su aplicación práctica. Este enfoque multidisciplinario no solo enriquece la experiencia educativa, sino que también posiciona a los estudiantes y futuros ingenieros biomédicos en la vanguardia de la innovación responsable, promoviendo un enfoque de la enseñanza que prioriza el desarrollo de tecnologías sostenibles y éticas. La sinergia entre la investigación en biomateriales y la docencia universitaria representa un cambio de paradigma en cómo se abordan los desafíos científicos y tecnológicos, proporcionando a los estudiantes las herramientas necesarias para liderar avances significativos en salud y tecnología. Así, la integración de los biomateriales en la docencia no solo impulsa la innovación académica, sino que también asegura que las nuevas generaciones de ingenieros estén equipadas para enfrentar los retos éticos y científicos del futuro, contribuyendo a un mundo más consciente y tecnológicamente avanzado.

Modelo biomecatrónico canino para entrenamiento

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Resumen

La educación basada en simulación en las áreas de la salud permite la adquisición de habilidades y destrezas, tanto técnicas como no técnicas, en ambientes controlados. Esto favorece un aprendizaje efectivo ya que se aprende haciendo; además, se disminuye el riesgo de cometer errores cuando el profesional se vea enfrentado a casos reales. La resucitación cerebro-cardiopulmonar es una habilidad básica que todo médico veterinario recién graduado debe tener, para salvar la vida de pacientes en casos de urgencia. Por tal motivo, se formó un equipo de trabajo de ingenieros del Instituto Tecnológico Metropolitano (ITM) y médicos veterinarios de la Universidad de Antioquia (UdeA), para trabajar en la elaboración de un simulador para resucitación cerebro-cardiopulmonar, integrando técnicas de plastinación con dispositivos mecatrónicos para la simulación de diversas variables fisiológicas. Se obtuvo un simulador plastinado con alta fidelidad anatómica, con los siguientes parámetros: pulso femoral, presión arterial, registro de electrocardiografía, tiempo de llenado capilar, color de mucosa lingual, medición de oximetría y reflejo pupilar. Mediante una interfaz de control el profesor puede modificar los parámetros fisiológicos según el escenario de simulación elegido. Tiene una interfaz de visualización donde los estudiantes pueden ver los parámetros fisiológicos establecidos. Se realizó una validación técnica con médicos veterinarios del Hospital Veterinario de la Universidad de Antioquia, donde se evaluaron los parámetros en tres escenarios simulados: paciente normotenso, paciente hipotenso, paciente hipertenso y paciente sin signos vitales. El simulador obtuvo patente de invención de la Superintendencia de Industria y Comercio (Resolución No. 59195 de septiembre de 2023).

Modelos alternativos para entrenamientos en técnicas y prácticas veterinarias

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Resumen

Desde comienzos de siglo, nuevas tendencias han generado un cambio en los modelos de enseñanza en las carreras envueltas dentro de las ciencias de la salud incluidas la medicina veterinaria, que ante el advenimiento de nuevos paradigmas éticos y morales en cuanto al aprendizaje de diversas técnicas médico-quirúrgicas que, en aquel entonces, implicaba la utilización de animales de laboratorio y otros modelos vivos de práctica. Muchos de estos procesos requieren para el afianzamiento de la técnica curvas de aprendizaje variables según la complejidad de la técnica y que ilustaradas e implementadas en pacientes en los espacios clínicos - quirúrgicos podían incrementar los riesgos vitales inherentes a la realización del acto médico. Es por esto que aunque el uso de sistemas de simulación físicos o virtuales se ha incrementado, los costos de adquisición pueden llegar a ser una limitante en países en desarrollo al momento de implementar ejercicios de simulación clínica en los diferentes pensums académicos. Sin embargo existen herramientas de ingeniería como el diseño y la impresión 3D que implementados en el entorno médico han permitido incursionar en campos de modelado a partir, por ejemplo, de imágenes diagnósticas avanzadas. En la Universidad de la Salle hemos incursionado en la apropiación de dichas tecnologías para la fabricación de simuladores a pedido, con base a réplicas realistas y con diferentes niveles de sensibilidad háptica que cumplan con características específicas para la enseñanza en cátedras de cirugía, medicina y emergentologia, así como su implementación en el área de preparación y planificación quirúrgica en la clínica veterinaria.

Propuesta de proyecto de ley que reglamenta las actividades de investigación, educación y estudios biológicos que involucran el uso de animales vivos

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Resumen

El proyecto de ley tiene por objeto regular las actividades de investigación científica, las pruebas de seguridad (testeo), los ensayos biológicos y las actividades de educación en las que se utilicen animales vivos vertebrados y cefalópodos, con el fin de garantizarles a los animales un trato respetuoso y digno, en razón de su sintiencia. Es de resaltar que, actualmente, Colombia cuenta con diferentes leyes y normas que reglamentan la investigación con animales vivos en el territorio nacional. Sin embargo, la falta de eficacia en la aplicación e implementación de este marco normativo es una constante, el poco liderazgo por parte del Gobierno Nacional, especialmente del Ministerio de Salud, según lo establecido en la Ley 84 de 1989, la desactualización normativa frente a los nuevos lineamientos internacionales sobre bienestar animal, y la ausencia de una reglamentación clara y precisa respecto a los requisitos que deben cumplir las instituciones o los investigadores para realizar investigaciones con animales vivos son algunas de las causas que hacen necesario que el Congreso de la República tome medidas para actualizar estos aspectos normativos. En ese sentido, el PL se basa en la implementación de las 3Rs, en la reglamentación de esta ley por parte del Ministerio de Ciencia, Tecnología e Innovación, la creación y habilitación el Registro Único de Investigación con Animales Vivos vertebrados y cefalópodos -RUIAVVC, la creación del Comité Institucional del Cuidado y Uso de Animales -CICUA y crear estímulos, incentivos y facilidades para el fortalecimiento de las capacidades de los laboratorios e instituciones de investigación nacionales que implementen modelos alternativos para evitar el uso de animales vertebrados y cefalópodos en actividades de investigación, educación y ensayos biológicos. Finalmente, se busca imponer sanciones ambientales, penales o civiles según correspondan, conforme a la normatividad vigente, con el único fin de garantizarles a los animales un trato digno.

Estrategia para modernizar la investigación biomédica en Latinoamérica

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Resumen

La comunidad científica que reconoce el fracaso de los experimentos en animales es cada vez más grande. Este reconocimiento ha resultado en el desarrollo de métodos de investigación sin animales en múltiples áreas. Con esta transformación como contexto, el equipo científico de People for the Ethical Treatment of Animals (PETA) creó un plan para modernizar la investigación biomédica, que sugiere lo siguiente: a) poner fin al uso de animales en áreas en las que se ha demostrado que son "modelos" deficientes de los humanos; b) realizar reseñas científicas de la eficacia del uso de animales para identificar áreas adicionales en las que dicho uso podría eliminarse; c) redirigir los fondos al uso y desarrollo de métodos confiables sin animales; d) aplicar un sistema de análisis de daños y beneficios que incluya una perspectiva ética y la consideración del daño permanente que se inflige a los animales; e) promover la armonización y la aceptación internacional de métodos sin animales para pruebas de toxicidad; y, f) educar y capacitar a los grupos de investigación y a las agencias reguladoras sobre los beneficios y el uso de los métodos de experimentación sin animales. Independientemente del tamaño del presupuesto existente para investigación biomédica en las fuentes financiadoras y las universidades de América Latina, la asignación de dichos recursos a proyectos que usen métodos basados en la biología humana representa una inversión responsable. El equipo científico de PETA está a disposición de las instituciones y los grupos de investigación que deseen asesoría en la transición al uso de métodos sin animales.

Percepción social y prácticas de transparencia sobre el uso de animales en investigación, educación e industria

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Resumen

El uso de animales como modelos de investigación es una práctica científica que genera tensiones sociales y bioéticas con márgenes diversos de aceptabilidad. Existen grupos que apoyan la investigación animal bajo el imperativo moral de curar enfermedades y salvar vidas, y opositores que centran su argumentación en el sufrimiento animal. Estas posiciones representan el 30% de la esfera social y para entender cuáles son las actitudes del público restante se han desarrollado investigaciones en diferentes entornos. Los resultados muestran que la aceptabilidad de usar animales en la ciencia es del 68 al 80% cuando se busca curar enfermedades, no existen métodos alternativos y se toman medidas para mitigar el sufrimiento. No obstante, el margen de aceptación disminuye entre las poblaciones más jóvenes. Las fuentes de información sobre el uso de animales de investigación son principalmente medios de comunicación (53%) y en menor medida canales oficiales (4%). Esto se relaciona con la preocupación que tienen la mayoría de los participantes (60%) sobre la falta de información en el desarrollo de métodos alternativos y las estrategias para mitigar el sufrimiento. El 82% de los individuos cree que las instituciones deben ser más transparentes y el 54% abogan por una democratización de la ciencia que favorezca la participación del público en la toma de decisiones. Los resultados de los estudios de percepción social muestran la importancia de desarrollar sistemas de transparencia institucional. Para ello, la adopción de acuerdos de transparencia que permitan la construcción de relaciones con el público es un requerimiento social. Europa (EARA), Oceanía y Canadá vienen adelantando mecanismos participativos y es fundamental que los países latinoamericanos adopten pautas similares.

Metodologías de reemplazo o Nuevas Aproximaciones Metodológicas (NAMs)

Las NAM en la evaluación del riesgo toxicológico

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Resumen

La evaluación del riesgo toxicológico por muchos años tuvo un importante sustento en las pruebas *in vivo* que están dirigidas a valorar efectos adversos puntuales sobre los animales de experimentación. Esto dio lugar a protocolos de evaluación toxicológica (p.ej: irritación/corrosión ocular primaria, sensibilización dérmica, letalidad en la prueba de toxicidad aguda, entre otras) que se aplican a agentes químicos con los cuales el ser humano puede entrar en contacto. De esta manera, se definieron los peligros intrínsecos de muchas sustancias. Sin embargo, los cuestionamientos éticos asociados al uso de animales experimentales, las regulaciones que prohíben su uso, las controversias relacionadas con la escasa extrapolación entre especies y la disponibilidad tecnológica actual que permite valorar ampliamente cambios moleculares y celulares, han motivado un cambio en el paradigma construido en torno a la evaluación tradicional. Ahora, más que determinar una manifestación clínico-patológica en un animal, se dispone de herramientas que permiten valorar los mecanismos o vías que conducen a la toxicidad. El cambio en el modelo de evaluación se ha sustentado en los avances científicos que han permitido el surgimiento de pruebas que no emplean animales y que no simplemente duplican las existentes en estos, sino que suministran nuevas bases científicas para la evaluación de la seguridad. Surge así el concepto de las nuevas aproximaciones metodológicas (NAM), por primera vez empleado en 2018 por la EPA que incluye cualquier metodología, tecnología, enfoque o combinación de estos, que pueda ser usado para suministrar información del peligro químico y la evaluación de riesgo que evite el uso de animales completos. Las NAM pueden incluir métodos *in vitro*, *ex vivo*, *in chemico* e *in silico* así como la combinación de estos; actualmente existe disponibilidad de NAM validadas para valorar efectos locales tóxicos agudos con propósitos regulatorios. El empleo de estos nuevos enfoques metodológicos no solamente es útil para determinar efectos adversos también ha sido relevante en diversas investigaciones biomédicas.

Transición de cultivos 2D A 3D

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Resumen

La transición del cultivo de células en dos dimensiones (2D) a sistemas tridimensionales (3D), como esferoides y organoides, presenta múltiples desafíos técnicos significativos que deben abordarse para maximizar sus ventajas en la investigación biomédica. A diferencia de los cultivos 2D, donde las células crecen en una capa sobre una superficie plana con un acceso uniforme a nutrientes y oxígeno, los modelos 3D requieren optimizar el microambiente tridimensional para mantener la viabilidad, proliferación y diferenciación celular de forma adecuada. Esto implica diseñar matrices extracelulares, tanto sintéticas como naturales, que ofrezcan soporte estructural y señales bioquímicas y mecánicas apropiadas, un reto que demanda un profundo conocimiento de la biología de los tejidos y de los materiales biocompatibles. Otro desafío es el control de los gradientes de nutrientes, oxígeno y factores de crecimiento dentro de los cultivos 3D, ya que, a diferencia de los cultivos 2D, estos factores no se distribuyen homogéneamente, lo que puede provocar hipoxia y necrosis en los esferoides u organoides, complicando la interpretación de resultados experimentales; para ello, son necesarias técnicas avanzadas como la perfusión dinámica y la ingeniería de microfluidos. Además, se requiere desarrollar métodos específicos de caracterización y análisis, ya que las técnicas convencionales para cultivos 2D, como la microscopía de luz y los ensayos de viabilidad, son inadecuadas para abordar la complejidad de los modelos 3D, demandando tecnologías avanzadas y costosas, como la microscopía de sección óptica y la imagenología basada en inteligencia artificial. También, la reproducibilidad y escalabilidad de los cultivos 3D representan retos importantes debido a la variabilidad en tamaño, forma y composición celular, que dificultan su uso en estudios de alto rendimiento. Finalmente, la compatibilidad con herramientas de alta tecnología, como las técnicas ómicas y la edición genética, es crucial para integrar estos modelos en investigaciones avanzadas. Superar estos desafíos es esencial para realizar la transición a modelos tridimensionales más precisos, lo que aceleraría el desarrollo de terapias más efectivas y personalizadas.

Organoid intelligence

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Summary

Understanding brain function remains challenging as work with human and animal models is complicated by compensatory mechanisms, while *in vitro* models have been too simple until now. With the advent of human stem cells and the bioengineering of brain microphysiological systems (MPS), understanding how both cognition and long-term memory arise is now coming into reach. We suggest combining cutting-edge AI with MPS research to spearhead organoid intelligence (OI) as synthetic biological intelligence. The vision is to realize cognitive functions in brain MPS and scale them to achieve relevant short- and long-term memory capabilities and basic information processing as the ultimate functional experimental models for neurodevelopment and neurological function and as cell-based assays for drug and chemical testing. By advancing the frontiers of biological computing, we aim to (a) create models of intelligence-in-a-dish to study the basis of human cognitive functions, (b) provide models to advance the search for toxicants contributing to neurological diseases and identify remedies for neurological maladies, and (c) achieve relevant biological computational capacities to complement traditional computing. Increased understanding of brain functionality, in some respects still superior to today's supercomputers, may allow to imitate this in neuromorphic computer architectures or might even open up biological computing to complement silicon computers. At the same time, this raises ethical questions such as where sentience and consciousness start and what the relationship between a stem cell donor and the respective OI system is. Such ethical discussions will be critical for the socially acceptable advance of brain organoid models of cognition. Based on the *Baltimore Declaration Toward Exploring Organoid Intelligence*, a community is forming to realize this vision.

Aplicación de sistemas microfluídicos como alternativa a la experimentación animal

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Resumen

La investigación preclínica, tradicionalmente basada en modelos animales *in vivo* y en modelos *in vitro* de cultivos celulares bidimensionales, ha permitido generar múltiples avances en la biomedicina. Sin embargo, su capacidad predictiva de los resultados clínicos a veces se ha visto comprometida por las diferencias fisiológicas con los animales de experimentación o por la excesiva simplicidad de los modelos *in vitro*. Para avanzar en la mejora de los modelos preclínicos se han desarrollado nuevas herramientas como los modelos *in silico* basados en modelos computacionales o los modelos de cultivo celular tridimensionales. Estos últimos comprenden varios tipos de modelos entre los que destacan los esferoides/ organoides, la ingeniería de tejidos, los cultivos ex vivo o la tecnología Organ on Chip. Esta última se basa en el uso de dispositivos microfluídicos (conocidos como chips) para el cultivo celular *in vitro* avanzado. Gracias a la miniaturización y la recreación fidedigna de la organización de los tejidos en el interior de los chips se puede simular en el laboratorio el contexto biológico, químico y mecánico de las células dentro de los órganos y tejidos. Al imitar el microentorno celular (vascularización, estimulación mecánica y/o eléctrica, etc.), las células se comportan de una manera similar a cómo lo harían en los tejidos de los pacientes, ya sea en condiciones fisiológicas o patológicas. Además, es posible unir varios chips que contienen diferentes tipos de órganos y tejidos para generar sistemas más complejos creando lo que se denomina *body on chip*. Una de las principales ventajas de esta tecnología es la posibilidad de emplear células propias de los pacientes, como por ejemplo las células del sistema inmune o de los diferentes tejidos, avanzando así hacia modelos cada vez más personalizados.

Uso de herramientas *in silico* en toxicología

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Resumen

En los últimos años, se ha trabajado mucho para encontrar alternativas a los métodos tradicionales de experimentación con animales por razones éticas, económicas o científicas. En particular para garantizar la seguridad de los medicamentos, una alternativa es el uso de herramientas in silico. En línea con este propósito, el Consejo Internacional para la Armonización de Requerimientos Técnicos para Medicamentos de Uso Humano (ICH, por sus siglas en inglés) creó la guía ICH M7. Esta fue desarrollada como respuesta a la preocupación por la presencia de impurezas potencialmente mutagénicas en productos químicos farmacéuticos y recomienda el uso de herramientas de predicción toxicológica in-silico complementarias para clasificar las impurezas genotóxicas: Una basada en reglas y otra basada en estadística. También sugiere estudiar los caminos de degradación relevantes para identificar no solo las impurezas reales, sino también las impurezas potenciales que pueden surgir durante el proceso de síntesis o debido a la interacción con excipientes. Lhasa Limited es una organización sin fines de lucro que ofrece soluciones de softwares para apoyar la toma de decisiones en seguridad química, minimizando la necesidad de realizar pruebas con animales. Actualmente, Lhasa cuenta con un amplio repertorio de herramientas que se pueden utilizar en distintos estadios del ciclo de vida de un producto farmacéutico. En esta presentación se mostrará la versatilidad y aplicabilidad de las soluciones in silico de Lhasa Zeneth, Derek Nexus y Sarah Nexus en un enfoque regulatorio aplicando la guía ICH M7.

ONTOX project

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Summary

The 3Rs concept, calling for replacement, reduction and refinement of animal experimentation, is receiving increasing attention around the world, and has found its way to legislation, in particular in the European Union. This is aligned by continuing high-level efforts of the European Commission to support development and implementation of 3Rs methods. In this respect, the European project called "ONTOX: ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment" was initiated in 2021 with the goal to provide a functional and sustainable solution for advancing human risk assessment of chemicals without the use of animals in line with the principles of 21st century toxicity testing and next generation risk assessment. ONTOX will deliver a generic strategy to create new approach methodologies (NAMs) in order to predict systemic repeated dose toxicity effects that, upon combination with tailored exposure assessment, will enable human risk assessment. For proof-of-concept purposes, focus is put on NAMs addressing adversities in the liver, kidneys and developing brain induced by a variety of chemicals. The NAMs each consist of a computational system based on artificial intelligence and are fed by biological, toxicological, chemical and kinetic data. Data are consecutively integrated in physiological maps, quantitative adverse outcome pathway networks and ontology frameworks. Supported by artificial intelligence, data gaps are identified and are filled by targeted *in vitro* and *in silico* testing. ONTOX is anticipated to have a deep and long-lasting impact at many levels, in particular by consolidating Europe's world-leading position regarding the development, exploitation, regulation and application of animal-free methods for human risk assessment of chemicals.

Rutas para evaluar la permeación cutánea utilizando el modelo experimental de piel humana nativa

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Resumen

Los principios activos utilizados en el tratamiento de las dermatosis ejercen sus efectos biológicos en los tejidos más profundos de la piel. Para eso, deben poder atravesar el estrato córneo. Por otra parte, por razones de seguridad, la permeación de la piel es indeseable para sustancias como, por ejemplo, protectores solares y repelentes de insectos, que ejercen su efecto sólo en la superficie de la piel. El método estandarizado para evaluar la permeación de la piel se describe en la directriz del ensayo 428 de la OCDE y consiste en aplicar la sustancia en estudio, en la superficie de una piel humana acoplada al aparato de difusión de Franz. La permeación se puede evaluar analizando la presencia de la molécula objetivo en las diferentes capas de la piel, utilizando técnicas analíticas cuantitativas, como cromatografía, espectrometría de masas, ensayo inmunoenzimático, entre otras. Para ampliar y diversificar las pruebas de permeación cutánea, utilizamos la técnica de espectroscopia infrarroja (FTIR-ATR), basada en el principio de absorción de IR, característicos de la molécula objetivo. Esta técnica se presenta como una herramienta menos costosa y más rápida para mediciones cualitativas, donde el objetivo es evaluar si la molécula permea o no el estrato córneo, alcanzando la epidermis viable. Otro recurso disponible consiste en evaluar la permeación marcando la molécula con un fluoróforo. Con este recurso es posible visualizar la profundidad de permeación de la molécula y semi cuantificar la intensidad de fluorescencia de esta permeación. El uso de piel humana nativa ex vivo es una ruta experimental bien establecida en nuestro laboratorio, es una herramienta estándar de oro para predecir pruebas de permeación y otras relacionadas con la seguridad y eficacia de productos tópicos, ya sean cosméticos, farmacéuticos, sanitarios o otro, por representar fielmente la diversa fisiología de la piel humana.

Últimos Avances en Pruebas de Sensibilización Cutánea con Métodos sin uso de Animales

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Resumen

Un sensibilizador cutáneo se refiere a una substancia que lleva a una reacción alérgica después de estar en contacto con la piel en repetidas ocasiones. Las manifestaciones clínicas provocadas por estas sustancias están caracterizadas por urticaria, erupciones cutáneas y enrojecimiento de la piel, comúnmente denominado dermatitis de contacto atópica. La identificación de estos sensibilizadores es un importante criterio de valoración en el área de toxicología, y es requerido por las agencias regulatorias para garantizar la seguridad de los productos y químicos que están en contacto con la piel. Históricamente, las pruebas para identificar sensibilizadores cutáneos han sido basadas en métodos que usan animales como modelo de estudio, por ejemplo, el ensayo del nódulo linfático local (LLNA, en inglés), que se realiza en ratones. El uso de modelos animales en el contexto de sensibilización cutánea, además de generar preocupaciones éticas, ha demostrado tener una baja reproducibilidad y relevancia limitada con humanos. En las últimas décadas, métodos alternativos que no usan modelos animales han sido desarrollados basándose en el mecanismo celular y molecular de sensibilización cutánea en humanos. Estos incluyen una combinación de ensayos *in vitro*, *in chemico,* así como *in silico*. Varios de estos ensayos han pasado por una rigurosa validación y han sido adoptados por la Organización para la Cooperación y el Desarrollo Económico (OCDE), la cual ha publicado protocolos estandarizados para su uso regulatorio. En esta plática se presenta un análisis detallado sobre los métodos alternativos establecidos para la predicción de sensibilizadores cutáneos, las estrategias definidas para la identificación de estas substancias, así como los avances biotecnológicos más importantes para el desarrollo de nuevos métodos.

Experiencia en el uso de alternativas para valorar agroquímicos

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Resumen

La racionalidad en el uso de animales de experimentación ha ido modelando las prácticas científicas para adecuarlas al principio de las 3Rs formulado por Russel y Burch en 1959. Pero desde finales del siglo pasado esta tendencia fue adquiriendo rango normativo aplicable al registro de productos que requieren de ensayos biológicos para la evaluación de su seguridad. Los ensayos de evaluación de toxicidad aguda forman parte del proceso de desarrollo, aprobación y registro de agroquímicos. Distintas organizaciones internacionales, tales como la EPA, la Organización Mundial de la Salud (OMS), la Autoridad Europea de Seguridad Alimentaria (EFSA), han elaborado guías detallando los datos que se exigen para el registro de plaguicidas. Los requisitos regulatorios globales para el registro de ingredientes activos y formulados, se conoce como *six-pack* y exige evaluar las siguientes seis toxicidades: toxicidad oral aguda, toxicidad inhalatoria aguda, toxicidad dermal aguda, irritación y corrosión ocular, irritación y corrosión dérmica, y sensibilización dérmica. Tradicionalmente estos ensayos son realizados en animales, sin embargo, existe una tendencia mundial hacia el uso de métodos alternativos y los organismos reguladores de plaguicidas la han adoptado. La toxicología convencional se basa en observar los efectos adversos en un animal como principal fuente de evidencia, pero la toxicología moderna busca determinar cuál es la combinación o secuencia más efectiva de métodos, basados en los mecanismos toxicológicos subyacentes que provoca una sustancia, mediante enfoques integrales para el testeo y evaluación (IATA). La combinación de métodos de un IATA puede ser estandarizada y ha sido nombrada como Enfoque Definido (DA). Un DA consiste en un procedimiento aplicado a datos generados por métodos no basados en animales con el propósito de generar una predicción. En esta presentación recorreremos las herramientas actuales disponibles aplicadas para la evaluación de toxicidad aguda de agroquímicos, sus limitaciones y sus ventajas.

An introduction to NGRA

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Summary

The International Collaboration on Cosmetics Safety (ICCS) is a global multi-stakeholder not-for-profit organization focused on advancing the adoption of animal-free assessments of cosmetics, and their ingredients, for human health and environmental safety. ICCS has a diverse cross-sectorial membership, including cosmetic product and ingredient manufacturers, cosmetic and chemical trade and research associations and non-governmental organizations (NGOs), who collaborate to increase the uptake and implementation of New Approach Methodologies (NAMs) and Next Generation Risk Assessment (NGRA) frameworks. The demand for non-animal safety assessments such as NGRA is increasing globally due to changes in societal attitudes, a need for better species relevance, and regulatory change. NGRA is defined as an exposure-led, hypothesis driven risk assessment approach that incorporates one or more NAMs to ensure that chemical exposures do not cause harm. The nine principles of NGRA have been described by the International Cooperation on Cosmetics Regulation (ICCR) and the application of NGRA described in a workflow developed by SEURAT-1, a European public-private research consortium working towards animal-free testing of chemicals. Despite this, many organizations and regulators have yet to adopt NGRA for safety assessment, and consequently, much work has been done to increase confidence in NGRA and provide examples of its application. To this end, many case studies on NGRA have been developed and published in peer-reviewed scientific journals or published by the Organisation for Economic Co-operation and Development (OECD) Working Party for Hazard Assessment's Integrated Approaches to Testing and Assessment (IATA) Case Studies Project.

AFSA Master Class in animal-free safety assessment of cosmetics and cosmetic ingredients

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Summary

Significant advances have been made over the last decade in science and regulations to end animal testing worldwide. Increasing confidence in non-animal methods is critical for promoting acceptance and use. Toward that aim, the Animal-Free Safety Assessment (AFSA) Collaboration – a global coalition of corporate, non-profit and science leaders - has developed a Master Class in animal-free risk assessment of cosmetics and cosmetic ingredients (chemicals). The AFSA Master Class is a free, in-depth, e-learning course based on the principles of Next-Generation Risk Assessment (NGRA), an exposure-led, hypothesis-driven risk assessment approach that integrates data from in silico, in chemico and in vitro approaches in a tiered framework for safety assessment for both internal company decision-making and regulatory safety assessments of products and ingredients. The tiered approach begins with assessment of exposure scenarios and existing or easily obtainable toxicity information and moves to more data-rich, time-intensive methods only if needed to fulfil the needs of the specific assessment. The problem-formulation based approach emphasizes the need to consider exposure at all assessment levels, and the evaluation of risk is based on a comparison of the likely exposure to the population of interest and the dose where toxicological response occurs. The overall goal of an NGRA is human safety, and the assessment is designed to prevent harm rather than predict specific toxicities. The AFSA Master Class modules comprise all steps of the NGRA: the framework, problem formulation, consumer exposure, predictive chemistry, exposure-based waiving, history of safe use, bioactivity (in vitro assay synthesis), internal exposure, and integration into risk assessment. A separate module discusses the global regulatory landscape for cosmetics and chemicals. AFSA invites all interested stakeholders to register and contribute to a more effective, efficient, and ethical safety paradigm for cosmetics and chemicals.

Education, training and application of non-animal methods (NAMs) in Latin America

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Summary

The approval of Law No. 11,794/2008, known as the Arouca Law, was a milestone for regulating animal use in scientific experimentation and education in Brazil and Latin America. This law led to the creation of the National Council for the Control of Animal Experimentation (CONCEA), which oversees the introduction of alternatives to animal testing. In 2012, the Brazilian National Network of Alternative Methods (RENAMA) was established with 51 members. RENAMA focuses on developing, validating, training, and disseminating new approach methodologies (NAMs) to reduce dependence on external technology. In 2015, the Regional Platform of Alternative Methods to the Use of Animals (PReMASUR) was created, relying on RENAMA for education and training activities in Argentina, Brazil, Paraguay, and Uruguay. More recently, CONCEA No. 58/2023 imposed a partial ban on cosmetic testing on animals, prohibiting the use of vertebrate animals in the scientific research, development, and quality control of cosmetics. This presentation will highlight educational activities empowering innovators and regulators to apply modern research and regulatory assessment approaches. For example, the Laboratory of Education and Research in In Vitro Toxicology at the Federal University of Goiás, Brazil, has introduced new in vitro scientific concepts to hundreds of undergraduate and graduate students. Our team has trained professionals, scientists, and regulators from Brazil and other South American countries in non-animal methods through in-person lectures, networking opportunities, online webinars, and hands-on demos. By working cooperatively, we have demonstrated how non-animal safety science is best used to regulate the ethical use of chemicals, enabling sustainable innovation and ensuring human and environmental safety. We have focused on cross-sectoral collaboration with academia, industry, CROs, and regulators to advance knowledge of NAM-based chemical assessments throughout Brazil and Latin America, assisting researchers and communicating advanced NAMs to accelerate adoption and regulatory acceptance.

Capacity building as a strategic tool for strengthening national laboratory infrastructure in support of NAMs

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Summary

The National Institute of Metrology, Quality, and Technology (Inmetro) is a unique institution due to its diverse activities in metrology, conformity assessment (e.g., accreditation, Good Laboratory Practice (GLP) authority), and consumer product regulation. Since 2012, Inmetro has served as one of three Central Laboratories of the National Network of Alternative Methods (Renama), a pivotal initiative by the Ministry of Science, Technology, and Innovation (MCTI), established to promote the implementation, dissemination, and validation of alternative methods to animal experimentation. The network currently includes 39 members from academia and the private sector. In 2015, MERCOSUR's regional platform for alternative methods to animal experimentation (PReMASUR) was approved under the Specialized Meeting on Science and Technology (RECYT) and has been coordinated by Inmetro ever since. PReMASUR, another key initiative from MCTI, relies on RENAMA's experts for training activities primarily in Brazil, offering opportunities for researchers from MERCOSUR countries. To date, PReMASUR has organized 25 courses focused on in vitro methodologies, training over 250 professionals from Brazil and other MERCOSUR member countries. Additionally, Inmetro has conducted four interlaboratory comparisons, involving 17 participants, focused on OECD in vitro methods. Our findings strongly suggest that periodic evaluation of laboratory performance by an independent third party can significantly enhance the quality of results, even though such evaluations are not mandated under the Good Laboratory Practice (GLP) management system. Given the growing complexity of new technologies in this field, successful participation in interlaboratory comparisons—whether through a proficiency testing program or an external quality assessment (EQA) — can serve as credible evidence of a laboratory's quality and competence, recognized by key stakeholders such as regulators. This presentation will highlight the key advances and challenges encountered in building capacity for NAMs.

Advancing New Approach Methodologies (NAMs) in LATAM: From reconstituted skin and cornea to more complex models

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Summary

Latin America is demonstrating significant progress in adopting New Approach Methodologies (NAMs) for toxicological testing. This progress is evident in the region's increasing utilization of reconstructed human skin and cornea models to assess various toxicological endpoints. Over the past five years, these models have facilitated robust evaluations of skin irritation (OECD TG 439) and corrosion (OECD TG 431). Similarly, reconstructed human cornea, integrated into research protocols within the last two years, have enabled reliable eye irritation assessments (OECD TG 492 and 492B). Implementing OECD-accepted alternative models in countries like Brazil serves as a catalyst for broader NAM adoption within LATAM. In other geographies, progress was made in skin sensitization (OECD TG 497) with the development of defined approaches and genotoxicity with high tier 3D assays currently under ESAC peer review. To further advance the safety assessment of cosmetic ingredients and address critical adverse human health effects such as repeated dose systemic toxicity, current research endeavors are focused on implementing and building confidence on frameworks based on new approach methodologies. These efforts signify the region's commitment to refining regulatory toxicology and reducing reliance on animal testing. Crucial to this progress is continued collaboration between the scientific community and regulatory bodies, which will be essential to foster the development, validation, and widespread acceptance of these vital alternative methods within LATAM.

Canada's path to NAM's implementation in chemical regulatory programs: successes, challenges and shaping the future

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Summary

Health Canada, like many international regulatory agencies, is committed to integrating New Approach Methods (NAMs) to address information needs in different regulatory contexts and to promote the transition toward replacing animal toxicity testing methods for the implementation of Next Generation Risk Assessment (NGRA). This is underscored by the newly introduced amendments to the Canadian Environmental Protection Act (CEPA) requiring the timely incorporation of alternative methods and strategies, as science permits, for chemical toxicity testing and assessment. It is recognized that this effort will take time, needing a progressive and iterative approach. Focusing on the application of NAMs in the context of data poor industrial chemicals, an emphasis under Canada's Chemicals Management Plan (CMP) has been on the development and standardization of fit-for-purpose in silico and in vitro methods to increasingly support prioritization, grouping and read-across, and for use as quantitative risk-based approaches that can be as protective of human health in the absence of traditional in vivo hazard data. This presentation will highlight key advances in computational approaches for hazard identification and the use of methods for deriving NAM-based points-of-departure from in vitro bioactivity data using in vitro-in vivo extrapolation. To guide future efforts at achieving the goal of replacing, reducing or refining the use of vertebrate animal testing, a strategy is being developed within the context of CEPA; the proposed key elements to be addressed on the path to implementation will be discussed. Together, these efforts contribute to the development of robust methods for integration into larger NAM-based frameworks and support the global transition to a non-animal approach to chemical risk assessment.

Regulatory acceptance of non-animal methods (NAMs) for cosmetics in Brazil

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Summary

The Brazilian Health Regulatory Agency (Anvisa) is responsible for regulating and monitoring the compliance of cosmetics exposed to consumption in Brazil. The word "cosmetics" is used here to refer to cosmetics, personal hygiene products and perfumes. Resolution RDC 752, of 2022, from Anvisa defines and establishes technical requirements and procedures for the regularization of cosmetic products, among other requirements. The regularization of these products must be through registration or prior communication by notification. The company responsible for the regularization of cosmetic products must have complete data proving the safety of the product and guarantee that the product complies with current regulations. However, it is only when proof of specific safety is required by current regulations or when a safety attribute is expressed on the label, that it is mandatory to present a summary of data proving safety of use in the product regularization process. The Guide for Safety Assessment of Cosmetic Products, from 2012, and Resolution RDC 35, from 2015, which provides for the acceptance of alternative methods of animal experimentation recognized by the National Council for the Control of Animal Experimentation – Concea, published by Anvisa, establish general guidelines for Regulatory Acceptance of NAMs for Cosmetics in Brazil. Furthermore, publications from the International Cooperation on Cosmetics Regulation (ICCR), of which Anvisa is a member, and the Organisation for Economic Cooperation and Development (OECD), are also used as complements to Anvisa publications. Vertebrate animal testing is not required to assess the safety of cosmetics. It is possible to prove the required toxicological endpoints based on ingredient information, in vitro methods and/or clinical tests, following the Integrated Approaches to Testing and Assessment (IATA). The presentation will give an overview of the Regulatory Acceptance of NAMs for Cosmetics in Brazil, considering the publications mentioned in this summary.

Estrategias para la integración de información

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Resumen

Minimizar los riesgos ambientales y para la salud humana asociados a los productos químicos es un tema regulatorio crítico. Este ejercicio requiere la identificación, compilación e integración de información acerca de los peligros intrínsecos de las sustancias químicas, la exposición y las relaciones entre exposición, dosis y efectos adversos. Avances significativos en métodos de prueba, biotecnología y modelos computacionales permiten pruebas de toxicidad más rápidas, menos costosas y más relevantes para las respuestas humanas que los métodos tradicionales de prueba de toxicidad. Estos nuevos métodos se basan en enfoques que reducen la necesidad de pruebas en animales (métodos *in vitro*, *in chemico*, modelos computacionales).

La Organización para la Cooperación y el Desarrollo Económicos (OCDE) ha desarrollado estrategias para la integración de información con el fin de promover la adopción regulatoria de estas nuevas metodologías, contribuyendo así a una mayor eficiencia y ética en la evaluación de la seguridad de las sustancias químicas. Tres de estas estrategias clave son:

Integrated Approaches for Testing and Assessment (IATAs, enfoques integrados para la prueba y evaluación) combinan múltiples fuentes de información para concluir sobre la toxicidad de los productos químicos. Los IATA pueden incluir información existente de la literatura científica u otras fuentes, junto con datos recién generados como resultado de nuevos métodos de prueba de toxicidad o métodos tradicionales para llenar lagunas de datos. Estos enfoques se desarrollan para abordar un escenario regulatorio específico o un contexto de toma de decisiones.

Los Defined Approaches (enfoques definidos) son similares a los IATAs ya que hacen predicciones basadas en múltiples fuentes de datos, pero los enfoques definidos están basados en reglas. Proporcionan un marco estructurado y reproducible.

La integración de datos de diferentes métodos y fuentes puede ser compleja, especialmente al considerar diferentes niveles biológicos y las relaciones entre eventos clave y efectos adversos. Para abordar esto, se utilizan los Adverse Outcome Pathways (AOP, vías de resultados adversos) como un marco para organizar y evaluar datos recopilados de diferentes métodos y relevantes para diferentes niveles biológicos.

Perspectivas regulatorias para el uso de animales de experimentación

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Resumen

El uso de animales como modelos de investigación genera dilemas éticos por el estatus moral animal que reconocen las sociedades contemporáneas, lo que ha impulsado la generación y validación de nuevas herramientas que Reemplacen el uso de animales, Reduzcan el número utilizado o Refinen las técnicas en pro del bienestar animal (3R), sin afectar el progreso científico, académico e industrial. Es así como en diferentes países y regiones se han desarrollado normativas para impulsar la adopción de métodos alternativos (MA) con la creación de centros que validen y regulen su implementación. En Colombia se constituyeron nuevas leyes que reconocen a los animales como seres sintientes y se prohíbe su uso en ciertas áreas como la evaluación de la seguridad y eficacia de productos cosméticos. Sin embargo, es necesario reglamentar la ley con el fin de implementar los cambios regulatorios requeridos para realizar la transición a MA en los casos necesarios. Con este fin se deben articular las partes interesadas (entidades del estado, investigadores, academia, industria y gremios profesionales) y construir de manera conjunta planes de acción, considerando la experiencia de países de referencia sobre las mejores formas para desarrollar los marcos regulatorios, los mecanismos para establecer centros o laboratorios que puedan ofertar pruebas con MA, las estrategias para orientar la validación de MA y la consolidación de un repositorio de información de pruebas alternativas válidas, junto a capacitaciones para que los interesados puedan encontrar la información técnica y regulatoria que les permita tomar decisiones para aplicar adecuadamente

Presentaciones orales

El valor de la certificación cruelty free en la industria cosmética (COL010)

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Resumen

El mercado cosmético latinoamericano crece a paso firme, se espera que su valor para el año 2025 alcance los 45.440 millones de dólares. Sin embargo, esta tendencia implica un aumento de animales utilizados para experimentación. Si bien algunos países han fortalecido sus leyes para regular el testeo animal en cosmética, mientras éstas no se implementen y se permitan estas prácticas en otras zonas del mundo, la única herramienta efectiva que poseen los consumidores para asegurarse de que los productos no han sido testeados en animales, es la certificación Cruelty Free. Este proceso inspecciona la cadena de suministro de las empresas para verificar que los proveedores de ingredientes y productos no encargan ni realizan pruebas en animales. Esta acreditación resulta valiosa para los consumidores ya que, una encuesta realizada el año 2019 en Chile, mostró que el 74% de los encuestados no compraría productos testeados en animales, y en consecuencia, hoy el atributo Cruelty Free está consolidado en los principales retails del país. Marcas como Petrizzio y Ballerina se vieron impulsadas a acreditarse por las demandas de la ciudadanía, y ahora destacan como pioneras en certificarse con Te Protejo, única organización de origen latinoamericano que ofrece este sello. Más de 500 marcas en la región son Cruelty Free, entre las que destacan Natura, L'bel, Ésika, Cyzone; y a nivel internacional, empresas o conglomerados como Lush, The Body Shop y Unilever, han demostrado un gran compromiso con la causa: generan instancias de aprendizaje, fomentan el avance de los proyectos de ley (campaña financiada por Lush en 2021 para impulsar la aprobación boletín 19.966-11 que busca prohibir el testeo animal para cosméticos en Chile), campañas de concientización (campaña Forever Against Animal Testing en 2017 financiada por The Body Shop), y apoyan el desarrollo de métodos alternativos (Unilever ha desarrollado al menos 8 pruebas ensayos NAM'S para la evaluación de la seguridad de sus ingredientes, desde 1990 se cuenta con el SEAC, equipo de 160 científicos que

utilizan el Enfoque Basado en Evidencia para acreditar la seguridad de productos e ingredientes, desde el 2023 es miembro de ICCS, coalición que evalúa y desarrolla enfoques de evaluación de seguridad libres de testeo animal). Los métodos alternativos. Éstos últimos son altamente respaldados por los consumidores de cosmética, es por ello que resulta fundamental seguir impulsando el cambio y ayudar a las marcas a transitar hacia una cosmética segura para las personas y respetuosa hacia los animales.

Simulator as a tool to support training in the surgical technique of extracapsular cataract extraction in canines (COL026)

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Summary

The increasing presence of domestic animals in homes has generated the need for specialized veterinary care, such as veterinary ophthalmology. Cataracts, a common cause of blindness in canines, may have hereditary aetiology or be associated with other diseases, and their timely diagnosis and treatment are crucial to preserve the animal's quality of life. Although surgery is the primary treatment, its success depends mainly on the surgeon's skill; the extracapsular extraction technique, one of the most used, presents intraoperative complications in up to 10% of cases. Because training with cadavers does not adequately replicate ocular biomechanical properties, a simulator prototype has been developed for training this technique, allowing realistic practices and improving professional competence. The development of the simulator involved the characterisation of the eye and the surgical technique through a bibliographic and experimental review, as well as a biomechanical analysis of the eye under stress and pressure, taking into account the forces generated by the extraocular muscles and the force required for the initial incision in the sclera (2.36 N), which an electronic system will measure during the procedure. In the design stage, 3D modeling of the mechanical and electronic systems was performed, using 3D printing and casting techniques to obtain the parts that simulate the tissues and the mesocephalic head of the canine. For the electronic design, strain gauges were proposed, specifically the BF120-10AA model, which allows measuring the established force. A first evaluation showed improvements in the simulator design concerning the size and arrangement of the interchangeable parts. In conclusion, the characterisation, design, and implementation process allowed for the optimal sizing of the defined skull type and the understanding of the corresponding biomechanical behavior. Although the calibration process of the electronic system is ongoing, preliminary results indicate that the location selected for the sensor allows for reducing dead zones and minimizing the margin of error in measurements.

Implementación de un programa de seguimiento del movimiento animal para investigar tratamientos innovadores en la dependencia a cocaína fumable (COL015)

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Resumen

Algunos estudios preclínicos sugieren el uso de cannabis para el tratamiento de la adicción a la cocaína. El objetivo de este trabajo fue determinar el potencial terapéutico de un extracto del cannabis no psicoactivo y CBD aislado como una nueva perspectiva para tratar la dependencia de la cocaína fumable en un modelo murino, siguiendo el paradigma de condicionamiento de preferencia de lugar (CPL). Durante toda la experimentación (56 días) fue usado un software especializado (AnyMaze®) que permitió medir con precisión el tiempo de exploración para 9 grupos experimentales de 10 individuos y un grupo control de 24 individuos (n=114). Gracias al uso de este software se pudo utilizar un número mayor de individuos en un ensayo a la vez, y esto permitió reducir el porcentaje de error comportamental. De este modo, con este software se obtuvieron datos paramétricos con un número reducido de individuos, lo cual supone una mejora en la técnica de recolección de datos si se compara con otras técnicas más antiguas. Al concluir los experimentos de CPL se observó un comportamiento más tranquilo de los individuos experimentales al no estar presente el investigador durante el ensayo. Además, el uso del software facilitó la rápida estandarización de los experimentos y protocolos de CPL ahorrando tiempo de experimentación. Todas estas mejoras se traducen en un refinamiento de las técnicas utilizadas en el manejo de los animales y evita el empleo de más unidades de experimentación asociadas a errores durante el desarrollo de los diferentes ensayos. Esto aumenta la confiabilidad de los datos obtenidos y supone una reducción sustancial en la repetición de ensayos. Con esta metodología propuesta fue posible identificar que el extracto de cannabis no psicoactivo es mejor que el CBD como tratamiento a la dependencia de la cocaína fumable.

Artemia salina: modelo alternativo al ensayo de letalidad en ratones inducida por venenos de serpiente y neutralización por antivenenos ofídicos (COL008)

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Resumen

El modelo en ratones es la principal herramienta en investigación de venenos de serpiente al evaluar la toxicidad de venenos y la eficacia preclínica de antivenenos. Sin embargo, estas pruebas demandan muchos ratones e implican mucho sufrimiento. El ensayo de toxicidad en *Artemia salina* puede ser una alternativa al empleo de ratones por su fácil manejo, bajo costo, hallazgos rápidos, beneficio ético y amplio uso en toxicidad ambiental. Sin embargo, su aplicación en venenos y antivenenos ha sido limitada y se desconoce si la misma se correlaciona con la prueba de letalidad en ratones. El objetivo fue caracterizar la utilidad del ensayo en *A. salina* como modelo sustituto para evaluar la toxicidad de venenos de serpiente y la eficacia de antivenenos. Se determinó la letalidad de venenos de 22 serpientes de importancia médica en África Sub-Sahariana, en ratones (dosis letal media, DL_{50}) tanto por vía intraperitoneal (i.p) como intravenosa (i.v) y en *A. salina* (concentración letal media, CL_{50}). Subsecuentemente, se determinó la capacidad de un antiveneno para neutralizar la toxicidad de estos venenos (dosis eficaz media, DE_{50}) en ambos modelos.

Se observó correlación moderada entre las DL_{50} vía intravenosa e intraperitoneal en ratones, entre DL_{50s} intravenosa entre ratones y CL_{50s} en *A. salina* y entre DL_{50s} intraperitoneal en ratones y CL_{50s} en *A. salina*. Se observó una fuerte correlación entre las DE_{50s} intraperitoneales e intravenosas en ratones, intraperitoneales en ratones y en *A. salina*, e intravenosas en ratones y en *A. salina*. Estos hallazgos permiten proponer el ensayo de toxicidad empleando *A. salina* como candidato sustituto del ensayo en ratones especialmente en etapas del control rutinario de calidad de antivenenos, por ejemplo, al evaluar la capacidad neutralizante del plasma hiperinmune o al verificar el desempeño de lotes completos de antivenenos. Es necesario ampliar estas observaciones con otros venenos y antivenenos.

Larvas de *Tenebrio molitor* como protocolo experimental para avaliação de estresse oxidativo utilizando silibinina (COL039)

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Resumo

A exposição da pele de forma exacerbada pelas diferentes fontes de radiação UV gera danos cutâneos, como a formação de espécies reativas a oxigênio, que são evitadas na presença de antioxidantes. Dentre os produtos naturais que se destacam com essa atividade, encontra-se a silibinina, principal bioativo da silimarina, que apresenta atividades como: anti-inflamatória, citoprotetora, anticarcinogênica. Diante disso, o objetivo deste trabalho foi estudar o estresse oxidativo da silibinina em larvas de *Tenebrio molitor*. Para tal análise, o estresse oxidativo foi induzido pela administração de 2µL de peróxido de hidrogénio, após 1 hora de tratamento. Os grupos foram divididos da seguinte forma: *Shan* (larvas não tratadas e sem indução do estresse oxidativo), controle negativo (salina + peróxido de hidrogénio), controle positivo (Trolox (1µg/mL) + peróxido de hidrogénio), veículo (salina) e tratamento (20 mg/kg de silibinina + peróxido de hidrogénio). Após 1 hora das administrações, a linfa das larvas foi coletada por grupo (através de corte na larva), diluída em tubos Falcon com 2 mL de salina, filtrada e analisadaa por Espectroscopia de Infravermelho com Transformada de *Fourier*. Como resultado, o espectro da silibinina apresentou semelhança com as bandas do controle positivo, exibindo as mesmas bandas idênticas, como 1747, 1240 e 1158 cm^{-1}. A diferença entre eles se estabeleceu no deslocamento mais claro da maioria das regiões, como de 3602 a 3609, 3440 a 3444, 3328 a 3321,

1652 a 1650, 1590 a 1595, 1542 a 1540 e 1460 a 1465 cm^{-1}. Além disso, as bandas 2.930 e 2.854 cm^{-1} apareceram em ambos os espectros, mas na silibinina houve um claro aumento na intensidade do pico. Portanto, a substância silibinina apresentou atividade análoga ao estresse oxidativo em comparação ao controle positivo, sendo considerada uma substância promissora para a fotoproteção.

Evaluación de una matriz para un cultivo celular mixto en 3D y su aplicación como modelo de hipoxia renal (COL018)

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Resumen

Existen eventos que afectan al riñón trasplantado. El daño se denomina, daño por isquemia-reperfusión, es importante conocer los mecanismos fisiológicos involucrados. Han sido empleados modelos animales para replicar isquemia/reperfusión, sin embargo, debido a las tendencias en reducción del uso de animales, se han optado por estrategias libres de ellos. Actualmente los modelos de cultivo celular en 3D tienen un gran auge y popularidad, ellos proporcionan información de los eventos fisiológicos *in vivo*, pero sin utilizar organismos vivos. Es por ello, que evaluar la funcionalidad de una matriz de cultivo en 3D permitirá crear un modelo de hipoxia renal empleando un cultivo mixto de células renales. La matriz desarrollada permite desarrollar un modelo de hipoxia empleando células tubulares renales (HK2), mediante construcción de hidrogeles de 800 mm^3 base gelatina 7.5% y alginato 3.75%. Fue evaluada la reproducibilidad y características fisicoquímicas. Finalmente, fueron embebidos esferoides de células HK2 obtenidos por cultivo en suspensión orbital 90rpm, 80%h y 5%CO_2, así mismo también células de endotelio vascular (EA.hy926) libres, esta construcción de matriz con células se estimuló con 100 μM $CoCl_2$. Se evaluaron matrices en 9 réplicas diferentes y en 5 días diferentes, se obtuvieron datos homogéneos y reproducibles, peso y altura promedio de 0.7 g y 0.6 cm respectivamente, con CV<7% por cada día para ambos parámetros, su capacidad de captación de agua alcanza un pico máximo a las 24h. Estas matrices fueron adecuadas para el crecimiento de células renales bajo condiciones estándar de normoxia, así como por hipoxia inducida con cloruro de cobalto a 100 μM sin afectar su viabilidad comparadas ante el control *(P<0.05)*. En conclusión, fue posible formular matrices replicables de gelatina/alginato, con parámetros fisicoquímicos adecuados y se demostró biocompatibilidad con células renales humanas, permitiendo el potencial desarrollo de modelos fisiopatológicos libres de animales.

La hipertermia potencia la actividad citotóxica del paclitaxel en un modelo tridimensional de cáncer de seno tipo esferoide (COL049)

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Resumen

El cáncer de seno es una patología con una de las tasas más altas de incidencia y mortalidad en Colombia y el mundo. Diferentes estrategias terapéuticas han sido empleadas para su manejo, sin embargo, el desarrollo de quimiorresistencia y la aparición de metástasis han obligado a explorar nuevas alternativas terapéuticas. Recientemente, se ha demostrado que terapias multimodales basadas en hipertermia mejoran la eficacia de tratamientos antitumorales, sin embargo, la evidencia en modelos 3D es limitada. En este trabajo se evaluó si la hipertermia aumentaba la actividad citotóxica del paclitaxel en un modelo tridimensional de cáncer de seno tipo esferoide. Los esferoides fueron generados a partir de células MCF-7 empleando placas de 96 pozos de adhesión ultrabaja Corning®, cultivados en 200 µL de DMEM suplementado con SFB al 10% y gentamicina (25 µg/mL); 72 horas después de su formación, fueron tratados durante 48h con paclitaxel y luego expuestos a la hipertermia (45°C) por 5min. La viabilidad de los esferoides se evaluó mediante el ensayo de reducción de resazurina, el kit de fluorescencia Live/Dead y la actividad de la enzima lactato deshidrogenasa. Adicionalmente, se evaluaron cambios morfológicos y la capacidad de formar clones. Los ensayos se realizaron en triplicado en tres experimentos independientes. Los resultados mostraron que la hipertermia (45°C por 5 min) potencia el efecto citotóxico del paclitaxel (0,160 µM y 0,190 µM) aumentado porcentajes de citotoxicidad a 51,6% y 59,8% respectivamente, en comparación con la hipertermia sola (20,9%) y el paclitaxel solo 0,160 µM (22,6%) y 0,190 µM (37,0%). Estos resultados fueron comparables a los encontrados en el ensayo clonogénico, observándose una clara disminución en la capacidad para proliferar y conformar colonias bajo las condiciones de ensayo establecidas. Por otro lado, los esferoides expuestos a ambos tratamientos disminuyeron la compactabilidad, lo que en consecuencia generó la

desagregación del esferoide parcial o totalmente, acompañado de disminuciones en la circularidad, esfericidad, convexidad y solidez. Con base en lo anterior se puede concluir que la hipertermia potenció el efecto citotóxico del paclitaxel por un sinergismo entre ambos tratamientos.

Desarrollo de un modelo 3D de artritis reumatoide para evaluar efecto de fármacos modificadores de la enfermedad (COL007)

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Resumen

La artritis reumatoide (AR) afecta alrededor del 1% de la población mundial, está caracterizada por daño articular que lleva a pérdida de la función en etapas avanzadas. Actualmente no existe cura y hay aspectos de la enfermedad que aún se desconocen. Para entender la fisiopatología y encontrar tratamientos efectivos se utilizan modelos de la enfermedad. Los modelos en cultivo 3D permiten interacciones célula-célula, célula-matriz y hacer pruebas directamente en células humanas. Los modelos *in vitro* de AR se han concentrado en etapas tempranas para evaluar el daño en cartílago. En el presente trabajo se desarrolló un modelo de AR para simular el daño avanzado en la articulación, cuando hay afectaciones en el hueso subcondral. En el cultivo se utilizó un soporte de ácido poliláctico e hidrogel compuesto por gelatina-colágeno sin entrecruzar para simular las condiciones de una articulación con inflamación. Para simular las principales células responsables de este daño, se realizó un cocultivo con la línea celular HFF-1 de fibroblastos y monocitos humanos activados con LPS para obtener el fenotipo M1. En este cocultivo se expresa el TNF-α, citocina clave en esta patología, que fue detectada por ELISA y Western blot. En el modelo desarrollado se probaron fármacos ampliamente utilizados; un fármaco modificador de la enfermedad (metotrexato) y un antiinflamatorio (celecoxib) donde los resultados coincidieron con lo reportado anteriormente por otros autores, además de asemejarse al efecto *in vivo*. Adicionalmente, se comparó el efecto de fármacos entre el modelo desarrollado contra el cocultivo en monocapa, el primero demostró que se simulan de manera más adecuada las condiciones y respuestas que se dan *in vivo*. En conclusión, se desarrolló un modelo para simular daño avanzado en AR que permitió evaluar el efecto de fármacos y simuló de manera más adecuada la respuesta a éstos en comparación con el cultivo en monocapa.

Cuantificación de la fusión de agregados celulares: uso de un modelo *off-lattice* con parámetros geométricos extraídos de datos experimentales (COL029)

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Resumen

La ingeniería de tejidos ha avanzado significativamente con el desarrollo de técnicas para crear estructuras tisulares complejas. Este estudio analiza la dinámica de fusión de agregados celulares mediante enfoques experimentales y simulaciones, comparando tres metodologías establecidas: Yalla, MecaGen y Oriola. Usando Julia y CUDA junto con nuestro programa CellAggregate.jl, investigamos parámetros clave como la fuerza de atracción-repulsión, las fuerzas contráctiles y el coeficiente de difusión del vector de polarización. La hipótesis principal es evaluar si estas metodologías pueden predecir con precisión tanto la fusión de agregados celulares como la estabilidad del proceso. Para evaluar esta hipótesis, se extrajo información sobre contornos de fusión de agregados de la literatura y se llevaron a cabo simulaciones y experimentos con datos estructurados. Se analizó cómo las fuerzas mencionadas afectan la dinámica de fusión y se compararon los resultados con las predicciones de los modelos. Los resultados muestran que el modelo biofísico es eficaz para identificar fases discretas de coalescencia y que la aproximación del vector de polarización describe adecuadamente las interacciones celulares. La calibración in-silico y las simulaciones revelan diferencias significativas en la velocidad y estabilidad de la fusión entre los enfoques de Oriola y MecaGen. Además, el estudio profundiza en la estabilización de un solo agregado celular bajo condiciones extremas, evaluando la pérdida de células y el desplazamiento cuadrático medio. La fusión de agregados celulares también se evaluó mediante la prueba de Chi-cuadrado, ofreciendo una visión integral de los aspectos topológicos en la bioimpresión de tejidos. En conclusión, la capacidad para cuantificar con precisión la fusión de agregados celulares es esencial en Ingeniería de tejidos. Este estudio subraya que una medición adecuada y modelización de estos procesos puede optimizar las técnicas de bioimpresión, mejorar el diseño de tejidos complejos y avanzar significativamente en el campo de la ingeniería de tejidos.

Explorando la expresión diferencial de genes en toxicología forense de opioides: un enfoque alternativo al uso de animales de experimentación (COL012)

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Resumen

El abordaje convencional para estudiar la causa de la muerte debido a una intoxicación por opioides involucra el uso extensivo de animales de experimentación, planteando no solo preocupaciones éticas, sino limitaciones en la interpretación de resultados desde un contexto legal. El objetivo de esta investigación fue identificar posibles biomarcadores y rutas metabólicas asociadas al uso de opioides como alternativa al uso de modelos animales, mediante técnicas de bioinformática para analizar la expresión diferencial de genes asociada a la toxicidad de dichas sustancias. El estudio se centró en datos obtenidos a través de repositorios como GEO DATASETS, que reúne información de muestras animales, humanas y de líneas celulares expuestas a diferentes concentraciones de opioides, utilizando plataformas de microarreglos y secuenciación de próxima generación para obtener perfiles detallados de expresión génica. Los datos recopilados, se analizaron mediante algoritmos avanzados para la identificación de patrones de expresión, mediante técnicas estadísticas robustas para la validación de la significancia de los genes diferencialmente expresados, aplicando a su vez, un análisis completo de enriquecimiento funcional, para explorar las vías biológicas afectadas. Este enfoque permitió identificar genes clave como DDR2, importante para la vía de la recompensa y algunos asociados a la interacción con los receptores opioides mu que pueden estar involucrados en la respuesta tóxica a los opioides. Además de proporcionar una metodología mucho más acorde con los lineamientos éticos en el uso de animales en la investigación, los resultados promueven la comprensión de la toxicología de los opioides a nivel molecular,

facilitando la interpretación de casos forenses relacionados con muerte por opioides. Este proyecto no solo subraya la viabilidad de los métodos alternativos en la investigación toxicológica, sino que también establece un precedente para futuros estudios en diversas áreas de la farmacología y toxicología que tradicionalmente dependen del uso de modelos animales.

Identificación *in silico* de moléculas con potencial actividad para el cáncer de seno triple negativo (COL025)

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Resumen

Entre los diferentes cánceres de seno, el triple negativo (TNBC) persiste como el más desfavorable por su elevada propensión a desarrollar metástasis. Dentro de las subclasificaciones del TNBC, destacan los tumores mesenquimales por su menor cobertura terapéutica; así como una alta asociación con marcadores metastásicos. En este contexto, la modulación de vías como la Wnt-Betacatenina, a través de Tankirasa 1 y 2 podría ser una opción terapéutica relevante. Por ende, el presente trabajo consistió en una fase inicial de investigación de inhibidores de Tankirasas mediante un cribado virtual basado en la estructura. Para ello, se obtuvo una librería de productos naturales derivados de microorganismos a partir del Lichen DB, el NPAtlas, y moléculas del grupo de investigación GRIQUIMED. Se efectuó un tamizaje basado en reglas extendidas de Lipinski y PAINS, seguido de un acoplamiento molecular empleando AutodockVina y Glide en un ensamble de 4 estructuras; finalizando con un filtro de cardiotoxicidad, hepatotoxicidad y mutagenicidad. De las 33863 moléculas iniciales, se destaca la Fellutanina A (NPA012964) por su afinidad estimada, así como sus características ADMET.

Rutas para evaluar la permeación cutánea utilizando el modelo experimental de piel humana nativa (COL001)

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Resumen

Los principios activos utilizados en el tratamiento de las dermatosis ejercen sus efectos biológicos en los tejidos más profundos de la piel. Para eso, deben poder atravesar el estrato córneo. Por otra parte, por razones de seguridad, la permeación de la piel es indeseable para sustancias como, por ejemplo, protectores solares y repelentes de insectos, que ejercen su efecto sólo en la superficie de la piel. El método estandarizado para evaluar la permeación de la piel se describe en la directriz del ensayo 428 de la OCDE y consiste en aplicar la sustancia en estudio, en la superficie de una piel humana acoplada al aparato de difusión de Franz. La permeación se puede evaluar analizando la presencia de la molécula objetivo en las diferentes capas de la piel, utilizando técnicas analíticas cuantitativas, como cromatografía, espectrometría de masas, ensayo inmunoenzimático, entre otras. Para ampliar y diversificar las pruebas de permeación cutánea, utilizamos la técnica de espectroscopia infrarroja (FTIR-ATR), basada en el principio de absorción de IR, característicos de la molécula objetivo. Esta técnica se presenta como una herramienta menos costosa y más rápida para mediciones cualitativas, donde el objetivo es evaluar si la molécula permea o no el estrato córneo, alcanzando la epidermis viable. Otro recurso disponible consiste en evaluar la permeación marcando la molécula con un fluoróforo. Con este recurso es posible visualizar la profundidad de permeación de la molécula y semi cuantificar la intensidad de fluorescencia de esta permeación. El uso de piel humana nativa ex vivo es una ruta experimental bien establecida en nuestro laboratorio, es una herramienta estándar de oro para predecir pruebas de permeación y otras relacionadas con la seguridad y eficacia de productos tópicos, ya sean cosméticos, farmacéuticos, sanitarios o otro, por representar fielmente la diversa fisiología de la piel humana.

Evaluación de la permeación transepidérmica de productos lipolíticos con cafeína en Colombia: impacto en la seguridad del consumidor (COL009)

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Resumen

En Colombia se comercializan productos de aplicación tópica que deben permear la piel para cumplir su propósito en capas más profundas. Es el caso de algunos productos con proclamas lipolíticas que contienen cafeína, un activo que actúa sobre los mecanismos de acumulación de grasa en los adipocitos, lo que además implica que la cafeína pueda llegar a la circulación sistémica, contribuyendo al riesgo de alcanzar su concentración tóxica. El objetivo de esta investigación fue estudiar el comportamiento de permeación de la cafeína incluida en estos productos, en un modelo basado en difusión pasiva empleando como barrera piel de cerdo. Se seleccionaron siete productos del mercado colombiano que prometen un efecto lipolítico para evaluar la liberación de la cafeína y su permeación empleando celdas de difusión de Franz. Previo a estos ensayos, los productos se caracterizaron fisicoquímicamente evaluando pH, contenido de cafeína, parámetros reológicos, microestructura, para establecer relaciones entre el comportamiento de permeación y las características del producto. Los resultados muestran que tres de los productos contienen cafeína en un porcentaje inferior al 0,009 [%w/w], mientras que los restantes tiene concentraciones superiores al 0,39 [%w/w]. Los ensayos de liberación y permeación demostraron que, en la mayoría de los casos, la cafeína se libera del producto, pero menos del 1% de la dosis aplicada alcanza la hipodermis. Este valor es menor a la concentración de cafeína reportada *in vitro* que causa un efecto lipolítico, lo que podría significar una ausencia de la actividad prometida y al mismo tiempo, aportar al aumento de los niveles plasmáticos del activo. La caracterización de los productos y el comportamiento de permeación de la cafeína a capas más profundas de la piel contribuyen a un mejor entendimiento de la relación entre la eficacia del producto y el riesgo toxicológico asociado a su uso.

Eye damage reversibility in an in vitro model of bovine cornea to replace the Draize test completely (COL011)

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Summary

One of the requirements for the registration of substances such as agrochemicals is to provide evidence about their potential eye damage. The Draize test performed in rabbits allows the products to be classified into four categories, considering both the severity of the lesions produced in the animal's eye as well as its healing time. The available alternative methods to this live animal test do not allow documenting the damage reversibility, nor the time necessary for such reversibility to occur, as required by the Global Harmonized System classifications (GHS). Our proposal is to complement the *in vitro* model that uses the bovine cornea as a substrate to predict whether a substance is irritating or non-irritating (BCOP), with a strategy that allows predicting if the observed irritation is reversible and the time it takes to revert. This is necessary to finally replace the Draize test completely. Limbal stem cells play a crucial role in repairing corneal injury. Therefore, we isolated these cells from bovine corneas to evaluate their sensitivity to reference chemicals. A wound healing assay was performed to determine whether these substances affect the replication and migration capacities of the cells. Additionally, a limbal tissue explant culture model was implemented to study if chemical exposure differentially alters cell replication, migration, and overall wound healing. These were evidenced using crystal violet staining and histopathological analysis. In both, the wound healing assay and the limbal tissue explant culture, differences were observed among the different reference chemical treatments. However, these assays alone were insufficient to discriminate effectively between the four categories. While these methods individually were inadequate for accurate category discrimination, a comprehensive approach is being implemented to replace the Draize test. We are evaluating a combination of these methods along with others to assess their effectiveness in detecting the different categories of GHS.

El valor de la certificación cruelty free en la industria cosmética (COL010)

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Resumen

El mercado cosmético latinoamericano crece a paso firme, se espera que su valor para el año 2025 alcance los 45.440 millones de dólares. Sin embargo, esta tendencia implica un aumento de animales utilizados para experimentación. Si bien algunos países han fortalecido sus leyes para regular el testeo animal en cosmética, mientras éstas no se implementen y se permitan estas prácticas en otras zonas del mundo, la única herramienta efectiva que poseen los consumidores para asegurarse de que los productos no han sido testeados en animales, es la certificación Cruelty Free. Este proceso inspecciona la cadena de suministro de las empresas para verificar que los proveedores de ingredientes y productos no encargan ni realizan pruebas en animales. Esta acreditación resulta valiosa para los consumidores ya que, una encuesta realizada el año 2019 en Chile, mostró que el 74% de los encuestados no compraría productos testeados en animales, y en consecuencia, hoy el atributo Cruelty Free está consolidado en los principales retails del país. Marcas como Petrizzio y Ballerina se vieron impulsadas a acreditarse por las demandas de la ciudadanía, y ahora destacan como pioneras en certificarse con Te Protejo, única organización de origen latinoamericano que ofrece este sello.

Más de 500 marcas en la región son Cruelty Free, entre las que destacan Natura, L'bel, Ésika, Cyzone; y a nivel internacional, empresas o conglomerados como Lush, The Body Shop y Unilever, han demostrado un gran compromiso con la causa: generan instancias de aprendizaje, fomentan el avance de los proyectos de ley (campaña financiada por Lush en 2021 para impulsar la aprobación boletín 19.966-11 que busca prohibir el testeo animal para cosméticos en Chile), campañas de concientización (campaña Forever Against Animal Testing en 2017 financiada por The Body Shop), y apoyan el desarrollo de métodos alternativos (Unilever ha desarrollado al menos 8 pruebas ensayos NAM'S para la evaluación de la seguridad de sus ingredientes, desde 1990 se cuenta con el SEAC, equipo de 160 científicos que utilizan el Enfoque Basado en Evidencia para acreditar la seguridad de productos e ingredientes, desde el 2023 es miembro de ICCS, coalición que evalúa y desarrolla enfoques de evaluación

de seguridad libres de testeo animal) . Los métodos alternativos. Éstos últimos son altamente respaldados por los consumidores de cosmética, es por ello que resulta fundamental seguir impulsando el cambio y ayudar a las marcas a transitar hacia una cosmética segura para las personas y respetuosa hacia los animales

Rutas para evaluar la permeación cutánea utilizando el modelo experimental de piel humana nativa (COL001)

Barbara de Freitas Carli

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Resumen

Los principios activos utilizados en el tratamiento de las dermatosis ejercen sus efectos biológicos en los tejidos más profundos de la piel. Para eso, deben poder atravesar el estrato córneo. Por otra parte, por razones de seguridad, la permeación de la piel es indeseable para sustancias como, por ejemplo, protectores solares y repelentes de insectos, que ejercen su efecto sólo en la superficie de la piel. El método estandarizado para evaluar la permeación de la piel se describe en la directriz del ensayo 428 de la OCDE y consiste en aplicar la sustancia en estudio, en la superficie de una piel humana acoplada al aparato de difusión de Franz. La permeación se puede evaluar analizando la presencia de la molécula objetivo en las diferentes capas de la piel, utilizando técnicas analíticas cuantitativas, como cromatografía, espectrometría de masas, ensayo inmunoenzimático, entre otras. Para ampliar y diversificar las pruebas de permeación cutánea, utilizamos la técnica de espectroscopia infrarroja (FTIR-ATR), basada en el principio de absorción de IR, característicos de la molécula objetivo. Esta técnica se presenta como una herramienta menos costosa y más rápida para mediciones cualitativas, donde el objetivo es evaluar si la molécula permea o no el estrato córneo, alcanzando la epidermis viable. Otro recurso disponible consiste en evaluar la permeación marcando la molécula con un fluoróforo. Con este recurso es posible visualizar la profundidad de permeación de la molécula y semi cuantificar la intensidad de fluorescencia de esta permeación. El uso de piel humana nativa ex vivo es una ruta experimental bien establecida en nuestro laboratorio, es una herramienta estándar de oro para predecir pruebas de permeación y otras relacionadas con la seguridad y eficacia de productos tópicos, ya sean cosméticos, farmacéuticos, sanitarios u otro, por representar fielmente la diversa fisiología de la piel humana.

Presentaciones póster

Integración académica de la 3Rs en la educación superior: Implementación y desafíos en la formación en Ciencias Farmacéuticas (COL022)

M. Pilar Vinardell*, Adriana S. Maddaleno, Montserrat Mitjans

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Resumen

A pesar de que en Europa desde 2010 el seguimiento del principio de las 3Rs es un requisito previo a cualquier experimentación con animales, aún persiste un considerable desconocimiento en el ámbito académico ya que actualmente no existe ninguna asignatura dedicada a las 3Rs en los programas de grado, lo que subraya la necesidad de una formación adecuada sobre el tema. En este contexto, hemos trabajado para integrar este principio en cursos de máster y en algunas asignaturas del grado de Farmacia. Hemos impartido clases y/o cursos de máster entre 2 y 30 horas de duración para un total de entre 15 y 30 estudiantes. Todos los estudiantes superaron satisfactoriamente la asignatura. A nivel del grado de Farmacia, hemos incorporado el principio de las 3Rs en los trabajos de fin de grado consistentes en trabajos bibliográficos. En cada curso participaban 2-3 estudiantes bajo nuestra directa supervisión. Los estudiantes debían realizar búsquedas sobre metodologías alternativas a experimentos tradicionalmente realizados con animales de laboratorio. El resultado ha sido exitoso ya que los estudiantes implicados obtuvieron muy buenas calificaciones además de manifestar un elevado grado de satisfacción por tener la oportunidad de aprender sobre un tema del que no tenían formación previa. Asimismo, hemos formado a estudiantes en una asignatura experimental optativa denominada "Trabajo Dirigido". En esta asignatura los estudiantes se incorporan durante un semestre a un grupo de investigación colaborando en las tareas del grupo y son evaluados mediante una presentación oral y la memoria escrita. En nuestro laboratorio, los estudiantes (2 por curso) se formaron en técnicas in vitro alternativas en estudios de toxicología y en el principio de las 3Rs. Se continuará con estos cursos que constituyen la única manera que existe actualmente ya que no existen asignaturas de grado donde se trate el tema, ni se han planteado a nivel institucional para un futuro plan de estudios.

Creación de un centro especializado en métodos alternativos en la Universidad de Costa Rica (COL017)

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Resumen

El Laboratorio de Ensayos Biológicos de la Universidad de Costa Rica (LEBi-UCR) es una unidad especial de investigación que tiene más tres décadas de trayectoria en la conducción de ensayos empleando diversos modelos animales, como ratas, ratones, conejos y hamsters. Se realizan una gran variedad de pruebas que incluyen modelos de seguridad y eficacia de medicamentos para humanos o animales, agroquímicos y productos naturales. En el LEBi, se creará la sección de métodos alternativos, la cual tendrá un laboratorio de cultivo celular, un acuario de investigación y un laboratorio de toxicología computacional e inteligencia artificial. La creación de estos laboratorios permitirá reducir el número de animales que se requieren para pruebas de toxicidad. Por medio de modelaje molecular, algoritmos de predicción de log P, pruebas *in vitro* se podrá obtener perfiles de seguridad en menor tiempo y también con una cantidad de animales menor. Además de las predicciones *in silico* y pruebas *in vitro*, el uso de peces cebra permitirá reducir el uso de roedores. Se podrá sustituir el uso de conejos para la determinación de la irritabilidad dérmica y ocular. Los laboratorios realizan acciones tendientes a disminuir el uso de animales, sin embargo, es necesario una integración en el funcionamiento y desarrollo de modelos que sustituyan de manera certera los modelos biológicos *in vivo*. Dentro de los principales retos está la necesidad de capacitación en distintas técnicas. Desarrollar modelos utilizando inteligencia artificial requiere equipos y software especializado, pero también colaboración de muchas instancias nacionales e internacionales. Para seguir avanzando en el desarrollo de métodos alternativos, se requiere adaptaciones de las estructuras administrativas y organizacionales, además de inversiones que deben ser incorporadas en la planificación estratégica de la Universidad. Indudablemente las inversiones que se realicen en esta línea, mejorará la calidad de las investigaciones y el bienestar animal.

Validación de sistema de simulación de intubación orotraqueal en caninos (COL034)

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Resumen

La aplicación de múltiples técnicas de intervención médicas requiere entrenamiento repetitivo y curvas de aprendizaje para validar el dominio e implementación por parte del profesional en su práctica clínica. En Medicina Veterinaria el uso de animales vivos para la formación, presenta desafíos éticos, logísticos y económicos, además de necesitar supervisión especializada en pro del bienestar animal. Por esta razón la búsqueda de opciones sustitutas para el aprendizaje de estas técnicas, se enfoca cada vez más en el uso de herramientas alternativas en el ámbito de la simulación clínica. Amparados en tecnologías como la segmentación de imágenes médicas y manufactura aditiva se pueden fabricar simuladores que permiten replicar de manera realista modelos anatómicos, que emulen técnicas y procedimientos médicos, facilitando el aprendizaje práctico y la mejora continua de habilidades específicas en quienes los usen. Este estudio evalúa un simulador de sondaje oro-traqueal canino fabricado con impresión 3D, con el cual 60 Médicos Veterinarios y estudiantes de último semestre de pregrado con experiencia en clínica veterinaria, realizaron la técnica antes descrita para luego proceder a completar una encuesta tipo Likert. Los resultados preliminares indican que el 21% de los encuestados (13 participantes) no están familiarizados ni capacitados en la técnica de intubación oro-traqueal. También se destaca la dificultad en comprender el modelo anatómico, en contraste con aquellos que ya conocen y están familiarizados con la técnica, quienes consideran útil el simulador para adquirir destreza. Se concluye que los simuladores como método de enseñanza alternativa pueden optimizar el aprendizaje a través de la repetición controlada, fomentando la adquisición de patrones precisos y memoria muscular, cruciales en la atención de pacientes caninos, aunque es necesario realizar estudios longitudinales para evaluar la efectividad de los simuladores clínicos, analizando cómo impactan en la precisión técnica, la toma de decisiones clínicas y los resultados en pacientes.

The AFSA Master Class provides instruction to address information needs in animal-free safety assessment of cosmetics and cosmetic ingredients (COL050)

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Summary

The Animal-Free Safety Assessment (AFSA) Collaboration Master Class in animal-free risk assessment of cosmetics and cosmetic ingredients (chemicals) is an in-depth, free, online course — avaliable at www.afsacollaboration.org/masterclass — that covers all aspects of performing a human health safety assessment for cosmetics and cosmetic ingredients (chemicals). The course is designed in 9 modules: an introduction on animal-free risk assessment; problem formulation, including developing an aim, hypothesis, and initial literature review; consumer exposure including oral, dermal, respiratory exposure, deterministic and probabilistic modeling, and aggregate exposure; predictive chemistry, which is in two parts - in silico tools and read-across –and includes detailed descriptions of the types of data and modeling techniques used; exposure-based waiving for the three major routes of exposure, oral, dermal and respiratory; safety of botanicals (complex mixtures/extracts), including history of safe use; bioactivity assessment including descriptions and examples of various types of in vitro methods and how to combine these methods to address the major toxicological information needs; internal exposure (dosimetry) including a description of the data and modeling used in physiologically-based kinetic modeling, in vitro-to-in vivo extrapolation, and integration into risk assessment; and a module discussing the process of reaching a risk conclusion with a detailed walk-through of eleven case examples describing different problem formulations (chemicals, products, toxicological endpoints). A separate module discusses the global regulatory landscape for cosmetics and chemicals. AFSA invites all interested stakeholders to register and contribute to a more effective, efficient and ethical safety paradigm for cosmetics and chemicals.

Physiology-Based Pharmacokinetic modeling of tafenoquine: Promoting alternatives to animal and clinical testing (COL036)

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Summary

Physiology-Based Pharmacokinetics (PBPK) utilize models and simulations combining physiology, population, drug substance, and product characteristics to mechanistically describe the pharmacokinetic behaviors of a drug. PBPK has become a promising tool to answer some pharmacological questions, to promote the use of *in silico* approaches as alternative methods for animal and clinical studies. Malaria is an acute febrile infectious disease transmitted by the bite of the female *Anopheles*. In South America cases are related to the etiological agent *Plasmodium vivax*. This species has evolutionary forms that remain dormant in the liver. Tafenoquine (TQ) is an 8-aminoquinoline analog of primaquine that appears promising due to its single-dose administration. However, being a prodrug, there are concerns regarding its drug-drug interactions. A PBPK model for TQ was developed in the PK-SIM® software(v.11) utilizing previously published studies of physiological characteristics and pharmacokinetic studies of plasma concentrations of tafenoquine in humans and animals. The model was created based on a healthy male European individual considering CYP2D6 as the predominant metabolizing enzyme. For some parameters that could not be determined or were found to be relevant after sensitivity analyses, parameter identification based on plasma concentration-time profiles was performed using a subset of the available clinical studies (training dataset) for model optimization. The model was evaluated qualitatively by comparing observed and predicted data with good data fitting, and quantitatively by calculating mean relative deviations (MRD) with a mean value of 0.18, and geometric mean fold errors

(GMFE) of the area under the concentrations-time curve (AUC) and maximum concentration(Cmax) of all the simulations, which were 1.67 for AUC and 1.83 for Cmax. The mean values were all within the acceptance range of 0.5-2.0. In summary, the model showed satisfactory descriptive and predictive power and could be used to evaluate drug-drug interactions, reducing the need for animal use and new clinical studies

PBPK Modeling as an approach to support the 3Rs in interspecies extrapolation: A systematic review (COL037)

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Summary

The use of animals in biomedical research is a practice that continues to prompt ethical and scientific debate due to animal suffering, reliability, and limitations for extrapolation of data to humans. An alternative to traditional methods for interspecies extrapolation is Physiologically Based Pharmacokinetic (PBPK) modeling. This approach simulates the anatomical structure of species using a computational model in which organs or tissues are represented as compartments interconnected by arterial and venous flows and are described by differential equations to predict the change in the concentration of the evaluated molecule. The aim of this systematic review was to analyze published articles that used PBPK models for interspecies extrapolation in drug development and health risk assessment. For this, a systematic search was performed in PubMed using the following search terms: "PBPK" and "Interspecies extrapolation," and the review was conducted following PRISMA guidelines. It was determined that the main application of the PBPK models was to evaluate the dose-response assessment for toxic agents (47%). The main source for obtaining the anatomical and physiological parameters required for the development of the models was literature data (64%), and rats and mice were the most used animal models (82%). PBPK modeling demonstrated a comprehensive application of the 3R concept (Replacement, Reduction, and Refinement). The use of animals was replaced by employing *in vitro* or *in silico* methodologies and utilizing parameters reported in the literature. *In vivo* studies were conducted only when necessary to validate the model or obtain input parameters that could not be acquired by other methods, thereby reducing the number of animals. Finally, PBPK models allowed the determination of toxicity endpoints and the establishment of humane endpoints, refining methodologies in animal studies for interspecies extrapolation.

Efecto neuroprotector en células CAD del incarnatósido y el stachysósido C aislados de las raíces *Scutellaria incarnata* (COL024)

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Resumen

La muerte neuronal es un proceso central en patologías neurodegenerativas como la enfermedad de Alzheimer y de Parkinson. Las terapias existentes para su prevención o tratamiento son limitadas en la actualidad. La C2-ceramida es un análogo de la ceramida que induce cambios apoptóticos observados en determinadas enfermedades neurodegenerativas y es empleada en modelos in vitro para la evaluación del efecto neuroprotector de nuevos agentes terapéuticos, permitiendo el screening de extractos, fracciones y/o moléculas con potencial actividad neuroprotectora. Especies del género *Scutellaria* han demostrado ser biosintetizadoras de metabolitos secundarios con efecto neuroprotector. Sin embargo, la acción farmacológica de los metabolitos presentes en especies colombianas de *Scutellaria* está por ser explorada. Esta investigación tiene como objetivo evaluar en células CAD (Cath.-a-diferenciadas) la citotoxicidad y el efecto neuroprotector frente a la C2-ceramida de extractos, fracciones y metabolitos aislados de las raíces de la especie vegetal *Scutellaria incarnata*. Las estructuras de metabolitos aislados se elucidaron mediante resonancia magnética nuclear, espectrometría de masas y espectroscopia infrarroja. El pretratamiento con el extracto etanólico de la raíz (25-200 µg/mL), con las fracciones polares (25-200 µg/mL) y con los feniletanoides glicosilados incarnatósido y stachysósido C (12,5-50 µg/mL), exhibieron un efecto neuroprotector en células CAD expuestas a C2-ceramida, sin inducir citotoxicidad. Este es el primer reporte de aislamiento del incarnatósido y del efecto neuroprotector de ambos feniletanoides glicosilados. Este estudio fue apoyado por la Universidad Nacional de Colombia-Sede Bogotá [proyecto 41529, 2018] y por el Ministerio de Ciencia, Tecnología e Innovación de Colombia-Colfuturo [Beca de Doctorado 647-2015].

Análisis conformacional de la proteína CBX4 canónica y su interacción con ligandos antioxidantes mediante simulación por dinámica molecular (COL019)

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Resumen

Las proteínas CBX de Polycomb regulan la expresión génica al dirigir el complejo represor de Polycomb 1 (PRC1) a sitios de las histonas H3K27me3 mediante sus cromodominios, desempeñando un papel clave en el desarrollo de diversas enfermedades como la presbiacusia y el cáncer. En este trabajo se evaluó mediante docking y dinámica molecular, los cambios conformacionales de la proteína CBX4 inducidos por antioxidantes y el inhibidor UNC3866. Para ello se realizó un estudio *in silico*, iniciando con la búsqueda de la estructura cristalográfica de CBX4, seguido por la identificación de ligandos con actividad comprobada. Los ligandos fueron optimizados mediante el método de Hartree-Fock 6-31G, utilizando Gaussian. Se realizó acoplamiento molecular usando Auto Dock Vina. Por último, se llevaron a cabo simulaciones por dinámica molecular con trayectoria de 1 microsegundo con análisis de energía libre, RMSD, RMSF, SASA, ROG y PCA usando el software AMBER. El acoplamiento molecular y la dinámica molecular determinaron que el oridonin y la curcumina son los ligandos con mayor afinidad y estabilidad conformacional incluso superior al inhibidor natural UNC3866, lo cual fue demostrado en los cálculos de energía libre. Además, los valores de RMSD (1,5-3 Å) indicaron un comportamiento similar de CBX4 a otras proteínas de Polycomb CBX2 y CBX7, validando el uso de dinámicas moleculares como un método robusto y ético para estudiar interacciones proteína-ligando. Se logró evaluar los cambios conformacionales de la proteína CBX4 mediante Dinámica Molecular. La curcumina y el oridonin demostraron una mayor afinidad y estabilidad conformacional con CBX4 en comparación con UNC3866, evidenciando su potencial como alternativas terapéuticas. Los resultados subrayan la eficacia de los métodos computacionales como un enfoque alternativo y ético frente a modelos murinos.

Empleo de esferoides como modelo para evaluar la acción anticancerígena de venenos de origen animal y moléculas derivadas. Revisión 2010-2022 (COL005)

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Resumen

El cáncer es una de las principales causas de muerte a nivel mundial y regional, con índices de incremento elevados, siendo esta morbilidad un gran desafío para el sistema de salud. Actualmente se han encontrado fármacos oncológicos que cumplen con las especificaciones de las fases de esta patología, aunque presentan altos niveles de toxicidad ocasionados por poca especificidad. Para enfrentar esta problemática se ha dado relevancia a la búsqueda de terapias naturales que puedan ser útiles en el tratamiento de las neoplasias, entre estas destaca el veneno animal, ya que contienen moléculas bioactivas con inminente capacidad terapéutica en el tratamiento del cáncer. Recientemente, se ha optado por emplear modelos de cultivo 3D como los esferoides para evaluar moléculas con actividad anticancerígena, ya que tienen la capacidad de simular microambientes semejantes a entornos tumorales in vivo. Por lo que este estudio pretende establecer la importancia de los esferoides para la identificación de moléculas provenientes de venenos animales como alternativa terapéutica al cáncer, a partir de una revisión sistemática comprendida entre 2010-2022. Se realizó una búsqueda y selección de artículos a partir de la guía PRISMA en cuatro bases de datos con los términos: "Spheroid" y "Venom". Se seleccionaron los artículos según criterios de inclusión y exclusión, para posteriormente organizar la información relevante para la revisión. La búsqueda arrojó 81 artículos y se definieron 14 como base para la construcción de la revisión. Sobresalieron las moléculas derivadas de venenos de serpientes, arañas, escorpiones y abejas. Estas moléculas fueron evaluadas en diversos métodos de obtención de esferoides y destacaron efectos citotóxicos que inhibieron la formación de esferoides e incluso afectaron procesos de invasión y migración. La evidencia encontrada sugiere que los modelos de cultivo 3D en combinación con sustancias derivadas de venenos representan una estrategia preclínica prometedora para el desarrollo de terapias alternativas.

Resultados del ensayo fenotípico *in vitro* para la búsqueda de nuevos agentes antimaláricos en el Departamento de Farmacia (COL044)

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Resumen

La malaria es la enfermedad parasitaria más importante del mundo. En el 2022 se estimaron 249 millones de casos y en el periodo comprendido entre 2020 y el 2022 se calcularon 2.1 billones de casos de malaria y 11.7 millones de muertes [1]. Medicamentos, seguros y efectivos para tratar lograr su control y eventual eliminación son indispensables [2] y la necesidad de encontrar nuevos fármacos sólo puede suplirse mediante el proceso de investigación y desarrollo (I&D). Una de sus primeras etapas es la realización de tamizajes. Para el caso particular de enfermedades parasitarias, el tamizaje fenotípico *in vitro* ha sido mucho más exitoso que otras aproximaciones, como lo es el uso de dianas farmacológicas [2]. En el presente trabajo se presenta parte de la experiencia que el Departamento de Farmacia, de la Universidad Nacional de Colombia ha tenido en el tamizaje de plantas medicinales, con usos etnofarmacológicos relacionados con la malaria, para encontrar compuestos y/o extractos, mediante el ensayo fenotípico de inhibición del desarrollo de *Plasmodium falciparum in vitro*. Se presentan, en particular, dos ejemplos, *Abuta grandifolia* y *Curarea toxicofera,* utilizadas en la región amazónica para el tratamiento de fiebres, que mostraron actividades promisorias en el tamizaje fenotípico y lograron ser seleccionadas para realizar estudios posteriores, dentro del proceso de I&D, con el fin de avanzar a la potencial estandarización de su extracto.

HaCat and human fibroblast spheroid: strategies to reduce the fetal bovine serum in cell culture (COL002)

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Summary

While monolayer cell culture offers valuable insights into cell behavior, it fails to replicate crucial *in vivo* conditions, such as cell-cell and cell-extracellular matrix interactions. To address this limitation and reduce reliance on animal experimentation, emerging models with greater physiological relevance, such as 3D cell culture, particularly spheroids, are garnering attention. Spheroids, formed by single or multiple cell types, offer enhanced relevance by facilitating those interactions. Despite the progress achieved with spheroids, there is a pressing need to move away from animal-derived reagents, notably Fetal Bovine Serum (FBS), due to ethical concerns and practical limitations. In this study, we propose a co-culture spheroid system involving HaCaT and human fibroblast cells, formed through auto-aggregation in non-adherent U-bottom shaped plates. To address FBS dependency, we systematically reduce its concentration (from 10% to 2.5%) during spheroid formation and evaluate resultant structures quality using viability parameters while investigating the potential delay in spheroid formation due to Fetal Bovine Serum deprivation. The results indicate the feasibility of the auto-assembling spheroid formation process, with a noted retardation in the absence of Fetal Bovine Serum. Different approaches have been made to optimize the spheroids' formation using smaller Fetal Bovine Serum concentrations. The integration of these approaches could lead to the replacement of animal experimentation and more reproducible *in vitro* research.

Efecto del tratamiento de *Mycoplasma* sp. en la proliferación y citotoxicidad de la línea celular de Fibroblastos L-929 (COL027)

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Resumen

Los cultivos celulares en las industrias cosméticas y farmacéuticas requieren un control estricto de parámetros, especialmente para garantizar la ausencia de *Mycoplasma* sp. En caso de contaminación, *Mycoplasma* sp. puede afectar la viabilidad celular, alterar características genómicas y producir resultados erróneos en ensayos de citotoxicidad y producción de biofármacos. Por ello, este estudio evaluó el efecto del tratamiento para erradicar *Mycoplasma* sp. en células L-929 contaminadas. Se utilizaron tres métodos de detección (PCR, luminiscencia bioquímica y tinción DAPI) para detectar *Mycoplasma* sp. y determinar la concentración necesaria del tratamiento. Además, se comparó la velocidad de crecimiento de las células tratadas y se realizó un ensayo de viabilidad con diferentes concentraciones de SDS para analizar la respuesta tras el tratamiento. Se detectó contaminación en células L-929 y se confirmó la efectividad de los tratamientos con Plasmocin® y Mycoplasma Removal Agent (MRA) para erradicar el patógeno. A pesar del éxito de los tratamientos, la presencia de micoplasma provocó disminución en la velocidad de crecimiento de las células, particularmente entre 120 y 144 horas. Durante este período, se observó una diferencia en la velocidad de crecimiento en las células tratadas con MRA, mientras que las tratadas con Plasmocin® lograron recuperar su velocidad de crecimiento. Además, el ensayo de viabilidad celular indicó que el tratamiento proporcionó protección adicional contra los efectos de la toxicidad celular, especialmente en concentraciones más bajas de SDS. Sin embargo, se observaron ligeros cambios en la morfología celular, lo que indica alteraciones irreversibles debido a la contaminación a pesar de la eficacia del tratamiento. Finalmente, estos resultados permitieron la selección de un método rápido para el uso rutinario en la detección de micoplasma en el laboratorio y la posibilidad de usar células tratadas en futuros estudios, considerando que puede conllevar a variaciones en los resultados de citotoxicidad y proliferación celular en respuesta al tratamiento.

In vitro study of the genotoxic and antigenotoxic effects against UVB radiation of bioactive compounds from *Rosa centifolia* (COL003)

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Summary

Photoprotection is a preventive strategy to defend human skin against cancer and photoaging. The use of sunscreens is among the most popular strategies in photoprotection. Plants are sources of sunscreen ingredients that prevent cellular mutations involved in skin cancer and aging**.** In this work, the genotoxic and antigenotoxic effects against UVB radiation of a *Rosa centifolia* hydroalcoholic extract (RCHE) and two major compounds into extract (kaempferol and kaempferol 3 glucoside) were studied. The chemical composition of the RCHE was determined using UHPLC-ESI$^+$-*Orbitrap*-MS. Genotoxicity and antigenotoxicity against UVB radiation of the RCHE (46.8 and 750 µg/mL), kaempferol (31.2 and 500 µg/mL) and kaempferol 3 glucoside (19.7 and 315 µg/mL), were studied in MRC5 human fibroblasts using Comet assays. The major compounds into RCHE were as follows: quercetin-3-rhamnoside (49 ± 2 µg/mL), kaempferol-3-glucoside (70 ± 12 µg/mL), kaempferol-rhamnoside (64 ± 8 µg/mL), quercetin (160 ± 26 µg/mL), and kaempferol (146 ± 5 µg/mL). The RCHE resulted genotoxic in human fibroblasts only at 750 µg/mL; while this extract was significant antigenotoxic at concentrations between 46.8 and 187.5 µg/mL. Kaempferol exhibited genotoxicity at concentrations up to 500 µg/mL, while significant antigenotoxic effects were observed between 31.2 and 125 µg/mL. On the other hand, kaempferol-3-glucoside resulted not genotoxic, but was antigenotoxic at all evaluated concentrations. Our study showed the antigenotoxic properties of the RCHE and their phytochemicals (kaempferol and kaempferol-3-glucoside) against UVB radiation. Therefore, these results support the potential of *Rosa centifolia* phytochemicals as ingredients for sunscreen formulations.

Evaluating *in vitro* the antigenotoxic and DNA repair-enhancing potential of *Posoqueria latifolia* flower extract (COL004)

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Summary

Introduction: Skin overexposure to solar radiation has been a serious public health concern because of its potential carcinogenicity. Therefore, preventive protection strategies using DNA protective agents are essential to counteract the harmful effects of solar radiation. Plants may be a source of photoprotective compounds. In this context, phytochemicals that modulate DNA repair mechanisms against UV radiation emerge as promising agents for mitigating these adverse effects. This work evaluated the antigenotoxic effects against UVB radiation of a *Posoqueria latifolia* flower extract (PLFE) and their potential to modulate DNA repair post-irradiation. The chemical composition of the PLFE was determined using UHPLC-ESI+-Orbitrap-MS. We studied the genotoxicity and antigenotoxicity against of PLFE (between 250 to 750 μg/mL) in MRC5 human fibroblasts using Comet assay. Cells were irradiated (875 mJ/cm^2) using irradiation chamber BS-02 with a UV radiation controller UV-MAT (Dr. Grobel UV-Elektronik GmbH, Etlingen, Germany). The PLFE ability for stimulate DNA repair post-irradiation of this PLFE was evaluated using the Comet technique. The major compounds in the extract of *P. latifolia* were as follows: chlorogenic acid (35 ± 1 μg/mL), ecdysterone (64 ± 8 μg/mL), rhamnetin-rutinoside (17 ± 1 μg/mL), cis-resveratrol-diglucoside (140 ± 7 μg/mL), and trans-resveratrol-diglucoside (280 ± 16 μg/mL). The PLFE was not genotoxic at the studied concentrations, obtaining in all the doses evaluated a genetic damage

index lower than 1.56 ± 0.09. However, it showed antigenotoxic effect between 250 and 375 µg/mL, reducing genetic damage caused by UVB in 62% and 58%, respectively. The PLFE prepared at 250 µg/mL increased DNA repair post-irradiation in human fibroblasts. The PLFE showed a significant antigenotoxic effect against UVB radiation and stimulated DNA repair after irradiation. Thus, its phytochemicals could be used as an ingredient in a sunscreen formulation.

Use of the alkaline comet assay for detecting photogenotoxic chemicals: An *in vitro* comparative analysis (COL023)

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Summary

Several chemical compounds used in pharmaceuticals and cosmetics have been reported to cause phototoxic side effects. Therefore, regulatory authorities require an assessment of the phototoxic potential of newly developed drugs and cosmetic products. Current legislation for photosensitive products, such as sunscreens, mandates ensuring safety against photogenotoxicity. Due to the limitations of animal models and recent international regulations emphasizing ethical considerations, there is an urgent need to develop in vitro assays to identify potential human safety hazards. The objective of this study was to develop a reliable and fast in vitro assay for photogenotoxicity testing using a commercial line of human keratinocytes (HaCat). Five chemicals with well-known toxic properties—sodium dodecyl sulfate, chlorpromazine, benzophenone-3, 8-methoxypsoralen, and chlorhexidine—were tested. Cells were incubated with the test compounds for 1 hour and irradiated with 4 J/cm^2 UV-A. After irradiation, treatment was removed and fresh medium was added. Cell viability was measured using the MTT and LDH assays, and a comet assay was performed 24 hours after irradiation at non-cytotoxic concentrations of the compounds and their vehicles, using a medium-high throughput format with 8 minigels per slide. Methyl methanesulfonate, a known alkylating agent, was used as a positive control for the comet assay. Sodium dodecyl sulfate and chlorhexidine were classified as non-phototoxic, while the other tested chemicals showed PIF values above 5 or MPE values above 0.15. None of the chemicals showed increased DNA damage 24 hours after irradiation, likely due to DNA repair during this period. As experimental design, including treatment conditions, can impact results, further studies are underway to establish the optimal conditions for detecting photogenotoxic chemicals. This assay shows promise as a straightforward method for identifying both photosensitizers and photogenotoxic chemicals, potentially enhancing the safety assessment of new pharmaceuticals and cosmetic products.

Estudio de metaloproteinasas como indicadores de fotosensibilidad *in vitro*: hacia una discriminación precisa entre sustancias fotoirritantes y fotoalérgicas (COL020)

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Resumen

Algunas de las sustancias químicas presentes en diversos productos (cosméticos, medicamentos, fragancias...) pueden inducir reacciones de fotosensibilidad desencadenadas por la exposición a la radiación ultravioleta. Entre ellas, la fotoirritación se manifiesta como una reacción aguda tras la aplicación tópica o sistémica de una substancia química, combinada con la exposición a radiación ultravioleta. Otra manifestación es la fotoalérgia, que se origina cuando una sustancia, después de la exposición a la luz ultravioleta, forma un hapteno produciendo una hipersensibilidad retardada de tipo IV. Actualmente no existen métodos alternativos a la experimentación animal para discriminar entre sustancias fotoirritantes y fotoalérgenas. Aunque son menos estudiadas que otras alteraciones producidas por la luz ultravioleta, también son importantes y representan un problema de salud pública en aumento. Además, el desarrollo de nuevas técnicas in vitro es crucial, dado que desde 2013, las pruebas de ingredientes y productos acabados cosméticos en animales de experimentación están prohibidas tanto en la Unión Europea como en otros países. En este estudio, presentamos una técnica *in vitro* basada en la guía de la OCDE 432, para discriminar entre sustancias fotoirritantes y fotoalérgenas mediante la identificación de metaloproteinasas como marcadores. Para ello, expusimos queratinocitos humanos HaCaT a diferentes substancias con carácter fotoirritante o fotoalérgeno conocido, seguido de una exposición a 4 J/cm^2 de luz UVA y se determinó la viabilidad celular. Los sobrenadantes se analizaron con una matriz multiplex con el objetivo de identificar y medir semicuantitativamente los niveles de metaloproteinasas y estudiar su utilidad como marcadores para discriminar entre fotoirritantes y fotoalérgenos. Los resultados indicaron que las sustancias fotoalergenas incrementaban la secreción de MMP-1 y MMP-10, al contrario que la sustancia fotoirritante, lo que sugiere que estas metaloproteinasas podrían ser potenciales marcadores para tal discriminación.

Avaliação fotoprotetora de flavonóides *in vitro* (COL030)

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Summary

Os testes *in vivo* são considerados padrão ouro para avaliação da atividade fotoprotetora, contudo envolvem a exposição dos indivíduos à radiação ultravioleta e apresentam como desvantagens, alto custo e limitações éticas. Sendo assim, os métodos *in vitro* surgem como uma alternativa econômica e ética para triagem de formulações fotoprotetoras e fornecem resultados padronizados sem a necessidade de testes em humanos. Diante do exposto, o objetivo desse trabalho foi avaliar o efeito fotoprotetor de formulações contendo flavonóides *in vitro*. Para tal, foi utilizado o teste de fotodegradação do resveratrol que analisa a capacidade das amostras em prevenir a fotodegradação do resveratrol sob radiação UV. Para o experimento, foram utilizadas placas de Petri protegidas da luz, exceto na parte superior. Na área desprotegida, foram aplicados homogeneamente 300 mg das formulações semissólidas (veículo, silimarina, silimarina + dióxido de titânio, hesperetina e hesperetina + dióxido de titânio). Dentro das placas de Petri, adicionou-se 10 mL de solução de resveratrol (10 μg/mL). Como o resveratrol é fotoinstável, a radiação UV que atravessar a formulação aplicada na placa provocará a degradação do composto. Nos tempos de 10 e 60 minutos, o teor de resveratrol na solução foi quantificado por Cromatografia Líquida de Alta Eficiência, com método previamente validado. Uma placa de Petri sem formulação foi usada como controle (sem proteção) para garantir a eficiência do método. A fotoproteção *in vitro* foi determinada em triplicata e calculada pela relação entre a concentração de resveratrol na amostra e a concentração de resveratrol no controle. Como resultado, diferenças significativas ($p < 0,001$) foram observadas para todas as formulações em comparação com o controle negativo e o veículo nos períodos de 10 e 60 minutos, indicando que as formulações apresentaram efeito fotoprotetor frente as radiações UVA e UVB. Portanto, os flavonóides silimarina e hesperetina possuem efeito fotoprotetor *in vitro*.

Implementación de la determinación del tamaño de partícula (TP) por microscopía electrónica de barrido como método para realizar la exención del ensayo de toxicidad aguda por inhalación (TAI) OECD 433 de sustancias sólidas (COL016)

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Resumen

Uno de los temas más importantes en la toxicología regulatoria actual es demostrar a los reguladores que no es necesario realizar un ensayo toxicológico debido a que este no es necesario para la evaluación de riesgo por las características intrínsecas de una sustancia, esto con un criterio que brinde seguridad al tomador de decisiones que la exención de un ensayo está debidamente justificada Las consideraciones sobre el tamaño de las partículas (determinadas, por ejemplo, mediante pruebas de granulometría, OECD TG 110) pueden ser útiles para determinar si es necesario o no realizar ensayos de TAI debido a que el tamaño de partícula supera los 4 µm de masa media aerodinámica (MMAD) o los 100 µm de tamaño de partícula pues no sería posible que la sustancia pudiera ingresar de forma uniforme en las vías respiratorias. Se siguió la metodología descrita en el documento guía 39 (OECD, 2009) documento de orientación sobre pruebas de toxicidad aguda por inhalación, para determinar el MMAD teórico y la determinación del tamaño de partícula se realizó usando un microscopio electrónico de barrido donde se tomaron imágenes de las partículas sólidas y estas fueron medidas su tamaño de partícula por el equipo. Con la implementación de la determinación del tamaño de partícula de las muestras sólidas se ha evitado realizar 6 ensayos de toxicidad aguda por inhalación pues la muestra presentaba partículas mayores de 100 µm, por lo que al calcular el MMAD teórico este era mayor de 4 µm y según los criterios de la norma OECD 433 el ensayo no debía ser ejecutado. Un resultado importante es que al no realizar este ensayo no se utilizaron al menos 66 animales Este método puede ser utilizado en un esquema de toxicidad por evidencia y así poder justificar el no realizar ensayos con animales para la toxicidad aguda por inhalación.

Innovative development of inclusion complexes with microbiological and toxicological assessment free of animal tests (COL038)

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Summary

Farnesol has demonstrated potential as an antibacterial agent in inhibiting the proliferation of certain microorganisms. Despite that, farnesol presents limited water solubility and inadequate retention, which has hindered its application in medicine. Recent advancements in pharmaceutical technology, such as inclusion complexes, have provided solutions to address the challenges related to its limitations. An essential aspect of introducing new drugs to the market is ensuring their safety. *The Tenebrio molitor* model has emerged as a reliable tool for investigating the impacts of drugs and obtaining initial insights into LD50 (median lethal dose) values. Therefore, this study aimed to prepare the farnesol inclusion complex, proposing its toxicity, and evaluating its *in vitro* antibacterial activity. Thus, farnesol (FAR) and 2-Hydroxypropyl-β-cyclodextrin (HP-β-CD) compounded the inclusion complex, prepared by the freeze-drying method. *T. molitor* larvae were used in toxicity tests. The inclusion complex and free FAR were solubilized in saline and injected on the hemocoel by administering each compound on the larvae dorsal side, and mortality was recorded daily for five days. For the antibacterial assay against *Staphylococcus aureus* ATCC, it was performed the Minimum Inhibitory Concentration (MIC) and defined as the lower concentration that inhibits the bacterial growth. Free FAR presented the toxic and LD50 doses as 200 mg/kg and 25mg/kg, respectively, and the inclusion complex presented a toxic dose of 1000 mg/kg and an LD50 of 350mg/kg. The inclusion complex exhibited a higher MIC (8 μg/mL)

against *S. aureus* ATCC 25923 compared to free FAR (16 µg/mL). Thus, HP-β-CD provided a higher improvement of the FAR solubility and reduced the volatile loss of the essential oil. Beyond that, the toxicity results proposed the higher safety of the inclusion complex compared to the free FAR, suggesting that the inclusion complex could be a better source for the pharmaceutical industries to develop new drugs.

Tiempo de observación de 6 horas en el ensayo de neutralización de letalidad para la eficacia preclínica de antivenenos ofídicos (COL031)

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Resumen

El ensayo de neutralización de letalidad en ratones es el estándar de oro para evaluar la eficacia preclínica y el cumplimiento de especificaciones de antivenenos ofídicos. Normalmente, los resultados de este ensayo se calculan a partir de la cantidad de ratones que sobreviven durante las 24/48 horas posteriores a la inyección de una dosis letal de veneno mezclada con diferentes cantidades del antiveneno que está siendo evaluado. Debido al sufrimiento animal, este ensayo es candidato para ser reemplazado por alternativas in vitro, o mejorado mediante la reducción del número de animales utilizados, la introducción de analgesia y el refinamiento de la prueba mediante la disminución del tiempo de observación. El objetivo principal de este estudio es reducir el sufrimiento animal asociado a este ensayo mediante la disminución del tiempo de observación. Para eso, los valores de dosis letal media (DL_{50}) de varios venenos y de dosis eficaz media (DE_{50}) de dos antivenenos fueron estimados usando tiempos de observación de 6, 24 y 48 horas. Se encontró una fuerte correlación entre los valores de DL_{50} y DE_{50} estimados en los tres tiempos de observación. La implementación del tiempo de observación de 6 horas no compromete la fiabilidad de los resultados. Por lo tanto, este estudio representa una contribución importante al refinamiento de los ensayos preclínicos de antivenenos ofídicos y puede servir como base para el desarrollo de métodos alternativos más éticos para la evaluación de antivenenos, avanzando en la reducción del sufrimiento animal en la investigación preclínica. No obstante, la reducción del tiempo de observación debe validarse para cada veneno y antiveneno antes de su implementación rutinaria.

Sustitución de la prueba de pirógenos en conejos por el ensayo de endotoxinas bacterianas para la evaluación de antivenenos ofídicos (COL032)

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Resumen

Los antivenenos ofídicos son inmunobiológicos de administración parenteral, por lo que su calidad microbiológica debe ser estrictamente controlada. Tradicionalmente, la evaluación del contenido de endotoxinas bacterianas en antivenenos se ha realizado mediante la prueba de pirógenos en conejos. Sin embargo, el mejoramiento de la sensibilidad en los reactivos para el ensayo de endotoxinas bacterianas por el método de LAL Gel Clot brindan la oportunidad de implementar un enfoque más responsable y ético en el uso de animales de experimentación, y promover los principios de Reemplazo, Reducción y Refinamiento. La comparación entre la prueba de pirógenos en conejos y la prueba de endotoxinas bacterianas por LAL Gel Clot, evidenció que ambos métodos no difieren significativamente en su capacidad para determinar concentraciones peligrosas de endotoxinas en solución salina estéril o antivenenos de origen equino, y que los antivenenos no contienen sustancias interferentes con ninguno de los dos ensayos, por lo que por el ensayo de LAL Gel Clot puede sustituir la prueba de pirógenos en conejos. Adicionalmente, la comparación de la prueba de LAL Gel Clot con la de LAL Recombinante sugiere que las propiedades analíticas de ambas metodologías permiten la adecuada determinación de endotoxinas en antivenenos ofídicos. Así, la prueba de LAL Recombinante se convierte en una valiosa alternativa que sustituye el uso de animales.

Wound healing assay to evaluate corneal damage reversibility in skinethic HCE model (COL033)

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Summary

Evaluation of chemicals for ocular toxicity is crucial for global product registration and labeling, ultimately safeguarding consumers across all sectors. The traditional Draize rabbit test, used for categorizing irritation severity, is deprecated due to ethical concerns, in addition to low reproducibility and species differences. Despite big advancements in alternative methods such as OECD TG 492 and 492B, a robust *in vitro* test for assessing damage reversibility remains a critical need. Developing an *in vitro* model for corneal wound healing could address these challenges, benefiting both regulatory compliance and testing ocular therapeutic products. The SkinEthic HCE model presents a promising solution, offering a 3D reconstructed tissue that mimics the structure, morphology, and functionality of the human cornea epithelium. Meanwhile, cell migration emerges as a pivotal factor in corneal wound healing. Our study aimed to investigate whether the SkinEthic™ HCE model could effectively assess damage reversibility following exposure to various reference chemicals known to induce different levels of ocular damage. To achieve this objective, a wound healing assay was implemented on the SkinEthic HCE model, incorporating both mechanical damage and chemical exposure to reference substances under the hypothesis that the *in vivo* healing capacity is related to the in vitro migration capacity. Cytotoxicity assays and histological tissue sections of the corneas at 0, 48, and 96 hours provided a detailed documentation of the wound closure process. In summary, the ability to observe damage reversibility in this three-dimensional epithelial model suggests a potential correlation with the four UN-GHS categories.

The obtained results highlight the potential of this innovative strategy as the initial step toward a novel human-relevant *in vitro* method for evaluating ocular damage, capable of distinguishing reversal times of the lesion and facilitating classification into all relevant categories without animal use.

Modelo experimental alternativo de indução de inflamação em larvas de *Zophobas morio* (COL041)

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Resumo

O crescente apelo por métodos experimentais alternativos ao uso de animais vem de encontro ao panorama internacional que fomenta e privilegia o princípio dos "3Rs" (Reduction, Refinement and Replacement); recomendando que mamíferos quando possível devem ser substituídos por animais com sistema nervoso menos desenvolvido, como invertebrados. Diante disso, a utilização de larvas de *Zophobas morio* se torna uma alternativa promissora em pesquisas experimentais, visto que o sistema imune tem similaridades com dos mamíferos. Foram utilizados 18 grupos (n=18) de larvas de *Z. morio*, e inoculação de 10µl de solução carragenina (0,5%); dexametasona (25µg/mL) e salina a 0,9%; nos tempos: 30min, 60min, 120min, 24h, 48h e 72h. A linfa foi extraída e analisada por microscopia para contagem de hemócitos. O perfil inflamatório foi determinado pelo quantitativo de hemócitos presentes nas amostras de linfa. Os grupos tratados com carragenina apresentaram valores médios (hemócitos/mL): 1458±671; 2467±321; 3158±1644; 7292±152; 8125±1426; 20808±9571, nos respectivos períodos de tempo supracitados. Já os grupos tratados com dexametasona apresentaram médias: 2017±428; 792±215; 1142±232; 875±499; 617±76; 4783±614, enquanto os grupos tratados com salina apresentaram médias: 2525±888; 3775±895; 3208±842; 2342±893; 3008±232 e 2767±52. A análise estatística por ANOVA de duas vias revelou significância ($p<0,0001$) na interação entre tratamentos e períodos de tempo. Em suma, verificou-se que a carragenina atuou como agente inflamatório, aumentando a quantidade de hemócitos nas larvas ao longo do tempo. Nos grupos de larvas tratados com dexametasona, houve redução esperada nas células de defesa, enquanto o grupo tratado

com solução salina não mostrou alterações na contagem de hemócitos. Conclui-se que a carragenina foi capaz de induzir inflamação nas larvas, diferentemente de dexametasona e salina que apresentaram diminuição e manutenção na contagem de hemócitos, respectivamente. Com isso, verifica-se que o experimento se encontra apto a ser testado frente a novas substâncias.

Tenebrio molitor (Coleoptera, Tenebrionidae) como huésped alternativo para estudiar la patogenicidad de *Candidas auris* (COL035)

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Resumen

Candida auris es un hongo multirresistente que representa una amenaza significativa para pacientes inmunocomprometidos y puede causar brotes en entornos hospitalarios. Los estudios in vivo son esenciales para comprender la patogenicidad y la resistencia antifúngica de *C. auris*. *Tenebrio molitor* es un modelo de infección ventajoso por ser fácil de criar en laboratorio, tener un sistema inmunológico bien caracterizado y permitir estudios de patogenicidad y respuesta inmune con costos relativamente bajos comparados con otros modelos. Este estudio investigó el potencial de la larva de *Tenebrio molitor* como modelo alternativo para estudiar los mecanismos de patogenicidad y virulencia de *C. auris*. Se utilizaron nueve cepas diferentes de *C. auris* de varias regiones: *C. auris* CBS 10913, *C. auris* Venezuela 150/23, *C. auris* Sudáfrica 155/23, *C. auris* Londres 156/23, *C. auris* Países Bajos 157/23, *C. auris* Kuwait 158/23, *C. auris* Corea 159/23, *C. auris* España 160/23 y *C. auris* Omán 161/23. Los experimentos incluyeron pruebas de supervivencia en larvas inoculadas con $1x10^4$, $1x10^6$ y $1x10^8$ de la cepa estándar, evaluación de la producción de biofilm, examen de melanización, recuento de colonias para determinar la carga tisular, análisis histopatológico y comparación de la virulencia de las diferentes cepas. Los resultados mostraron que la tasa de mortalidad de las larvas de *T. molitor* aumentó con la concentración del inóculo de *C. auris*, presentando diferencias significativas en comparación con el grupo de control con PBS. En cuanto a la virulencia, las cepas de Kuwait, Omán y Londres causaron mayor mortalidad en las larvas, con tasas de 100%, 93% y 87%, respectivamente, 24 horas después de la infección. Todas las cepas analizadas demostraron capacidad de formación de biofilm, con variaciones de virulencia entre los aislados de diferentes regiones geográficas. El análisis histopatológico confirmó la invasión de los tejidos larvales por *C. auris*. Además, se observó un aumento en la melanización de las larvas infectadas, lo que indica la activación del sistema inmunológico del insecto. Estos resultados destacan la eficacia de los modelos de inverte-

brados para evaluar la patogenicidad de *C. auris* y proporcionan información valiosa sobre la interacción patógeno-hospedador y la virulencia de este hongo emergente. Este estudio contribuye a la comprensión de la infección por *C. auris* y puede ayudar en el desarrollo de terapias más eficaces contra esta grave patología.

Evaluación *in vitro* de la seguridad biológica de productos cosméticos y de cuidado personal (COL040)

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Resumen

Convencionalmente, se han usado modelos animales para evaluar la seguridad de productos químicos con el fin de determinar efectos adversos para los humanos. Sin embargo, el dilema ético detrás de estas prácticas de experimentación ha resaltado la necesidad de desarrollar alternativas que reduzcan en lo posible el testeo en animales. Se ha abordado esta problemática mediante la implementación de ensayos *in vitro* en cultivos celulares, basados en la medición de la actividad metabólica de las células tras el contacto con la muestra cuya reactividad se desea evaluar, con la intención de reemplazar ensayos de Draize realizadas *in vivo* para evaluar irritación dérmica, además de estudios de toxicidad aguda. Estas medidas han representado diversas oportunidades de mejora para la estandarización de estos protocolos, incluyendo: la insolubilidad de muestras en soluciones acuosas y la reactividad de muestras con MTT. Se empleó la línea celular CCL-1 NTCN clon 929 para realizar los ensayos de citotoxicidad de contacto directo e indirecto con la muestra, utilizando los reactivos MTT (bromuro de 3-(3,4-dimetiltiazol-2-il)-2,5-difeniltetrazolio) y resazurina para observar la actividad metabólica de las células como marcador de viabilidad celular. Se concluyó que es más conveniente emplear resazurina en lugar de MTT en casos donde el reactivo requiere lectura de absorbancia, pues el pigmento de trazas de la muestra interfiere con los valores de absorbancia del formazán producto del MTT metabolizado, mientras que el producto obtenido de la resazurina al ser medido por fluorescencia reduce este error, sin mencionar que el MTT es más reactivo con las muestras para el tiempo en el que se hace la lectura. Por otra parte, es más favorable el uso de MTT en pruebas cualitativas de citotoxicidad por contacto indirecto, ya que al formar cristales insolubles permite observar el halo de reactividad a través del agar. Adicionalmente, se halló un posible abordaje para evaluar la citotoxicidad de muestras insolubles en medios acuosos, obteniendo aproximaciones del grado de citotoxicidad con "extractos" de las muestras, pues representaba una dificultad no poder preparar diluciones de la muestra en medio de cultivo sin la separación en dos fases imposibilitando el contacto entre las células y la muestra.

Potencial sensibilizante dérmico de formulaciones cosméticas empleando KeratinoSens™ (COL006)

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Resumen

La valoración del potencial sensibilizante de moléculas y formulaciones resulta de gran importancia durante el proceso de evaluación de seguridad de productos en la industria cosmética, ya que se trabaja con formulaciones complejas y no es fácil la determinación del potencial sensibilizante final mediante métodos *in silico*. Esta observación se hace especialmente relevante cuando se trata de productos con filtros solares de origen orgánico, los cuales dada su naturaleza y características químicas poseen un potencial intrínseco como desencadenantes de dermatitis atópica por contacto (DAC). Dichas características resultan retadoras cuando se emplean métodos alternativos de evaluación en animales tales como KeratinoSens™, ya que el riesgo real de estas fórmulas se puede ver incorrectamente estimado debido a un diseño experimental equivocado. Hemos implementado una modificación al protocolo estándar descrito por la OECD en la guía TG 422D, que nos permite hacer una mejor estimación del riesgo de sensibilización dérmica de manera específica para formulaciones que contienen filtros solares. Este ajuste metodológico facilita la inclusión de un estándar interno a las muestras con el objetivo de evaluar los efectos sinérgicos de las moléculas y los cambios dentro del potencial riesgo como sensibilizante cutáneo, enmarcado en la tendencia de que ningún consumidor utiliza solo un producto cosmético dentro de sus rutinas de cuidado personal, este hecho aumenta el riesgo de experimentar eventos adversos por acumulación biológica.

Desarrollo de un método alternativo para la evaluación de la adherencia epicuticular de formulaciones de bioplaguicidas (COL045)

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Resumen

La adherencia expresada como trabajo de adhesión utilizando formulaciones de plaguicidas, se ha venido trabajando ampliamente sobre la superficie de las hojas. Sin embargo, hasta el momento no se ha evaluado sobre la superficie de un insecto utilizando bioplaguicidas como producto de control biológico y tampoco se han desarrollado modelos *in vitro* de evaluación. Para la elaboración de la película se tuvo en cuenta los ácidos grasos presentes en la cutícula de las especies de *Diatraea saccharalis* y *Chloridea virescens*. Se elaboraron con fuentes de hidrocarburos (animal, vegetal y mineral), mediante la técnica casting húmedo; se usó una fuente derivada de celulosa y plastificante un alcohol. Para su evaluación se prepararon 18 emulsiones tipo (con y sin conidios de *Beauveria bassiana*), desde W/O hasta O/W. Se determinó el ángulo de contacto mediante el uso de la técnica de gota sésil, tensión superficial (gota pendante) y se calculó el trabajo de adhesión. Una vez determinado el trabajo de adhesión tanto sobre las cutículas de ambos insectos y sobre la película seleccionada para cada insecto se realizó una Correlación de Pearson. La tensión superficial se encontró en un rango de 33,91 – 41,58 mN/nm para las O/W, mientras que las W/O entre 22,64 – 39,03 mN/m. Las emulsiones con proporciones de aceite entre 70% (emulsión tipo 13) y más del 80% de aceite (emulsión 15 a 18), humectaron completamente las cutículas de las larvas de las 2 especies. Observando que la presencia de conidios disminuye los ángulos de contacto y aumenta el trabajo de adhesión independientemente del tipo de emulsión. Los valores encontrados sobre las películas desarrolladas se encontraron dentro del rango encontrado sobre las larvas de las 2 especies, Finalmente, se encontró una correlación de 0,69 y 0,66; indicando una nueva alternativa de reemplazo del modelo *in vivo*.

Bogotá, D. C., Colombia, septiembre 19 de 2024

A los Gobiernos de América Latina y el Caribe,

El Congreso Latinoamericano de Métodos Alternativos al Uso de Animales de Experimentación (COLAMA) es un espacio que desde 2012 reúne a la comunidad científica y al público en general interesado en reemplazar, reducir y refinar el uso de animales en diversas áreas de investigación y educación.

Los investigadores, estudiantes, profesionales, representantes de la sociedad civil y otros interesados reunidos en el V COLAMA realizado en Bogotá Colombia entre el 16 y 20 de septiembre de 2024, considerando el creciente interés colectivo por la aplicación de prácticas éticas y humanitarias en los ensayos con animales y también los progresos tecnológicos alcanzados por la ciencia, solicitan a todos los Gobiernos de América Latina y el Caribe que fomenten el desarrollo de métodos libres de animales para ser utilizados en la investigación y la docencia.

Cuando no es posible reemplazar completamente a los animales y necesitan ser utilizados, debe ser con rigor ético, se deben respetar como seres vivos y se debe garantizar su bienestar empleando los principios de refinamiento y reducción.

El estímulo al desarrollo solicitado debe ser entendido en sentido amplio, considerando los aspectos financieros, de formación, difusión, investigación y uso de estos métodos con el fin de garantizar el respeto a la vida de los animales.

Firman,

Participantes en el Quinto Congreso Latinoamericano de Métodos Alternativos al Uso de Animales de Experimentación (V COLAMA).

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Índice general por tipo de artículos

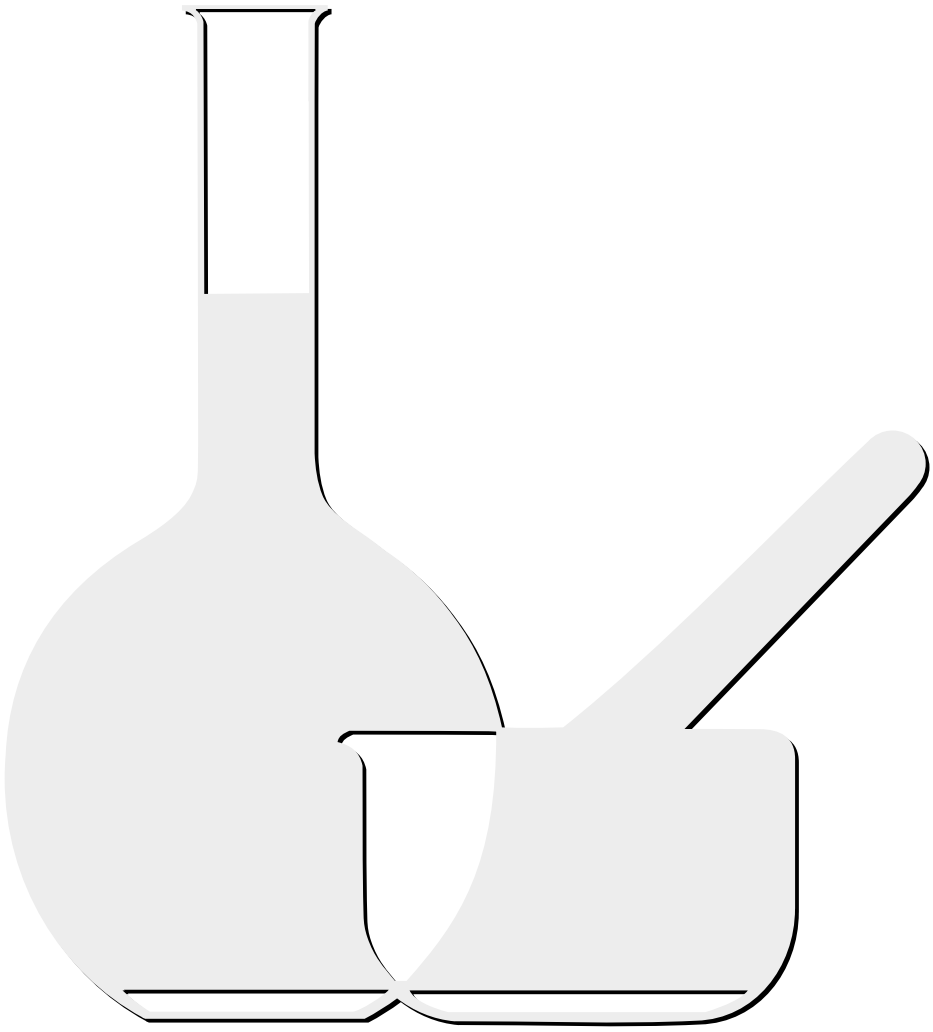
Artículos de investigación científica

Artículos de investigación clínica

Artículos de revisión

Eventos

Índice general de autores



NORMAS PARA PUBLICACIÓN

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- En los manuscritos en español y portugués se empleará la coma (,) como separador decimal, en inglés se utilizará el punto (.).

- Los artículos se dividirán en las siguientes secciones: Introducción, Metodología, Resultados y Discusión, Conclusiones (si las hay), Agradecimientos, Conflicto de intereses y Referencias. Los títulos deben estar separados por dos espacios en sus partes superior e inferior, centrados y en negrilla. Los detalles acerca de la metodología experimental utilizada deberán ser lo suficientemente claros como para repetir la experimentación.

- Las tablas deben llevar numeración arábiga de acuerdo con el orden de aparición en el texto. El título debe ir en su parte superior y las notas en la parte inferior. En los encabezamientos de las columnas se deben anotar los símbolos de las unidades utilizadas.

- Las fotografías, gráficas, dibujos y esquemas se denominan 'figuras' y deben llevar numeración arábiga de acuerdo con el orden de aparición en el texto. El título debe ir debajo de las figuras.

De otro lado, los artículos relacionados con experimentación con animales deben ajustarse plenamente a los lineamientos éticos trazados por la Organización Mundial de la Salud. Los extractos o fracciones evaluados *in vitro* o *in vivo* deben definirse químicamente, cuando menos en cuanto a la clase de constituyente. El material vegetal deberá estar clasificado botánicamente.

Las abreviaturas de pesos y medidas serán las indicadas por la Farmacopea de los Estados Unidos en su edición oficial o unidades SI. Los datos espectroscópicos se deben presentar de la siguiente manera:

UV λ max (solvente ε) nm (log ε). Ej.: UV λ max (MeOH) 275 (log ε 2,94).

IR ν max (medio) cm^{-1}. Ej.: IR ν max (KBr) 1740, 1720 cm^{-1}.

EM *m/z* (% intensidad relativa). Ej.: em *m/z* (%): 340 (M^+, 100), 295 (10), 134 (26) ...

RMN ^1H (solvente, frecuencia de registro) δ ppm (integración, multiplicidad, J en Hz, asignación). Ej.: RMN ^1H ($CDCl_3$, 400 MHz) 3,84 (1H, *d*, J = 10,3 Hz, H-30).

RMN ^{13}C (solvente, frecuencia de registro) δ ppm (multiplicidad, asignación). Ej.: RMN ^{13}C ($CDCl_3$, 600 MHz) 16,60 (t, C-12).

Las abreviaturas usadas para describir la multiplicidad de las señales en RMN son: *s* = singlete, *d* = doblete, *t* = triplete, *m* = multiplete, *dd* = doble de dobletes, *ddd* = doble de doble de dobletes.

Las abreviaturas para los solventes y reactivos más comúnmente usados son: EtOH= etanol, MeOH = metanol, $CHCl_3$ = cloroformo, C_6H_6 = benceno, AcOEt = acetato de etilo, EP = éter de petróleo, Me_2CO = acetona, DMSO = dimetilsulfóxido, AcOH = ácido acético.

Se evitará el uso excesivo de tablas y figuras que estarán numeradas y que se anexarán en hojas separadas con su respectiva descripción

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2. A.J.M. Leeuwenberg, Agricultural University, Wageningen, Holanda, comunicación personal, 1984.

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3. D.R. Morris, "The Biochemistry of Disease", Morris et Marton Eds., London, 1981, Vol. 8, p. 223.

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Tesis: Iniciales del nombre y apellido completo de los autores, *título en cursiva*, Tesis de..., institución, ciudad, año, páginas. Ej.:

5. F. Salcedo, *Contribución al estudio de las Cinchonas colombianas*, Tesis de Grado, Universidad del Valle, Cali, 1983, pp. 14-16.

Referencias de Internet: Iniciales del nombre y apellido completo del autor, título del documento, dirección URL y fecha de revisión. Ej.:

6. Lipidat, Lipid thermotropic phase transition database, Ohio State University, URL: http://www.lipidat.chemistry.ohio-state.edu, consultado en septiembre de 2001.

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