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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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Carta al editor

Boro y diseño de enzimas artificiales: Hacia una nueva era en la biocatálisis y el desarrollo de fármacos

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Estimado Editor,

La integración del boro en el diseño de enzimas artificiales representa un avance transformador en la biocatálisis, ofreciendo oportunidades sin precedentes para ampliar el repertorio catalítico de las enzimas naturales [1]. Aunque ha estado subrepresentado en los sistemas biológicos, el boro ha demostrado ser un grupo catalítico versátil y altamente eficaz cuando se incorpora en los sitios activos de las enzimas. Esta innovación no solo desafía los paradigmas convencionales del diseño enzimático, sino que también abre nuevas fronteras en la síntesis de compuestos con propiedades biológicas relevantes para la investigación farmacéutica.

El boro posee propiedades electrónicas únicas. Como ácido de Lewis, tiene una capacidad intrínseca para formar enlaces covalentes reversibles con nucleófilos, como los grupos hidroxilo y carbonilo, lo que lo convierte en un candidato ideal para catalizar reacciones que, de otro modo, serían inaccesibles para las enzimas sintetizadas a partir de aminoácidos naturales. Uno de los aspectos más prometedores de la catálisis mediada por enzimas artificiales es su capacidad para ampliar el alcance de las reacciones enzimáticas más allá de las limitaciones impuestas por los aminoácidos naturales. A través del diseño computacional de proteínas y la evolución dirigida, los investigadores pueden desarrollar sitios activos que contienen boro con una precisión notable, logrando altos niveles de selectividad y actividad. Este enfoque se puede utilizar para aplicaciones específicas, como la producción de fármacos quirales, un área clave en el desarrollo de medicamentos, donde la pureza enantiomérica impacta directamente en la eficacia terapéutica y la seguridad. Además, la catálisis enzimática basada en boro abre nuevas posibilidades para la biotransformación de precursores farmacéuticos, la síntesis de compuestos bioactivos innovadores y la eliminación de contaminantes farmacéuticos en el medio ambiente.

A pesar de estos avances, aún existen varios desafíos que deben superarse antes de aprovechar plenamente el potencial de las enzimas que contienen boro. La optimización de las interacciones entre el boro y la enzima, la estabilidad de los grupos funcionales que contienen boro en condiciones fisiológicas y la posible toxicidad de los compuestos a base de boro en sistemas biológicos son áreas críticas de investigación. Además, el desarrollo de métodos robustos para la incorporación de boro en estructuras enzimáticas y la elucidación de los mecanismos de catálisis mediados por boro requerirán una colaboración interdisciplinaria entre químicos, bioquímicos, biólogos estructurales y científicos farmacéuticos. La integración del

boro en el diseño enzimático podría redefinir el futuro de la ciencia farmacéutica y la investigación, ofreciendo soluciones innovadoras a algunos de los desafíos más apremiantes en química medicinal y biocatálisis.

CONFLICTO DE INTERESES

El autor no declara conflicto de interés

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Scientific research article

GDMSafe: Enhancing gestational diabetes prediction through visceral adipose tissue and ensemble learning models

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SUMMARY

Introduction: Globally, diabetes is common chronic disease, which occurs when the pancreas in the body cannot generate enough insulin or body cannot utilize the generated insulin. Particularly, Gestational Diabetes Mellitus is the frequent condition, associated with the high maternal and fetal and morbidity. It is significant to detect the disease earlier to evade consequences in the future. Traditionally, detection of gestational diabetes comprises of the GCT (Glucose Challenge Test), OGTT (Glucose Tolerant Test). Conversely, it is the time consuming, inconvenience and subjectivity process. **Purpose:** To address the issue, conventional researches used AI (Artificial Intelligence) technology to automate the detection procedure. Nevertheless, it is limited by accuracy, speed, handling of larger datasets and high error rate. To overcome the problem, proposed model aimed at developing a predictive model to ascertain GDM based on the Visceral Fat deposit by leveraging the benefits of Ensemble learning methods. Contradicting the principles of Occam's razor, ensemble models introduce complexity but still reduces the generalization error. **Methodology:** A group of 133 pregnant women upto 20 weeks of gestation from Physionet is utilized for this study and it has been already proven that there exists a strong correlation between VAT and GDM. **Results:** Convincing ROC and Accuracy is achieved and a comparison with PIMA Indian dataset demonstrate the robustness of the model for predicting Gestational Diabetes to move the knowledge in this field a little further along.

Keywords: Gestational Diabetes Mellitus; Visceral Adipose Tissue; Random Forest; Ensemble Methods

RESUMEN

GDMSafe: Mejora de la predicción de la diabetes gestacional mediante tejido adiposo visceral y modelos de aprendizaje conjunto

Introducción: A nivel mundial, la diabetes es una enfermedad crónica común, que ocurre cuando el páncreas del cuerpo no puede generar suficiente insulina o el cuerpo no puede utilizar la insulina generada. En particular, la Diabetes Mellitus Gestacional es una condición frecuente, asociada a alta morbilidad materna y fetal. Es importante detectar la enfermedad a tiempo para evitar consecuencias en el futuro. Tradicionalmente, la detección de la diabetes gestacional se compone de la prueba de provocación con glucosa (TCG) y la prueba de tolerancia a la glucosa (PTGO). Por el contrario, es un proceso que consume tiempo, es inconveniente y subjetivo. **Propósito:** Para abordar el problema, las investiga-

ciones convencionales utilizaron tecnología de IA (Inteligencia Artificial) para automatizar el procedimiento de detección. Sin embargo, está limitado por la precisión, la velocidad, el manejo de conjuntos de datos más grandes y una alta tasa de error. Para superar el problema, el modelo propuesto tuvo como objetivo desarrollar un modelo predictivo para garantizar la GDM basada en el depósito de grasa visceral aprovechando los beneficios de los métodos de aprendizaje de conjunto. Contradiciendo los principios de la navaja de Occam, los modelos de conjunto introducen complejidad pero aun así reducen el error de generalización. **Metodología:** Para este estudio se utiliza un grupo de 133 mujeres embarazadas de hasta 20 semanas de gestación de Physionet y ya se ha demostrado que existe una fuerte correlación entre VAT y DMG. **Resultados:** Se logra una ROC y una precisión convincentes y una comparación con el conjunto de datos indios PIMA demuestra la solidez del modelo para predecir la diabetes gestacional y hacer avanzar un poco más el conocimiento en este campo.

Palabras clave: Diabetes Mellitus Gestacional; Tejido adiposo visceral; Bosque aleatorio; Métodos de conjunto

RESUMO

GDMSafe: Melhorando a previsão do diabetes gestacional por meio do tecido adiposo visceral e modelos de aprendizagem de conjunto

Introdução: Globalmente, o diabetes é uma doença crônica comum que ocorre quando o pâncreas não consegue gerar insulina suficiente ou o corpo não consegue utilizar a insulina gerada. Particularmente, o Diabetes Mellitus Gestacional é uma condição frequente, associada a alta morbidade materna e fetal. É importante detectar a doença precocemente para evitar consequências no futuro. Tradicionalmente, a detecção do diabetes gestacional compreende o TCG (Teste de Provocação de Glicose) e o TTGO (Teste de Tolerância à Glicose). Por outro lado, é um processo demorado, inconveniente e subjetivo. **Objetivo:** Para abordar o problema, a pesquisa convencional usa tecnologia de IA (Inteligência Artificial) para automatizar o procedimento de detecção. Entretanto, é limitado pela precisão, velocidade, manuseio de conjuntos de dados maiores e alta taxa de erro. Para superar o problema, o modelo proposto teve como objetivo desenvolver um modelo preditivo para determinar o GDM com base no depósito de gordura visceral, aproveitando os benefícios dos métodos de aprendizagem do Ensemble. Contrariando os princípios da navalha de Occam, os modelos de conjunto introduzem complexidade, mas ainda reduzem o erro de generalização. **Metodologia:** Utilizou-se neste estudo um grupo de 133 gestantes com até 20 semanas de gestação da Physionet e já foi comprovado que existe uma forte correlação entre VAT e GDM. **Resultados:** ROC e precisão convincentes são alcançados e uma comparação com o conjunto de dados indiano PIMA demonstra a robustez do modelo para prever diabetes gestacional para levar o conhecimento neste campo um pouco mais adiante.

Palavras-chave: Diabetes Mellitus Gestacional; Tecido adiposo visceral; Floresta aleatória; Métodos de conjunto.

1. INTRODUCTION

Gestational Diabetes Mellitus (GDM) usually happens in the second half of the pregnancy [1, 2]. The excess blood glucose travels through the placenta, giving the fetal high blood glucose levels [3, 4]. This causes the baby's pancreas to make extra insulin to get rid of the blood glucose. During the prenatal visit, the risk assessment is made by the presence of fasting hyperglycemia and various other risk factors associated with GDM such as Overweight, Ethnic race, Blood Sugar levels, Age factor, Family history, Hypertension, etc. [5]. To reduce the risk factor of the disease, it is necessary to detect the disease earlier. Classically, GDM is functioned with the GCT (Glucose Challenge Test), OGTT (Glucose Tolerant Test). It is the time invasive and

in need in need of qualified doctors to process the results. Besides, it can be prone to human error. Congruently, technology based GDM detection is needed to overcome the problem. One such technology is AI (Artificial Intelligence), which uses ML (Machine Learning) and DL (Deep Learning) for the automatic detection of GDM [6].

Correspondingly, several conventional models attempted to attain better detection of diabetes mellitus with ML and DL techniques [7, 8]. For instance, in the existing model, CNN based architecture has been used for the detection of diabetes. It has processed with the data of diabetic retinopathy. The function involved in the classical model are data acquisition, preprocessing and classification. Accordingly, outcome of the classification signifies the better efficacy of the diabetic detection [9]. Similarly, diabetes detection has been designed with the SVM (Support Vector Machine) and hierarchical clustering. Besides, CNN has been used for the detection and examination of every part in the classification. The outcome of the detection signifies the better efficiency of the traditional model [10]. Likewise, enormous pioneering methods tried to achieve efficient detection of diabetes [11]. However, lacks in accuracy and speed. Moreover, researches on GDM is limited in the existing models [12]. Several Existing models is focused on the detection of diabetes mellitus but inadequate in the GDM based detection [13-16]. Accuracy is the essential factor, which determines the overall performance of the model where numerous classical mechanism lacks in accuracy [17].

In essence, the contributions of the works are:

- To utilize feature selection mechanism using recursive features elimination to select significant features in the data.
- To use data augmentation with the SMOTE based technique to alleviate class imbalance problem.
- To employ bagging and boosting model for the prediction of Gestational Diabetes Mellitus. Besides, it analyzes the best hyper parameters for enhancing the predictive accuracy.

In the conventional method, ML based model has been used for the classification of GDM. It has been processed with the sequential data which has been taken from the cell free DNA from the maternal plasma. Besides, classification has been processed using the CNN based architecture. The experimental results signifies that the classical model attained better performance with the accuracy of 88.14% [17]. Similarly, ML and data mining based model has been deployed for the detection of GDM. The input data has been collected from the pregnant women data through the data mining process. Further, it has functioned with normalization and dimension reduction method. The classification has been functioned with the CNN based architecture where the results of the classification signifies better performance with the better accuracy [18]. Congruently, convolutional LSTM based detection of diabetes. The database used for the project has been taken from the Pima Indians diabetes database. The experimental results signifies batter efficacy of the traditional models with the better accuracy [19]. In the same way, DL based GDM detection model has been conventional mechanism. To achieve this, three set of layers has been used such as cloud layer, fog layer and IoT layer. Besides, EPM (Explainable Prediction Algorithm) and data finding methodology. The outcome of the experiment represents the better performance of the GDM prediction [20].

Correspondingly, CNN and bidirectional LSTM based method has been used for the GDM detection [21]. It has been functioned with the PIMA dataset. It has been processed with the PIMA dataset. The efficiency of the classical model signifies the better performance with better accuracy [22]. Similarly, Deep LSTM model has been used for the prediction of diabetes mellitus. For that, diabetes dataset has been used in the classical method. The better efficacy of the

classification has been used in the traditional mechanism with better accuracy [23]. In the existing approach, SMOTE based oversampling method has been used in the conventional method. To attain this, Pima Indian diabetes dataset for the classification of diabetes mellitus. The better efficiency of the prediction has signified through the results [24]. Consistently, LSTM based diabetes mellitus prediction method has been designed in the traditional system. For that, PIDD dataset has been utilized in the conventional method. The experimental outcome signifies the better performance of the exiting model with better accuracy [25, 26].

Compatibly, weighted entropy based deep features has been used for the classification of diabetes mellitus [13, 14]. Besides, hybrid RNN with LSTM aided mechanism has been utilized to examine the blood glucose level and prediction of diabetes [15, 16]. Moreover, CNN has been used for the feature extraction [27]. The classification outcome indicates that the classical method attained better performance in the diabetes prediction [28]. Similarly, deep CNN and LSTM based mechanism has been utilized for the detection of diabetic retinopathy. To accomplish this, shark sell jaya optimization has been utilized to improve the performance of the prediction. The experimental outcome represents the better performance of the classification. In the exiting method, DL based diabetes mellitus on the terms of DNA sequences. To attain this, CNN and LSTM based mechanisms have been used in the classical method. The outcome of the classification represents better efficiency with better accuracy.

2. METHODOLOGY

The proposed model predicts the Gestational Diabetes Mellitus (GDM) using the Physionet Visceral Adipose Tissue dataset during pregnancy by employing a series of techniques to improve classification accuracy. It begins with data pre-processing, followed by feature selection using recursive feature elimination to identify key features. To enhance model efficiency, data augmentation is applied through the SMOTE technique. The classification process is then carried out using ensemble methods, specifically bagging and boosting. GDM, a serious health condition that can lead to complications such as kidney failure, blindness, and heart disease, requires early detection to minimize future risks. Although conventional models have struggled with accuracy and speed, the proposed system aims to address these issues by integrating these advanced procedures, as illustrated in Fig. 1.

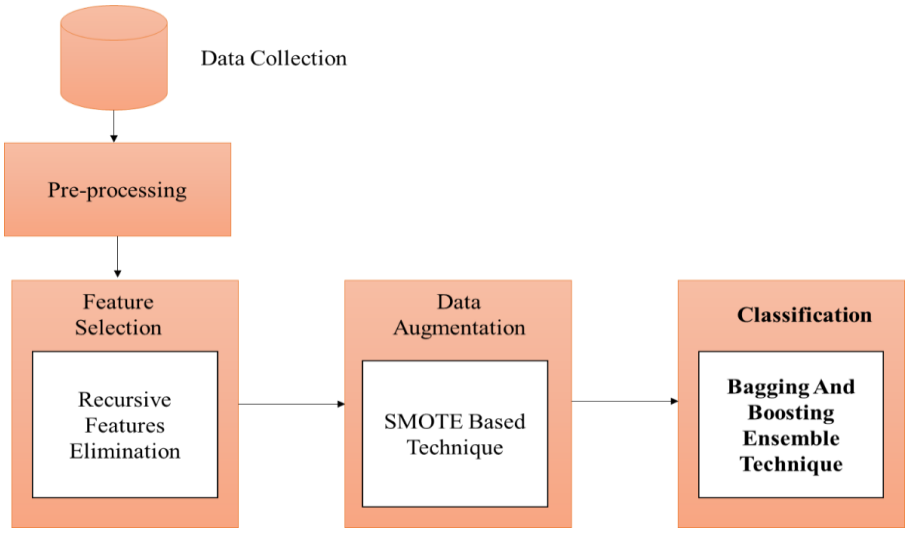


Figure 1. Methodological Flow of the Presented Model

Figure 1 depicts the design flow of the respective research. It is identified that the presented approach comprises of the following procedures.

- Data Collection
- Data Pre-processing
- Feature selection
- Data Augmentation
- Classification

Correspondingly, detailed description of the proposed model is signified in the following sub-sections.

2.1. Data collection

The proposed model is performed by adopting the machine learning methods using the Physionet Bank Visceral Adipose Tissue Measurement published on March 2020, Version 1.0 for ascertaining the presence of GDM among pregnant mothers. It is processed with the cohort analysis with the pregnant women for the period in gestation of 20 weeks. The analysis is followed with the 15 parameters until the delivery of the pregnant women. In the scan of consistent trimester, VAT evaluation is take placed where the biometric examination is carried out in terms of parental care data. The data for the analysis is processed with the medical records of the pregnancy women. The various attributes of the dataset are listed in Table 1.

Table 1. Attributes of the Dataset

Data in Early Gestational Period (20 Weeks)	
Number	Unique Id for the case
Age (years)	Age in years
Ethnicity	0 – White, 1-Not white
Diabetes Mellitus Previous	0 – No; 1- Yes
Mean Diastolic BP	Mean diastolic blood pressure in mmHg
Mean Systolic BP	Mean systolic blood pressure in mmHg
Central Armellini Fat	Maternal Visceral Adipose tissue measurement in mm
Current Gestational Age	Weeks and days of pregnancy
First fast glucose	First measured fasting glucose in mg/dl
BMI pregestational	Pregestational body mass index in kg//m
Data at the outcome of Delivery	
Gestational age	Age at birth (weeks and days)
Type of Delivery	0 – Vaginal Birth; 1 – Caesarean section
Gestational DM	0 – No; 1 -Yes

2.2. Preprocessing

Out of the 133 pregnancy patient records, there were 26 values missing values for the attribute First Fasting Glucose and 5 missing values for number of pregnancies. Missingvalues imputation is one of the biggest tasks in preprocessing and a suitable imputation will produce quality dataset for betteranalysis of clinical trials. To discover what works best for the dataset, each statistical techniques for examining the missing values in the dataset was tried and tested. Case deletion for the 5 missing values of 'Number of Pregnancies' attribute was made as this deletion will not have a greater impact in the precision, and the 26 missing values for the attribute fastglucose level is a good 'Line of Dignity' to observe and was imputed using multiple im-

putation methods. Predictive models can be computationally expensive, but repeated imputation would significantly increase this cost. Time and overhead. Yet it is crucial to have imputed values as close as possible to unobserved values. Given the stochastic nature of the algorithms, the algorithms were run for different parametric values. It is clearly evident that there is only a minor fluctuation in the mean performance. KNN is sensitive to outliers and involves expensive computations. The Expectation Maximization Imputation is better than Mean Imputation as they preserve the relationship with other variables (Algorithm 1).

Algorithm 1: MVIMPUTE(D,N)

Input: D - DataSet
Output : D′ - Preprocessed Dataset Procedure:
for each sample Ri in D do
for attributes A1,A2,A3..An Є Ri do
If Ai == NULL then Ci := Class(Ai)
(a) Compute Mean(Ai)= ($\sum^{N} Ai$)/N
i=1
(b) Compute Median(Ai)= A[(N+1)/2]
(c) Compute Mode(Ai)=most frequent value
(d) Compute ConceptMostMean(Ai)
= ($\sum^{N} Ai$)/N , where Class(Ai)=Ci
i=1
(e) for k=1..9 step 2 do
(i) Computer k-Nearest Neighbour using
n
$\sqrt{\sum}(qi - pi)(qi - pi)^2$
i=1
(f) Compute θ = argmaxlog(∑z p(x, z|θ))
(g)Impute the Missing Values with the computed values.
end
end
Fit the model and validate the performance of the classifier. D′=Perform imputation with the value that yields max{ Accuracy
Return D′

2.3. Feature Selection

Feature Engineering is a kind of Machine Learning itself that uses the process of domain knowledge to improve Machine Learning. A feature is a characteristic that is important to a predictive model and the best features selected greatly influence the results. Feature Selection considerably reduces the complexity of the model and faster training can be achieved. Figure 2 shows the feature selection methods.

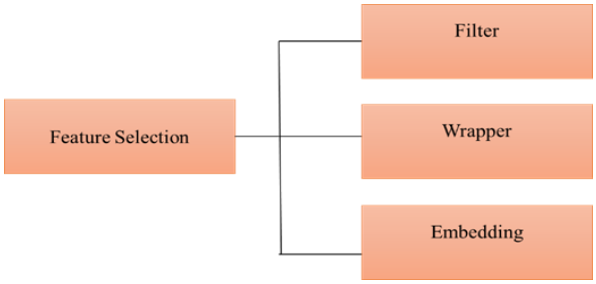


Figure 2. Feature Selection Models

Figure 2 depicts the mechanism in the feature selection. The model is built by considering the correlation among the independent variables and removing the irrelevant features to the model to improve the prediction performance of the predictors was performed. There are three categories for feature selection methods: filter, wrapper, and embedding. The Filter technique assigns a statistical weight to each prediction and quantifies each property. Wrapper techniques characterize feature selection as a gluttonous search for the best characteristics, in which a variety of combinations of subsets are gathered, assessed, and compared with one another. Embedded techniques assess the utility of the feature subset selected by the learning procedure. The model is built while the features that are most important to the model's accuracy are determined. In this study, a rapper method of feature selection that uses filters internally known as Recursive Feature Elimination was used to remove redundant attributes. Not all modes can be paired with RFE. The top nine features selected are VAT, Glucose, current gestation age, no. of pregnancies, BMI, diastolic and systolic BP, age, and ethnicity. The attributes gestational age, ethnicity and outcome attributes were not taken into consideration. Eventually, the zero-importance features were opted out for the prediction process. The features arranged according to their importance shows that VAT is the most influential attribute and also the cumulative feature importance plot shows the number of features selected that yields maximum accuracy. Algorithm 2 signifies the RFE(D') mechanism.

Algorithm 2: RFE(D')

Input: D' - DataSet, N-number of features Output : D'' - FS_Dataset
Procedure:
Initialize a pipeline of models, M={'LR','DT','RF','GB'} n_features=N ' Subset size
For i in 1..n_features do
Identify the Collinear features using Equ (1)

$$Rxy = \sum(xi - x)(yi - y / \sqrt{\sum(xi - x)^2 \sum(yi - y)^2}$$

Compute the feature importance score for each
feature end
Remove features with zero-feature score For each model mi in M
Fit mi using CrossValidation Determine Acc = TP/TP+TN
End For
Return FS_Dataset
* LR-Logistic Regression DT- Decision Tree RF-Random Forest GB-Gradient Boost

Correspondingly, Fig. 3 shows the feature importance of the proposed model.

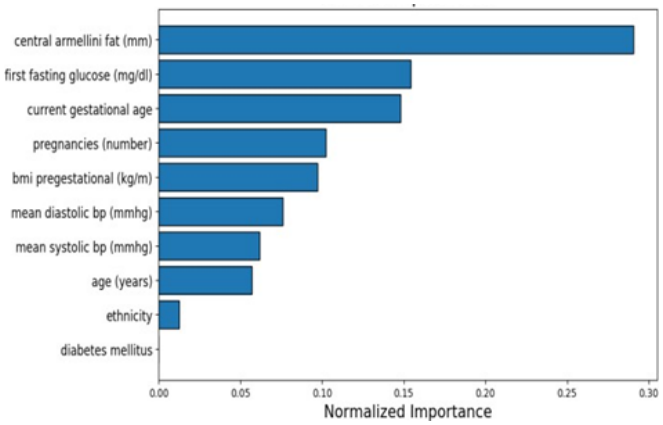


Figure 3. Feature Importance.

2.4. Data Augmentation

The understanding of the dataset, the class imbalance problem was dealt with suitably. Apparently, the success of the prediction model depends on the dataset; the problem of class imbalance greatly influences the performance of the classifier. The PhysioNet Bank dataset contains dichotomous records attributing to 1-Presence of GDM and 0-Absense of GDM. Out of the 133 records, 18 were only contributing to No GDM class and it is clearly a Class-imbalance problem to be addressed too. One of the approaches for this class imbalance challenge is to oversample the minority class. The SMOTE method concentrates on the feature space to create new instances with interpolation between the positive instances that are close together. It starts by selecting a minority class-instance and finding its k-nearest neighbors (typically k=5). A synthetic instance is created in the feature space between the two examples, one that is randomly selected from the k-nearest neighbor and the random sample itself. New plausible synthetic minority class samples were created, and the data set is augmented with GDM-1 {115 records} and GDM-0 {115 records} (Algorithm 3). SMOTE on the minority class is followed by an undersampling of the majority class. This pipeline performs better than just oversampling alone.

Algorithm 3: SMOTEVAT(FS_D)

Input: FS_D - Feature Selected DataSet Output : D′ - Synthesized Dataset Procedure:
A=Φ; B=Φ
for each sample Ri in D do if Ri.target=1
A=A U Ri
else
B=B U Ri
Initialize k=5; N=3 ‘ k-no of neighbours, N- sampling_rate Choose X=random(A)
for each x in A && x <> X do
Calculate the Euclidean distance :

$$d(p, q) = d(q, p) = \sqrt{(q1 - p1)^2 + (q2 - p2)^2 + (q2 - p2)2+ \quad +(qn - pn)(qn - pn)^2}$$
$$n$$
$$= \sqrt{\sum}(qi - pi)2$$
$$i=1$$

End for
Sort the distance, determine the k nearest neighbours, say K for i in 1..N do
kx= pick random(ki), k Є K and for i=(1..5)

Generate new synthetic instance using $x' = x + rand(0,1) * | x - xk |$
end for
D′= D U x′
Return D′

2.5. Classification

The dataset experimented initially using a simple MultiLayer Perceptron (MLP) sequential model with plain stack of layers starting with an input layer of 9 inputs attributes, followed by two hidden layers with 64 nodes, closely stacked by two consecutive hidden layers with 8 nodes each and a final output layer used for classification. The detailed architecture of the 6-Layer stacked network is presented in Table 2.

Table 2. Network Architecture

Layer	Output Shape	Parameters #
Dense	(None,64)	640
Dense	(None,64)	4160
Dense	(None,8)	520
Dense	(None,8)	72
Dense	(None,1)	9

Model: "sequential" Total params: 5,401
Trainable params: 5,401
Non-trainable params: 0

Table 2 represents the architecture of the proposed network. A stochastic gradient descent optimizer with a learning rate of 0.01 and a ReLu activation function was used to create this MLP Five-Fold Model repeated stratified cross-validation was performed on the dataset with a preferred loss function of binary cross entropy and an accuracy of 75.8% was achieved. The accuracy achieved was not optimal and in order to produce a better predictive model a set of experiments with the bagging and boosting methods were performed.

2.5.1. Bagging Based Ensemble Learning

A Bagging classifier is an ensemble meta-estimator that randomly picks subsets of data from the original set and constructs a tree-based classifier. Such trees generated are then combined to form a final prediction by fitting the base classifiers each on random subsets of the original dataset and then aggregate their individual predictions. Figure 4 represents the generation of trees and majority voting in the classification mechanism.

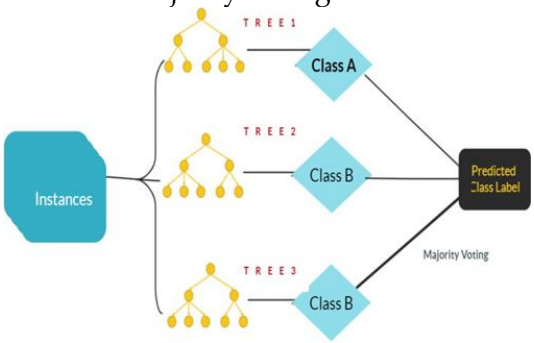


Figure 4. Generation of Trees and Majority Voting

2.5.2. Boosting Based Ensembles

To reduce bias and variance in a supervised learning, a family of machine learning algorithm is used to convert the weak learners to strong ones. The algorithm initiates by training a model with the full training set, and consequent models are constructed by fitting the residual error values of the initial model. In this way, Boosting method prepares itself to give higher weights to those instances that were wrongly estimated by the previous model. A final estimation is produced by combining the results of the weighting of the predictions made by the succession of developed models according to their accuracy scores by averaging to form a final prediction as given in Equation 1.

$$f(x) = \frac{1}{M} \sum_{m=1}^{M} f_m x \quad (1)$$

The equation represents the average of M functions $f_m(x)$, giving a single function f(x) that combines their values.

The base estimator chosen was a CART decision tree with different seed values and a KFold of 3. The accuracy obtained was 90.57%. Also, using a random forest as a base estimator with different seed values were experimented with a KFold of s10. The accuracy achieved was 92.59%. Clearly, for this dataset on GDM, the random forest bagging ensemble obtained high degree of accuracy in comparison to the CART ensemble.

2.5.3. Max_Features

Max_features is the number of features that a single tree in Random Forest may include. It is widely accepted that the more features there are, the more accurate a prediction will be. This is not always true, though, as it lessens the variability of each individual tree, which is the USP of random forests. But, a closer inspection reveals that we increase the computational time of the algorithm by increasing the max_features (Algorithms 4 and 5). Hence, we need to maintain a good balance between computational time and number of features used in the system.

Algorithm 4: GDMSafePred(D)

Input : DataSet - D
Number of Instances - N Output : M-Prediction Model Procedure:
(i) D'=MVIMPUTE(D, N)
(ii)FS_D=RFE(D')
(iii)D''=SMOTEVAT(FS_D)
(iv) M=RF(D'')
(v)Save M for Prediction
End;

Algorithm 5: RF(D'',i,n,k)

Input: DataSet - D''; i - max_depth; n-no_of_trees;
k-no of features Output: Model - M
Procedure:

1. Do
2. nt=1; count=0
1. do
2. count=count+1
3. Randomly pick 'k' features from 'm' features, where k<m
4. Calculate the best split using Entropy calculation

$$H(X) = -\sum_{x \in X} p(x) \log p(x)$$

5. Split node into child nodes
6. while (count <=i)
7. nt=nt+1
8. While (nt <= n)
9. For q in 1..n do
10. Evaluate the Predicted outcome Pq on Test data
11. vote=mode(Pq)
12. **Return M**

2.5.4. Key Parameters for Tuning

As parameters selection and optimization plays a vital role in a classifier, the best hyper parameter that was selected for this classifier implementation is discussed here.

2.5.4.1. Seed Values

Proper setting of Random Seed value in ML experiments are required. Non-determinism may impact your model's convergence rate, the stability of your results, and the final quality of a network.

2.5.4.2. Number of Trees

This feature determines the number of trees in the forest of a model. The number of trees parameter was set sufficiently large enough to stabilize the error rate. The rule of thumb is to have the number of trees to be 10 times the number of features. Trying with the different parameter values, it was set be 100 to have a good balance between ROC AUC and processing time.

3. RESULTS AND DISCUSSIONS

3.1. Exploratory Data Analysis

As the dataset contains more numerical data, to understand the different attributes of the dataset, exploratory data analysis (EDA) was made to have a thorough understanding of the underlying dataset. This exposes the trends, patterns, associations and correlations among the data, which are not readily apparent. This will consequently help in performing advanced analytics of the data. Figure 1 provides a visual data exploration of the tabular data, and a univariate analysis is made to get deeper understanding of the data. The measures of central tendency and the measures of dispersion are understood to identify the spread. Bivariate Analysis determines the empirical relationship between the variables and determines the association among them. Scatter plot that maps each variable to a point in 2D space is presented in Fig. 2. Few insights obtained on the dataset are: (a) The highest age range among the mothers is 25 years. (b) 57% are of white ethnicity and 43% are of black race. (c) All cases in the dataset show no presence of previous diabetes. (d) The maximum value of BMI is 55 kg and the average stands at 27 kg. (e) The VAT measurements averages to 44 mm and it has been proved that a 45 mm threshold was determined to be the best demarcation value to predict GDM with an accuracy of 66%. It clearly states that for less values of the Visceral Adipose Tissue Measurement, the risk of GDM is less and as the VAT measurement increases, there is a probability of GDM to occur. Finally, an attempt to elucidate the interrelationship between the variables was made through a correlogram by finding the correlation coefficients. In Fig. 5, the blue dots represent positive correlation and red represents negative correlation.

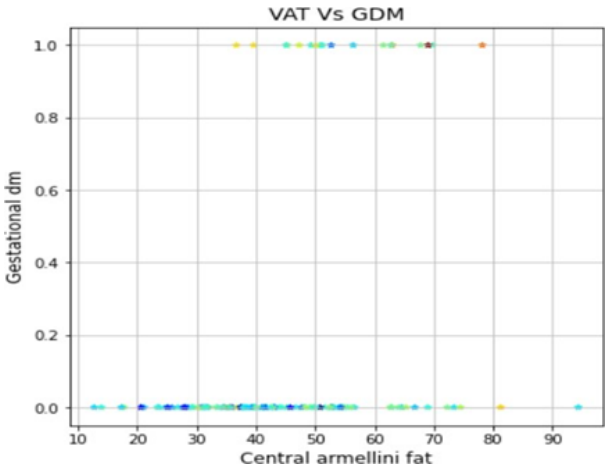


Figure 5. VAT Measurement Vs Gestational Diabetes

Figure 5 represents the VAT vs GDM analyzed in the respective model. Accordingly, Fig. 6 and Fig. 7 portray the correlation matrix of the projected system.

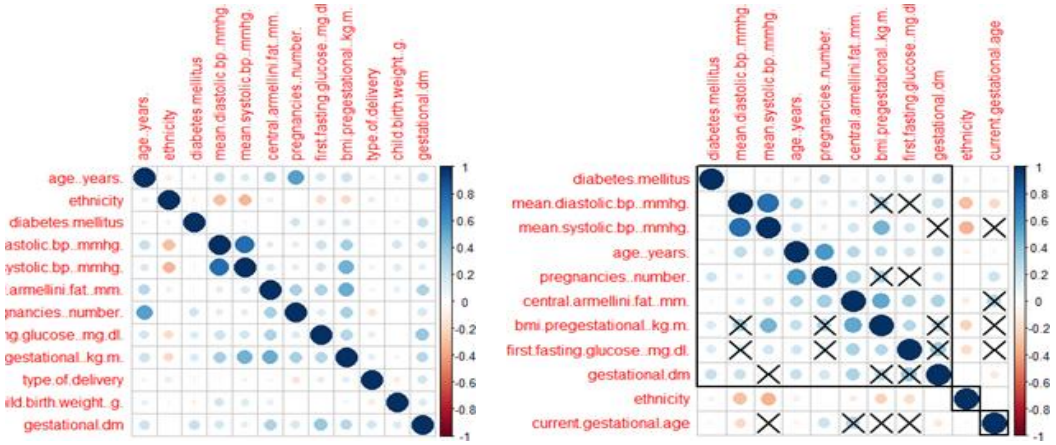
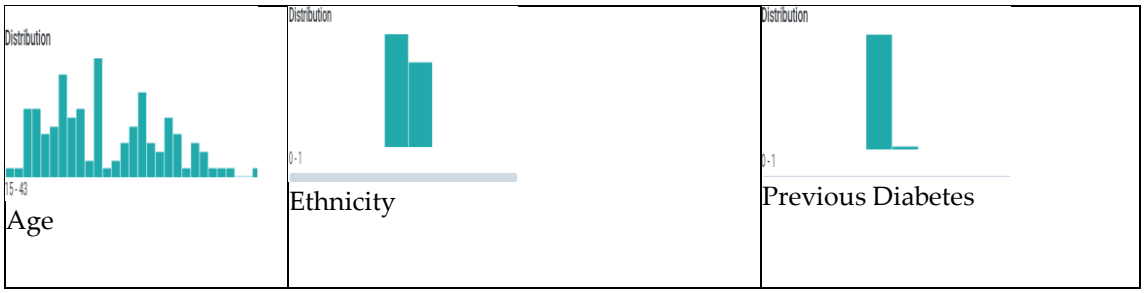


Figure 6. Correlogram-Correlation Matrix Plot.

Figure 6 represents the correlation matrix of the presented approach. Here, Blue indicates a positive correlation and red indicates negative. Figure 7 depicts the attributes distribution in the projected system.



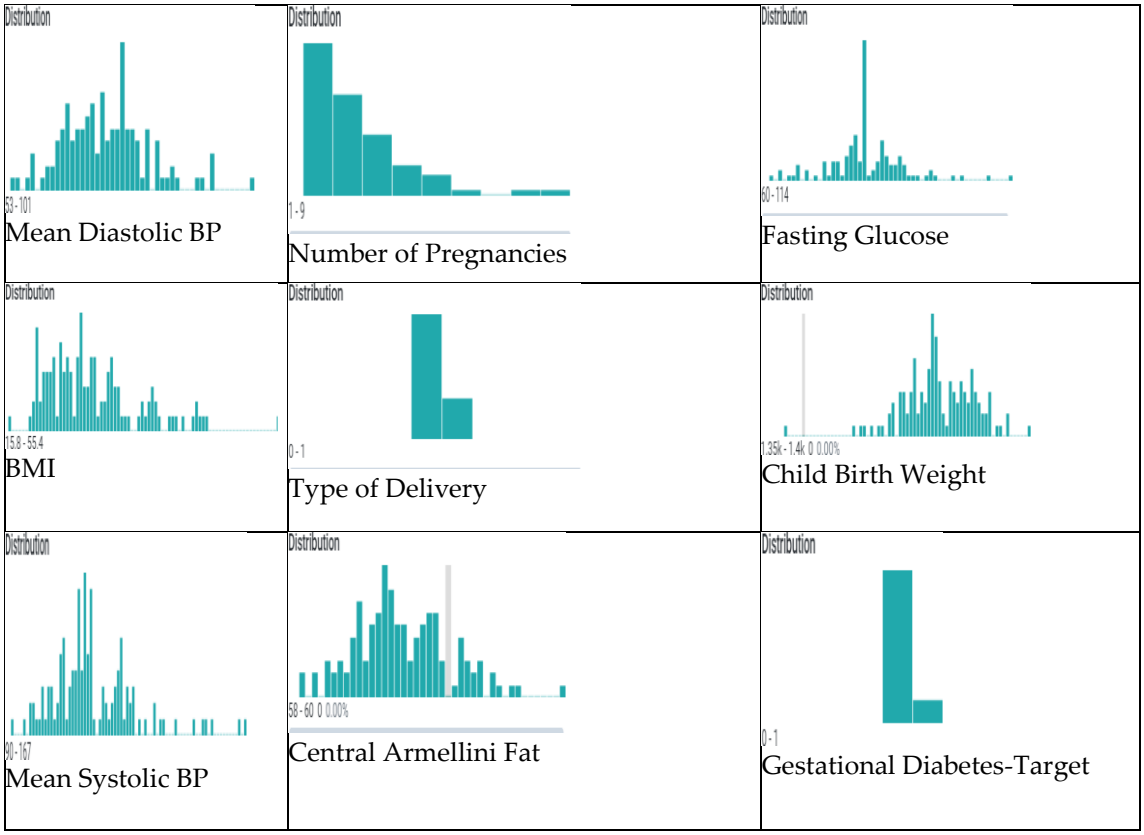


Figure 7. Distribution of Attributes - Univariate Analysis

Correspondingly, Fig. 8 depicts the Bivariate Analysis-Scatter Plot in the presented model.

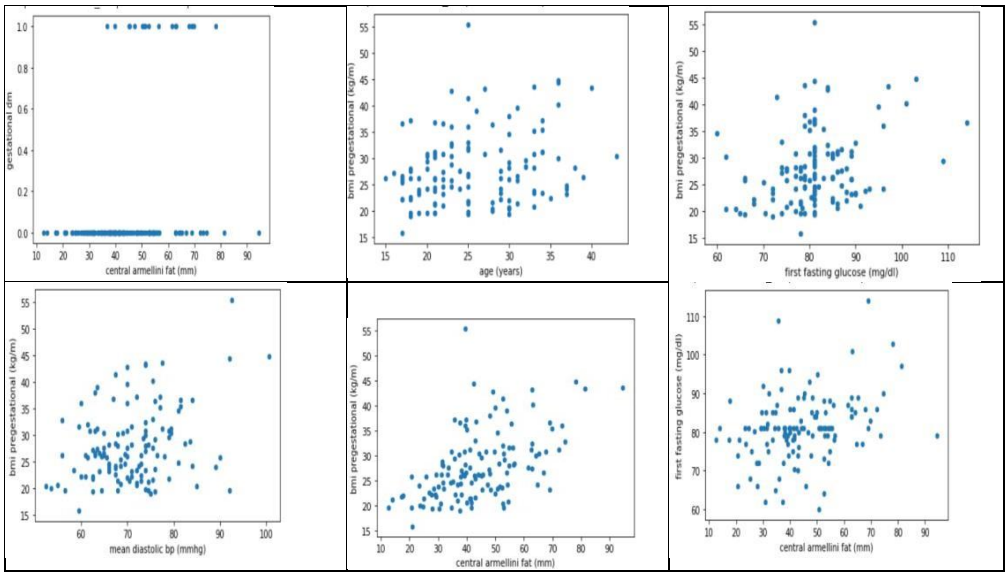


Figure 8. Bivariate Analysis-Scatter Plot

Otherwise, Fig. 9 represents the baseline model of the feature selection.

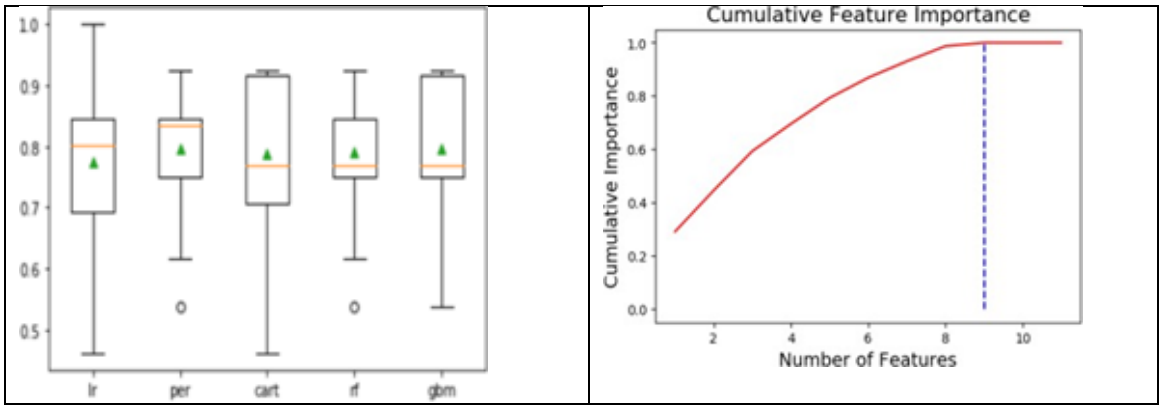


Figure 9. Baseline Models-BoxPlot

3.2. Performance Metrics

The factors utilized to measure the efficacy of the presented approach is represented in this section.

3.2.1. Accuracy

The accuracy is the essential metrics utilized to analyze prediction amount, which are accurately correct in the proposed model. The formula for accuracy is depicted in subsequent equation.

$$\text{Accuracy} = \frac{\text{TP+TN}}{\text{TP+FP+TN+FN}}$$

Where TP, TN, FP, FN are True Positive, True Negative, False Positive and False Negative.

4.2.2. AUC Curve

The AUC is utilized to compute the two-dimensional area that is under the ROC curve.

3.3. Global Results

The section represents the outcome attained by the proposed model. The different boosting algorithms like XGBoost, AdaBoost and GradientBoost were fit and tested on the dataset and the evaluation metrics are signified in Table 3. Thus, Table 3 illustrates the performance of the boost classifier; it is identified that the AdaBoost attained higher accuracy. Table 4 and Fig. 10 signifies the performance of the proposed model.

Table 3. Performance of Boosting Classifier

Classifiers	**Accuracy**	**CrossValidation**
XGBoost	89.19%	10 Fold CV
AdaBoost*	91.93%	10 Fold CV, no of trees=30
Gradient Boost	87.86%	10 Fold CV, no_trees=30

Table 4. Performance Chart

Classifier	**Accuracy**
RF- Gini	92.59
RF-Entropy	92.59
ExtraTrees Classifier-Gini	85.19
ExtraTrees Classifier Entropy	81.48

K-NN	85.19
NN Classifier	88.89
Weighted Ensemble	92.05

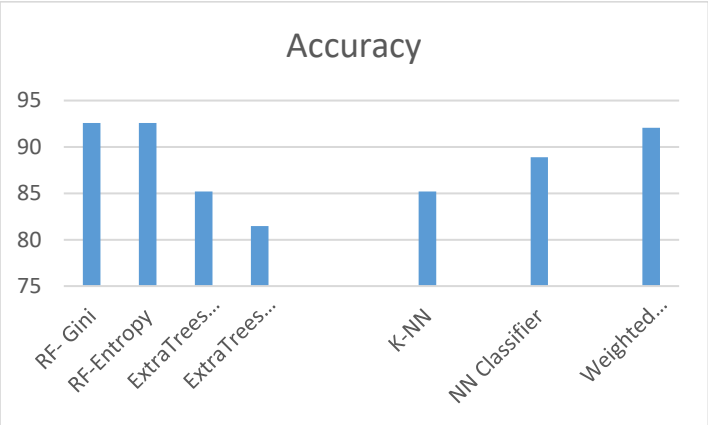


Figure 10. Outcome of the Presented Method

Figure 10 and Table 4 represent the outcome accomplished by the presented system. Here, multiple machine learning algorithms were wrapped up as voting ensembles by averaging the predictions of the arbitrary models. To gauge the predictive performance of the different models experimented; accuracy was used as the evaluation metric. Table 5 represents the accuracy yielded with the different classifiers and it is obvious that the Random Forest algorithm gives the highest accuracy comparatively.

In Random Forest, each of the trees built in the ensemble is formed with a random sample taken with replacement. Despite this fact of the randomness involved, a random subset of features is also picked up. The forest's bias somewhat increases in this situation, but its variance decreases as a result of the averaging of fewer connected trees, leading to a better model overall. The predictive performance on the dataset is 92.59%. Bagging enhances the benefits of the single model while minimizing its drawbacks, which could result in a combined model with reduced errors. The highest accuracy achieved is 92.59% with Random Forest Bagging Classifier.

Accordingly, the mean accuracy of each of the strategy was evaluated using a Decision TreeClassifier and is presented in Table 5.

Table 5. Statistical Strategies of Missing Value Imputation for Fast Glucose Attributes

Imputation Method	**Methods &Parameters**	**Accuracy(%) -Decision Tree**
Most CommonImputation	Mean: 81 Median: 80 Mode : 79	74.35 74.35 79.48
Concept MostCommon Imputation	Class: 1 Mean: 81 Class: 0 Mean: 81	74.35 74.35
K Nearest Neighbour imputation	k=1 to 9	87.40
Expectation Maximization Imputation	Loops=10	87.70

Table 5 represents the statistical Strategies of Missing Value Imputation for Fast Glucose Attributes. Similarly, RFE method was experimented with by hyper tuning the parameters such as Baseline model and Number of Features presented in Table 6.

Table 6. RFE - Hyper parameter Tuning

Baseline Model		**Mean Accuracy**			
Max_Features					
	5	6	7	8	9
Logistic Regression	0.776	0.78	0.76	0.77	0.79
Perceptron	0.797	0.79	0.80	0.80	0.80
CART	0.78	0.79	0.79	0.79	0.79
RF	0.79	0.796	0.80	0.78	0.79
GBM	0.79	0.807	0.79	0.80	0.80

Table 6 represents the RFE hyper parameter tuning. Accordingly, Tables 7, 8 and 9 signifies tuning seed values, tuning no of trees and tuning maximum number of features.

Table 7. Tuning Seed Values

Classifier	**seed**	**num_trees**	**max_features**	**kFold**	**Mean Accuracy**
RF	10	100	4	10	91.44
RF	15	100	4	10	91.46
RF	20	100	4	10	91.02
RF	25	100	4	10	91.48
RF*	**30**	**100**	**4**	**10**	**92.59**
RF	35	100	4	10	92.46

Table 8. Tuning No of Trees

Classifier	**seed**	**num_trees**	**max_features**	**kFold**	**Mean Accuracy**
RF	30	10	4	10	89.20
RF	30	25	4	10	89.68
RF	30	50	4	10	90.15
RF	30	75	4	10	91.91
RF*	**30**	**100**	**4**	**10**	**92.59**
RF	30	150	4	10	90.55

Table 9. Tuning Maximum No. of Features

Classifier	**seed**	**num_trees**	**max_features**	**kFold**	**Mean Accuracy**
RF	30	100	2	10	90.11
RF	30	100	3	10	90.57
RF*	**30**	**100**	**4**	**10**	**92.59**

Figures 7, 8 and 9 indicate the tuning seed values, tuning no of trees and tuning maximum number of features. It is identified that the RF attained higher accuracy than the other models. Correspondingly, the respective method is evaluated using a Decision Tree Classifier with Repeated Stratified KFold validation with the hyperparameters {nsplits=10, repeats=3, random=1} and obtained a mean ROC of 87.7%.

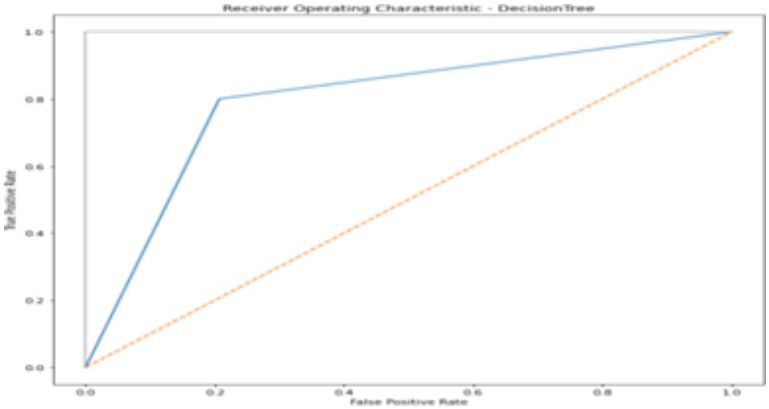


Figure 11. ROC curve

Figure 11 represents the ROC curve of the respective method. Building a decision support system is the core of machine learning and building a good model with optimal accuracy will be the solution to the prediction problem. This proposed work experiments with the different ensemble method, which is a potential technique to get a highly precise model. It is a computational knack of amalgamating different set of learners to improvise the predictive power of the model. Wide varieties of ensemble experiments were conducted and the results are recorded.

3.4. Comparative Analysis

The section represents the comparative analysis of the proposed model with the existing model.

3.4.1. Comparison with PIMA Indian Dataset

The proposed study on the prediction of Gestational Diabetes Mellitus was also performed using the benchmark PIMA Indian Diabetes dataset. The National Institute of Diabetes and Digestive and Kidney Diseases is the source of the PIMA Indian dataset. The primary goal for collecting this dataset was to predict diabetes from a cohort of 768 cases based on their diagnostic parameters. The attributes of PIMA datasets are No. of times Pregnant, Glucose, BP, Skin Thickness, Insulin, BMI, Pedigree, and Age. The PhysioNet Visceral Adipose Tissue Measurement Dataset with gestational outcome was used to execute the task of predicting GDM from maternal VAT at delivery time. The common attributes between PIMA and PhysioNet Visceral Adipose Tissue were taken into consideration to evaluate the prediction of diabetes. The results are tabulated with comparative analysis. With these six predominant and likely common attributes, the various ML bagging and boosting techniques were investigated and it was observed that high accuracy was achieved with RF classifier on PhysioNet VAT dataset and PIMA dataset as well for the prediction of Gestational Diabetes Mellitus. Evidently, in previous studies a 45 mm threshold of Visceral Adipose Tissue measurement was identified as the best cut-off value to predict GDM with an accuracy of 66%. The proposed model also ascertains that there exists a strong association between VAT depth and GDM. It is apparent that along with other important features, the presence of Adipose Tissue measurement aids in the prediction of GDM to a greater extent. Table 10 and Fig. 11 illustrates the common features in the PIMA and PhysioNet Dataset.

Table 10. Common Features - PIMA & Physionet

S. No	Physionet- Visceral Adipose Measurements	PIMA Indian Diabetes
1	Age	Age
2	Blood Pressure	Blood Pressure
3	Visceral Measurement	Skin Thickness
4	No. Of Pregnancies	No.of Pregnancies
5	Glucose Fast	Glucose Fast
6	BMI	BMI

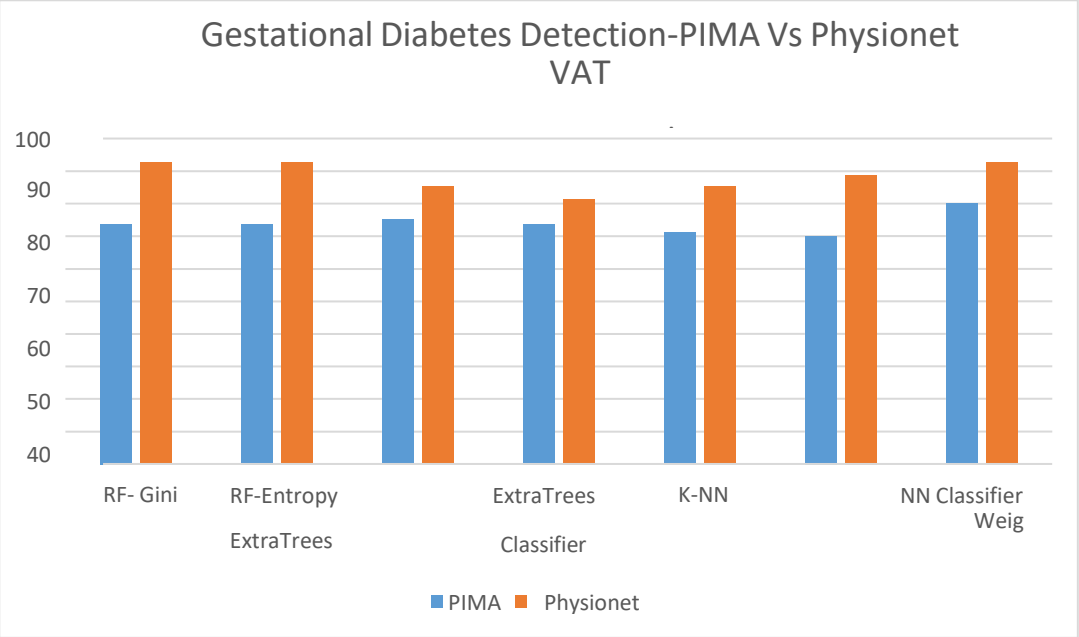


Figure 12. Comparison with PIMA Indian Dataset

Figure 12 illustrates the comparison of PIMA Indian dataset. Accordingly, Table 11 represents the efficiency comparison of the proposed model with the existing mechanism.

Table 11. Performance Comparison

Models	PIMA	Physionet VAT
RF- Gini	**73.75**	**92.59**
RF-Entropy	73.75	92.59
ExtraTrees Classifier-Gini	75	85.19
ExtraTrees Classifier Entropy	73.75	81.48
K-NN	71.25	85.19
NN Classifier	70	88.89
Weighted Ensemble	80	90.50

Table 11 shows the comparative analysis of the proposed model with the conventional model. It is identified that the presented method attained better efficiency than the classical methods.

3.5. Applications

Rapid diagnosis is necessary for Gestational Diabetes Mellitus (GDM), a public health issue that poses dangers to both the mother and the fetus. The work done is summarized in the development of a tool to be able to collect pregnant women data in the early weeks of gestation and also to infer the occurrence of GDM with high level of confidence. Further the interface can be extended by adding new symptomsand defining relationships between the new signs and the corresponding diseases. Figure 13 signifies the Prediction of Input Query Patient Using GDMSafe Tool.

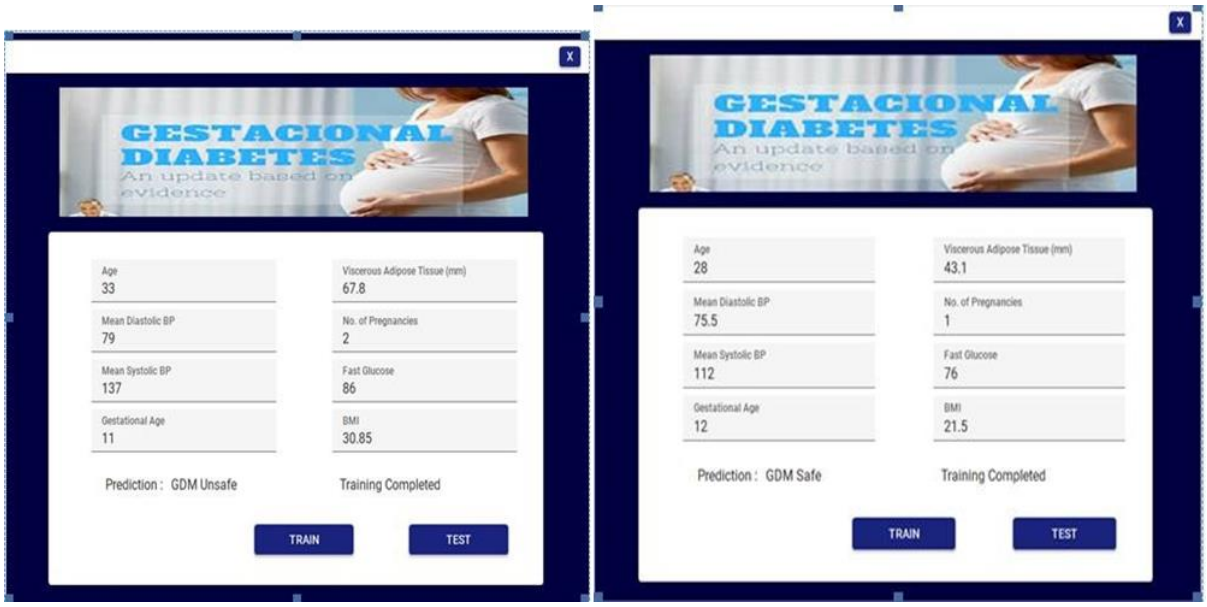


Figure 13. Prediction of Input Query Patient Using GDMSafe Tool

4. CONCLUSION

The implemented work was able to obtain insight on the Visceral Adipose Tissue data. Predicting well in advance the occurrence of GDM may help the mothers to take adequate measures and precautions to combat GDM. With Random Forest, one of the most common ensemble approaches that uses a decision tree as a classifier, the best accuracy is attained. By varying the hyperparametes like random state, number of trees, maximum number of features and performing cross validation, it has been successfully demonstrated that RF provides more reliable and accurate predictions. The prediction tool provides useful knowledge to health care professionals and can also serve as a reference in their decision to predict GDM. However, most of the studies for GDM Prediction have made use of PIMA Indian Diabetes dataset. To the best of the knowledge, this is the first predictive model using the Visceral Adipose Dataset published recently in the public repository of PhysioNet Bank. In our view, the result emphasis the validity of the model. This study paves way for further progress in this direction as clinical studies suggests that VAD measured by ultrasound in early pregnancy may predict the onset of GDM, substantiating the previous findings in the literature.

DECLARATIONS

- **Conflict of Interest**: The author reports that there is no conflict of Interest.
- **Funding**: None
- **Acknowledgement**: None
- **Data Availability Statement**: The author do not have permission to share data.
- **Ethics Approval**: Not Applicable

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Scientific research article

Thermodynamic analysis and preferential solvation of metronidazole solubility in methanol-water and ethanol-water cosolvent mixtures at different temperatures

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SUMMARY

Introduction: Solubility is one of the most important physicochemical properties because it is related to some industrial processes such as: formulation, preformulation, purification and quantification. **Objective:** This paper presents the thermodynamic analysis of the solubility of metronidazole in methanol-water and ethanol-water cosolvent mixtures at seven temperatures. **Methodology:** From solubility data, the thermodynamic functions of solution and mixture are calculated and analysed using the Perlovich graphical method. On the other hand, an enthalpy-entropy compensation analysis is performed, and the preferential solvation parameters are calculated using the inverse Kirkwood-Buff integral (IKBI) method. **Results:** The result of the calculations performed indicates that the dissolution process of metronidazole is endothermic with entropic preference, where the addition of methanol and ethanol has a positive cosolvent effect in intermediate and water-rich mixtures. With regard to preferential solvation, the results are not entirely conclusive, since, except in intermediate mixtures, the values of the preferential solvation parameter are less than 0.01, so that negligible preferential solvation takes place.

Keywords: Solubility, metronidazole, solution thermodynamics, enthalpy-entropy compensation, preferential solvation.

RESUMEN

Análisis termodinámico y solvatación preferencial de la solubilidad del metronidazol en mezclas cosolventes metanol-agua y etanol-agua a diferentes temperaturas

Introducción: La solubilidad es una de las propiedades fisicoquímicas más importantes, puesto que está relacionada con algunos procesos industriales, como: formulación, preformulación, purificación y cuantificación. **Objetivo:** Este trabajo presenta el análisis termodinámico de la solubilidad del metronidazol en mezclas de cosolventes metanol + agua y etanol + agua a siete temperaturas diferentes. **Metodología:** A partir de los datos de solubilidad, se calcularon y analizaron las funciones termodinámicas de la solución y la mezcla mediante el método gráfico de Perlovich. Por otro lado, se realiza un análisis de compensación entalpía-entropía y se calculan los parámetros de solvatación preferencial mediante el método de la integral inversa de Kirkwood-Buff (IKBI). **Resultados:** Los cálculos realizados indican que el proceso de disolución del metronidazol es endotérmico con favorecimiento entrópico, la adición de metanol y etanol tiene un efecto cosolvente positivo en mezclas intermedias y ricas en agua. En cuanto al parámetro de solvatación preferencial, los resultados no son del todo concluyentes, ya que, salvo en las mezclas intermedias, los valores del parámetro de solvatación preferencial son inferiores a 0,01, por lo que se concluye que se tiene una solvatación preferencial despreciable.

***Palabras Clave*:** Solubilidad, metronidazol, termodinámica de soluciones, compensación entalpía-entropía, solvatación preferencial.

RESUMO

Análise termodinâmica e solvatação preferencial da solubilidade do metronidazol em misturas de metanol-água e etanol-água a diferentes temperaturas

Introdução: A solubilidade é uma das propriedades físico-químicas mais importantes, pois está relacionada com vários processos industriais, nomeadamente formulação, pré-formulação, purificação e quantificação. **Objetivo:** O presente trabalho apresenta a análise termodinâmica da solubilidade do metronidazol em misturas de metanol + água e etanol + água em sete temperaturas diferentes. **Metodologia:** A partir dos dados de solubilidade, as funções termodinâmicas da solução e da mistura foram calculadas e analisadas com recurso ao método gráfico de Perlovich. Além disso, é efetuada uma análise de troca de entalpia-entropia e os parâmetros de solvatação preferenciais são calculados utilizando o método integral inverso de Kirkwood-Buff (IKBI). **Resultados:** Os cálculos indicam que o processo de dissolução do metronidazol é endotérmico com favorecimento entrópico, e que a adição de metanol e etanol tem um efeito positivo de co-solvente em misturas intermédias e ricas em água. No que se refere ao parâmetro de solvatação preferencial, os resultados não são totalmente conclusivos, uma vez que, exceto nas misturas intermédias, os valores do parâmetro de solvatação preferencial são inferiores a 0,01, pelo que se conclui que a solvatação preferencial é desprezível.

Palavras-chave: solubilidade, metronidazol, termodinâmica de soluções, compensação entalpia-entropia, solvatação preferencial.

1. INTRODUCTION

Metronidazole (MET) (Figure 1) is a widely used antibiotic and antiprotozoal medication. Since its introduction in France in 1960, this essential drug has become integral to treating a variety of infections, including bacterial vaginosis, pelvic inflammatory disease, and parasitic

infections such as giardiasis, trichomoniasis, and amebiasis. Its versatility is further demonstrated by its use in treating anaerobic infections, Crohn's disease, Helicobacter pylori infections, and as a prophylactic agent after surgery. With multiple formulations—oral, topical, and intravenous—metronidazole continues to play a critical role in modern medicine, ranking among the most commonly prescribed medications globally [1,2].

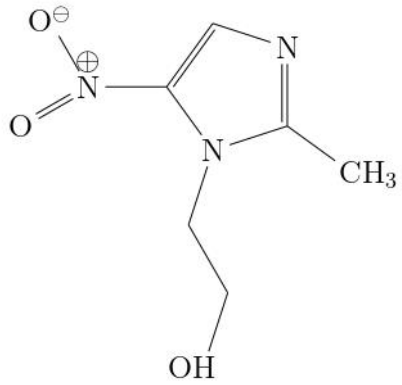

Figure 1. Molecular structure of metronidazole ($C_6H_9N_3O_3$; 2-(2-Methyl-5-nitro-1H-imidazol-1-yl)ethanol; CAS Number 443-48-1).

While the therapeutic potential of metronidazole is well-recognized, a critical factor that governs its clinical efficacy is its solubility and bioavailability [3, 4].

Water (solvent 2) is the most used solvent in pharmaceutical formulations, renowned for its safety, biocompatibility, and ability to dissolve polar compounds. As a universal solvent, water facilitates the dissolution of APIs, ensuring their bioavailability and therapeutic efficacy [5, 6]. It is particularly critical for oral drug formulations, where aqueous solubility governs the rate of drug dissolution and absorption in the gastrointestinal tract [7]. Additionally, water (W, solvent 2) is indispensable in parenteral, ophthalmic, and topical formulations, where purity and compatibility with physiological systems are paramount [8]. Methanol (MeOH, solvent 1^a), though less commonly employed than water due to its toxicity at higher concentrations, is a valuable cosolvent in pharmaceutical applications [9]. Its low molecular weight and high polarity make it an effective agent for dissolving hydrophobic drugs and enhancing the solubility of poorly water-soluble APIs. In laboratory settings, methanol is widely used in chromatographic analyses and quality control of drugs, including metronidazole, to ensure their compliance with pharmacopoeial standards [10, 11]. Ethanol (EtOH, solvent 1^b) serves a dual purpose in the pharmaceutical industry: as a solvent and as an antimicrobial preservative [12, 13]. Its compatibility with water and ability to dissolve both hydrophilic and hydrophobic substances make it a preferred cosolvent in liquid formulations, such as syrups, tinctures, and elixirs [14, 15]. Ethanol also plays a role in enhancing the bioavailability of drugs with poor aqueous solubility by modifying the solubility profile of APIs in mixed-solvent systems [6, 16–18].

The solubility of a drug is a key determinant of its therapeutic efficacy, particularly for oral dosage forms, which constitute many pharmaceutical products [19–21]. Poorly soluble drugs often face challenges such as low bioavailability, variable absorption, and suboptimal therapeutic outcomes. Factors influencing solubility, including pH levels, polarity, and dissolution dynamics, are critical considerations in drug formulation and delivery. Effective drug absorption and bioavailability hinge on the drug's ability to dissolve in gastrointestinal fluids

and traverse biological membranes. Poorly soluble drugs may exhibit erratic absorption patterns, leading to inconsistent therapeutic outcomes [22, 23].

The thermodynamic analysis allows us to evaluate the energetics related to the possible molecular interactions between the different molecules present in the solution; this information is relevant for the rational design of dosage forms, the optimization of industrial processes and the development of strategies for the decontamination of matrices such as water, soil and waste materials [24–26].

The preferential solvation analysis of drugs in co-solvent mixtures may be performed through the method of inverse Kirkwood–Buff integrals (IKBI). It expresses the local compositions near to the solute with respect to the different components present in the co- solvent solutions [18, 27]. This method depends on the value of the standard molar Gibbs energies of transfer of the solute from neat water to the cosolvent mixtures and the excess molar Gibbs energy of mixing for the solute-free mixtures. So, this treatment is of very importance in pharmaceutical sciences in understanding the molecular interactions between solute and solvent, because many solubility studies developed have been focused toward correlating or modelling the solubilities and the possible prediction in mixed solvents from the solubilities in the neat solvents [28]. Nevertheless, just a few of them have been intended to analyse the local environment around the drug molecules describing the local fraction of the solvent components (1 or 2) in the surrounding of solute [29].

2. METHODOLOGY

Experimental mole fraction solubility data (x_3) for metronidazole (component 3) in binary cosolvent mixtures of {methanol (1) + water (2)} and {ethanol (1) + water (2)} across a range of temperatures (specify range, e.g., from 293.15 K to 313.15 K) were sourced from a previously published scientific study [30].

Based on these solubility data, the apparent standard thermodynamic functions associated with the dissolution process were calculated [31]. The apparent standard Gibbs free energy of solution ($\Delta_{soln}G°$) it was determined according to Gibbs and van't Hoff equations based on the modification of Krug. The apparent standard enthalpy of solution ($\Delta_{soln}H°$) was derived using the modified van't Hoff equation by assessing the slope of the graphical representation of $\ln x_3$ plotted against $T^{-1} - T_{hm}^{-1}$. Where T_{hm} denotes the mean harmonic temperature over the experimental temperature interval. Consequently, the apparent standard entropy of solution ($\Delta_{soln}S°$) was computed at the temperature T_{hm} using the fundamental Gibbs relationship [32–36].

An enthalpy-entropy compensation analysis was conducted to explore the potential linear correlation between the enthalpy and entropy changes governing the solution process. This involved plotting $\Delta_{soln}H°$ against $\Delta_{soln}G°$ evaluated at the mean harmonic temperature T_{hm}. The linearity and the slope derived from this plot were analysed to characterize the compensation effect and potentially infer the molecular mechanisms driving the dissolution [37–39].

Furthermore, the preferential solvation of metronidazole by the constituent solvents within the mixtures (water and methanol/ethanol) was investigated employing the Inverse Kirkwood-Buff Integrals (IKBI) methodology [40–42].

3. RESULTS AND DISCUSSION

The solubilities of MET in mixtures of {MeOH (1[a]) + W (2)} and {MeOH (1[b]) + W (2)} (Figure 2) provided by Yu *et al.* [30].

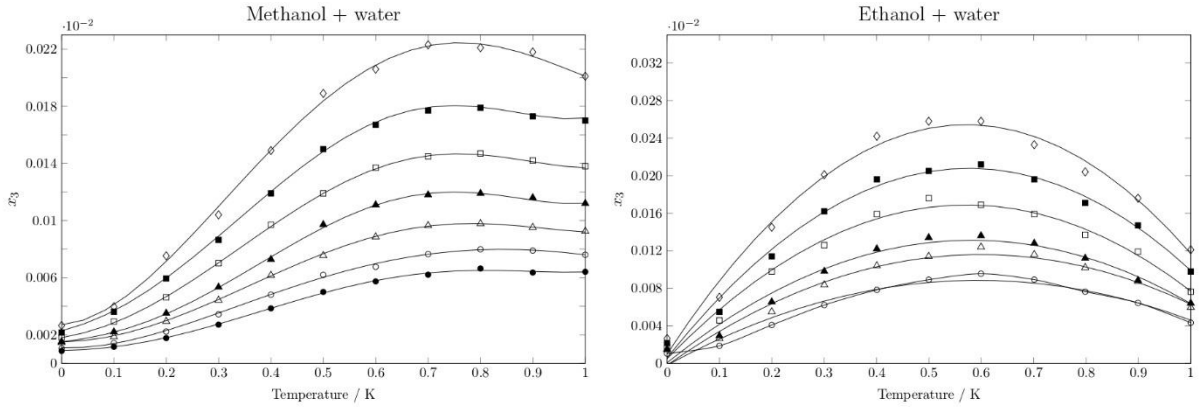

Figure 2. Solubility of MET in mole fraction (x_3) in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} cosolvent mixtures: ●= 293.15 K; ○= 298.15 K; Δ= 303.15 K; ▲= 308.15 K; ■= 313.15 K; □= 318.15 K; ◊= 323.15 K. From [30].

According to Figure 2, the solubility of MET in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} increases with increasing temperature, indicating an endothermic process [27, 28]. Furthermore, in both cosolvent mixtures, the cosolvent (MeOH and EtOH) influences the profile of the solubility isotherms. In mixtures {MeOH (1[a]) + W (2)}, except for 298.15 K, the maximum solubility is reached in a cosolvent mixture (between x_1=0.7 and x_1=0.9); in mixtures {EtOH (1[b]) + W (2)}, the maximum solubility is reached in a cosolvent mixture [43, 44].

When analysing the behaviour of the solubility isotherms as a function of the solubility parameter of the cosolvent system, a behaviour similar to that of sulfadiazine is observed at {MeOH (1[a]) + W (2)} [35] and {EtOH (1[b]) + W (2)} [31]. MET with a calculated solubility parameter of 25.6 $MPa^{1/2}$ (Table 1) reach it maximum solubility in {MeOH (1[a]) +W (2)} mixtures of δ_{1+2}=31-35 $MPa^{1/2}$ and in {EtOH (1[b])+W (2)} mixtures with δ_{1+2}=35.4 $MPa^{1/2}$.

Table 1. Fedor's methods applied to calculate partial and total solubility parameters of MET [45]

Group	#	$\Delta U°$ (kJ·mol^{-1})	$V°$ (cm^3·mol^{-1})
-CH_3	1	4.71	33.5
–CH_2–	2	2·4.94 = 9.88	2·16.1 = 32.2
–CH=	1	4.31	13.5
–OH	1	29.8	10
–NO_2	1	15.36	32
–N=	1	11.7	5
>N–	1	4.2	-9.0
Conjugation in ring	2	2·1.67=3.34	2·-2.2=-4.4
Ring closure [3] 5 atoms	1	1.05	16
	∑	84.35	128.80
		$\delta_2 = (84350/128.80)^{1/2} = 25.6$ $MPa^{1/2}$	

3.1. Thermodynamic functions of solution

From solubility data [30], the thermodynamic functions of solutions are calculated. Table 2 and Table 3 present the standard molar thermodynamic functions for dissolution of MET (3) in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} cosolvent mixtures.

The mathematical expressions for calculating the apparent thermodynamic functions of a solution according to the Gibbs and van't Hoff equations based on the modification of Krug *et al.* are [46–48]:

$$\Delta_{\text{soln}}H^{\text{o}} = -R\left(\frac{\partial \ln x_3}{\partial T^{-1} - T_{\text{hm}}^{-1}}\right)_P \quad (1)$$

$$\Delta_{\text{soln}}G^{\circ} = -RT_{\text{hm}} \times \text{intercept} \quad (2)$$

and

$$\Delta_{\text{soln}}G^{\circ} = \frac{(\Delta_{\text{soln}}H^{\circ} - \Delta_{\text{soln}}G^{\circ})}{T_{\text{hm}}} \quad (3)$$

where $\Delta_{\text{soln}}H^{\circ}$, $\Delta_{\text{soln}}G^{\circ}$ and $\Delta_{\text{soln}}S^{\circ}$ denote the enthalpy, Gibbs energy and entropy of the solution, respectively. T_{hm} denotes the harmonic average of the study temperatures, and R denotes the universal constant of the gases. The intercept corresponds to the linear equation of the plot of ln x_3 vs ($T^{-1} - T_{\text{hm}}^{-1}$) [49, 50].

The contribution of the energy factors (solution enthalpy) and organizational aspects (entropy) to the Gibbs energy of the solution can be obtained using equations 4 and 5 [51, 52].

$$\zeta_H = |\Delta_{\text{soln}}H^{\circ}|(|\Delta_{\text{soln}}H^{\circ}| + |T\Delta_{\text{soln}}S^{\circ}|)^{-1} \quad (4)$$

$$\zeta_{TS} = |T\Delta_{\text{soln}}S^{\circ}|(|\Delta_{\text{soln}}H^{\circ}| + |T\Delta_{\text{soln}}S^{\circ}|)^{-1} \quad (5)$$

Thus, Tables 2 and 3, presents the thermodynamic analysis results of the MET dissolution processes in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} mixtures.

Table 2. Standard thermodynamic functions of the MET solution in {MeOH (1[a]) + W (2)} mixtures at T_{hm} = 307.3 K

x_1[a]	$\Delta_{\text{soln}}G^{\circ}$/ kJ·mol^{-1}	$\Delta_{\text{soln}}H^{\circ}$/ kJ·mol^{-1}	$\Delta_{\text{soln}}S^{\circ}$/ J·mol^{-1}·K^{-1}	$T\Delta_{\text{soln}}S^{\circ}$/ kJ·mol^{-1}	ζ_H	ζ_{TS}
0.00	16.54	28.45	38.74	11.91	0.705	0.295
0.10	15.58	34.24	60.71	18.66	0.647	0.353
0.20	14.35	38.46	78.46	24.11	0.615	0.385
0.30	13.33	36.14	74.21	22.81	0.613	0.387
0.40	12.48	35.96	76.43	23.49	0.605	0.395
0.50	11.86	35.34	76.39	23.47	0.601	0.399
0.60	11.55	34.65	75.17	23.10	0.600	0.400
0.70	11.35	33.69	72.68	22.33	0.601	0.399
0.80	11.30	32.04	67.49	20.74	0.607	0.393
0.90	11.37	32.22	67.84	20.85	0.607	0.393
1.00	11.45	30.96	63.49	19.51	0.613	0.387
Ideal	7.87	22.97	49.13	15.10	0.603	0.397

[a]x_1 is the molar fraction of methanol in the cosolvent mixture free of solute.

Table 3. Standard thermodynamic functions of the MET solution in {EtOH (1^b) + W (2)} mixtures at T_{hm} = 310.0 K

x_1[a]	$\Delta_{soln}G°$/ kJ·mol^{-1}	$\Delta_{soln}H°$/ kJ·mol^{-1}	$\Delta_{soln}S°$/ J·mol^{-1}·K^{-1}	$T\Delta_{soln}S°$/ kJ·mol^{-1}	ζ_H	ζ_{TS}
0.00	16.44	28.53	39.00	12.09	0.702	0.298
0.10	14.43	44.03	95.51	29.60	0.598	0.402
0.20	12.49	42.44	96.64	29.96	0.586	0.414
0.30	11.55	38.63	87.37	27.08	0.588	0.412
0.40	11.00	37.25	84.68	26.25	0.587	0.413
0.50	10.78	34.89	77.81	24.12	0.591	0.409
0.60	10.71	32.30	69.66	21.59	0.599	0.401
0.70	10.90	31.35	65.99	20.46	0.605	0.395
0.80	11.25	31.64	65.77	20.39	0.608	0.392
0.90	11.69	32.71	67.84	21.03	0.609	0.391
1.00	12.69	32.34	63.41	19.66	0.622	0.378
Ideal	7.57	23.34	50.90	15.78	0.597	0.403

[a]x_1 is the molar fraction of ethanol in the cosolvent mixture free of solute.

The Gibbs energy of solution is positive in all cases and decreases as the polarity of the system decreases from pure water to pure methanol in the {MeOH (1^a) + W (2)} mixtures; in the {EtOH (1^b) + W (2)} mixture it initially has the same behaviour as in the {MeOH (1^a)+W(2)} mixture, decreasing from pure water to the mixture x_1=0. 6, from this mixture the Gibbs energy of the solution shows an increase up to pure EtOH, because the solubility decreases, possibly due to the effects of the change in polarity of the medium, making it less favourable in terms of MET. In mixtures {MeOH (1^a) + W (2)} the standard enthalpy of solution increases from pure water to the mixture x_1 = 0.2 and then decreases from this mixture to pure MeOH, possibly due to an increase in solute-solvent molecular interactions caused by the addition of MeOH; the initial increase may be due to the destructuring of the water around the non-polar groups of MET. It is important to note that the standard enthalpy of a solution is positive in all cases, indicating an endothermic process, which inhibits the dissolution process. However, unlike the positive enthalpy values, the standard entropy of the solution also has positive values, favouring the dissolution process [53, 54]. This favourability is lower in pure water, possibly because water adopts a highly organised structure around hydrophobic molecules, which maximises the interactions between its own molecules (hydrophobic hydration) and promotes the formation of 'clathrate-like structures', reducing the entropy of the system [55].

In terms of the contribution of energetic and organisational factors, the enthalpy of the solution is the most important contributor to the Gibbs energy values of the solution in all cases.

In mixtures {EtOH (1^b) + W (2)} the standard enthalpy of solution increases from pure water to the mixture x_1 = 0.1, then decreases from this mixture to the mixture x_1= 0.7, then increases, behaving randomly. As with the mixtures {MeOH (1^a) + W (2)}, the enthalpy is positive, which favours the solution process, but with a strong entropic favoring.

Analysis of the Perlovich plot (Figure 3) shows that all values are in the first sector ($\Delta_{soln}H° > T\Delta_{soln}S° > 0$), indicating that the standard enthalpy of solution is a major contributor to the Gibbs energy of solution in the two cosolvent systems [56–59].

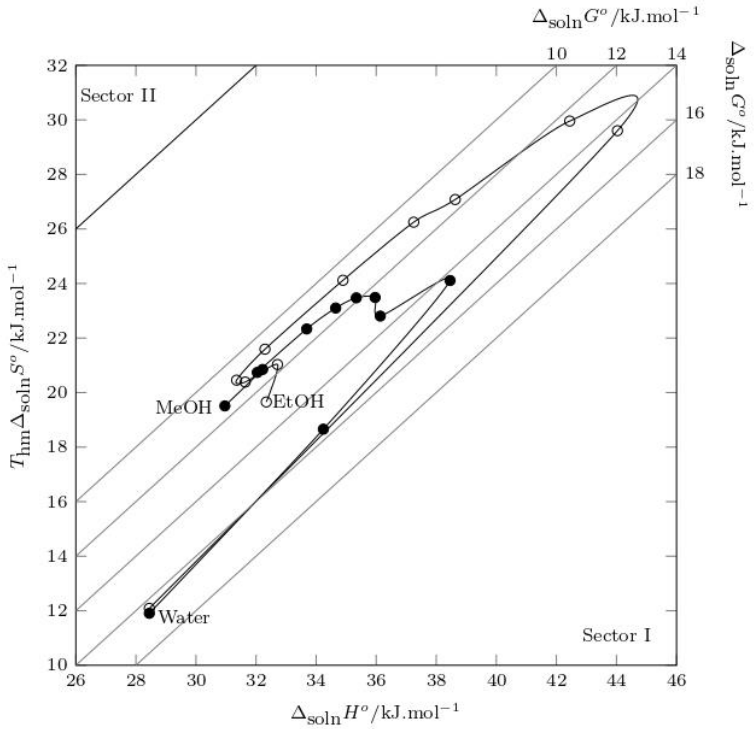

Figure 3. Relationship between the enthalpy and entropic terms of solutions functions of MET in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W(2)} cosolvent mixtures. The isoenergetic curves of $\Delta_{soln}G°$ function are marked by dotted lines.

3.2. Thermodynamic functions of mixing

In general, the solution process can be described by three sub-processes [60] (Figure 4):

- Drug fusion process: In a hypothetical process, the drug changes phase, transforming into a super-cooled liquid. Technically, this process requires energy supply, which is why it is unfavourable for the solution process.
- Cavity formation: Although the solvent does not present a phase change, the solvent molecules must disintegrate, forming a cavity to house the solute molecule; this process also requires energy investment (endothermic process) and is therefore unfavourable for the solution process.
- Mixing process: Once the drug is in a liquid state and the cavity has been formed in the solvent, the solute molecule is housed in the solvent cavity, forming the solution. This process is exothermic, which favours the solution process.

Mathematically, the solution process can be described as

$$\Delta_{soln}f^o = \Delta_{mix}f^o + \Delta_{fus}f^{Thm} \quad (6)$$

Thus, the thermodynamic mixing functions are calculated as follows:

$$\Delta_{mix}f^o = \Delta_{soln}f^o - \Delta_{fus}f^{Thm} \quad (7)$$

where f represents the Gibbs energy, enthalpy or entropy of mixing and f_{fus} represent the thermodynamic functions of the fusion of MET (3) and its cooling to the harmonic mean temperature, 307.3 K (MeOH) and 310.0 K (EtOH). As it has been described previously in the literature, in this research study, the $\Delta_{soln}f°$ values for the ideal solution processes were used instead of $\Delta_{fus}f^{Thm}$.

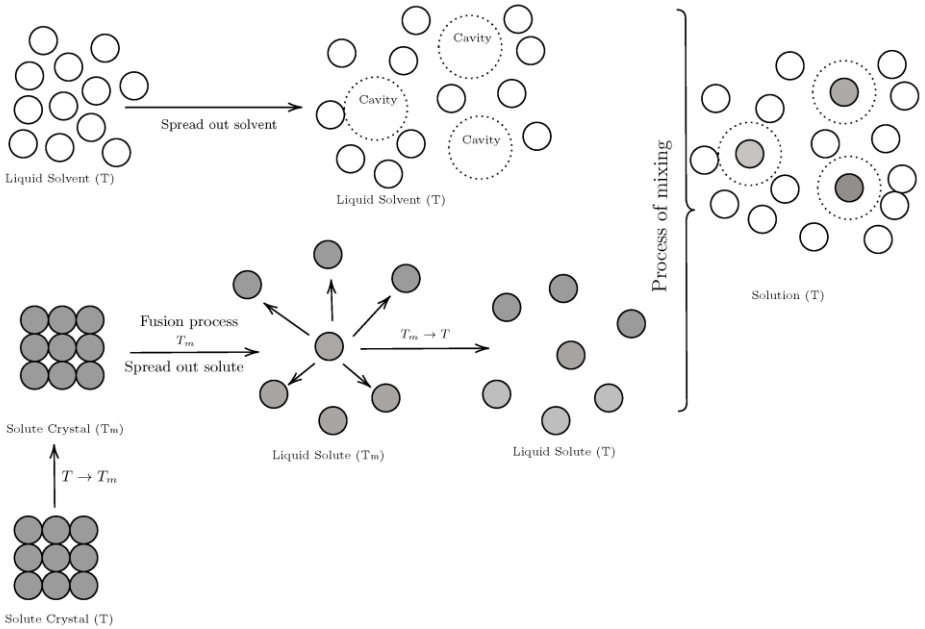

Figure 4. Diagram of hypothetical mixing process (solution formation) [25]

Table 4. Thermodynamic functions of mixing of MET (3) in {MeOH (1[a]) + W (2)} cosolvent mixtures at 307.3 K.

x_1 [a]	$\Delta_{mix}G°$/kJ·mol^{-1}	$\Delta_{mix}H°$/kJ·mol^{-1}	$\Delta_{mix}S°$/J·mol^{-1}·K^{-1}	$T\Delta_{mix}S°$/kJ·mol^{-1}
0.00	8.67	5.48	-10.39	-3.19
0.10	7.71	11.27	11.58	3.56
0.20	6.48	15.49	29.32	9.01
0.30	5.46	13.16	25.08	7.71
0.40	4.60	12.99	27.30	8.39
0.50	3.99	12.37	27.25	8.38
0.60	3.68	11.68	26.04	8.00
0.70	3.48	10.71	23.54	7.23
0.80	3.43	9.07	18.36	5.64
0.90	3.50	9.25	18.71	5.75
1.00	3.58	7.99	14.36	4.41

[a]x_1 is the mass fraction of MeOH (1[a]) in the {MeOH (1[a]) + W (2)} mixtures free of MET (3).

Table 5. Thermodynamic functions of mixing of MET (3) in {EtOH (1[b]) + W (2)} cosolvent mixtures at 310.0 K.

x_1 [a]	$\Delta_{mix}G°$/kJ·mol^{-1}	$\Delta_{mix}H°$/kJ·mol^{-1}	$\Delta_{mix}S°$/J·mol^{-1}·K^{-1}	$T\Delta_{mix}S°$/kJ·mol^{-1}
0.00	8.87	5.18	-11.90	-3.69
0.10	6.86	20.69	44.61	13.83
0.20	4.92	19.10	45.74	14.18
0.30	3.98	15.29	36.46	11.30
0.40	3.44	13.91	33.78	10.47
0.50	3.21	11.55	26.91	8.34
0.60	3.14	8.95	18.76	5.81
0.70	3.33	8.01	15.09	4.68
0.80	3.69	8.30	14.87	4.61
0.90	4.12	9.37	16.94	5.25
1.00	5.12	9.00	12.51	3.88

[a]x_1 is the mass fraction of EtOH (1[b]) in the {EtOH (1[a]) + W (2)} mixtures free of MET (3).

The thermodynamic mixing functions of MET in mixtures of cosolvents {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} at T_{hm} are given in Tables 4 and 5. The Gibbs energy is positive in all cases and decreases from pure water up to x_1 = 0.80 in mixtures {MeOH (1[a]) + W (2)} and x_1= 0.60 in mixtures {EtOH (1[b]) + W (2)}, then shows a slight increase up to pure alcohol (MeOH and EtOH). As for the enthalpy of the mixture, an increase is observed from pure water up to x_1 = 0.2 in mixtures {MeOH (1[a])+W(2)} and x_1 = 0.10 in mixtures {EtOH (1[b])+W(2)}; from this mixture up to x_1 = 0.80 in mixtures {MeOH (1[a]) + W (2)} and x_1 = 0.70 in mixtures {EtOH (1[b]) + W (2)}, the enthalpy decreases, indicating that the addition of MeOH or EtOH to the system favours the dissolution process by reducing the energy required to form a cavity between the solvent molecules. This is possibly due to the fact that water-water molecular interactions are much more energetic than W-MeOH, W-EtOH, MeOH-MeOH or EtOH interactions. Therefore, the formation of cavities in intermediate and alcohol-rich mixtures requires less energy. The entropy of the mixture is positive, except for pure water, due to the molecular interactions between the MET and water that cause the water to structure around the non-polar groups of this drug. Thus, starting from the cosolvent mixture x_1 = 0.1 to pure MeOH or pure EtOH, the solution process is favoured by the entropy of the mixture.

3.3. Enthalpy-entropy compensation

Changes in the free energy landscape upon tethering can be attributed to changes in entropy or enthalpy. Very often the changes in entropy and enthalpy are coupled. In many cases, a perturbation produced by a change in the solvent composition, leads to a change in the enthalpy of solution processes is correlated with a similar change in entropy in what is commonly referred to as "entropy–enthalpy compensation" [37, 46, 61, 62]. Entropy–enthalpy compensation is reported for many chemical processes and is often accounted for as a general thermodynamic principle. In this case, analysis has been used to identify the mechanism of the cosolvent action [63]. Figure 5 shows that MET in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} cosolvent mixtures at 307.3 K for {MeOH (1[a]) + W (2)} and 310.0 K for {EtOH (1[b]) + W (2)} presents a non-linear $\Delta_{soln}H°$ vs. $\Delta_{soln}G°$ curves. In cosolvent mixtures {MeOH (1[a]) + W (2)}, a positive slope is present in the interval from x_1= 0.70 to x_1=0.20, indicating that the driving mechanism of solubility is enthalpic; in the intervals from MeOH to x_1=0.70 and from x_1=0. 20 to pure water the trend has a negative slope, indicating entropic driving in these intervals; as for mixtures {EtOH (1[b]) + W (2)}, there are two continuous regions with enthalpic driving between pure EtOH and x_1=0.10, from this cosolvent composition to pure water the solution process is driven by entropy.

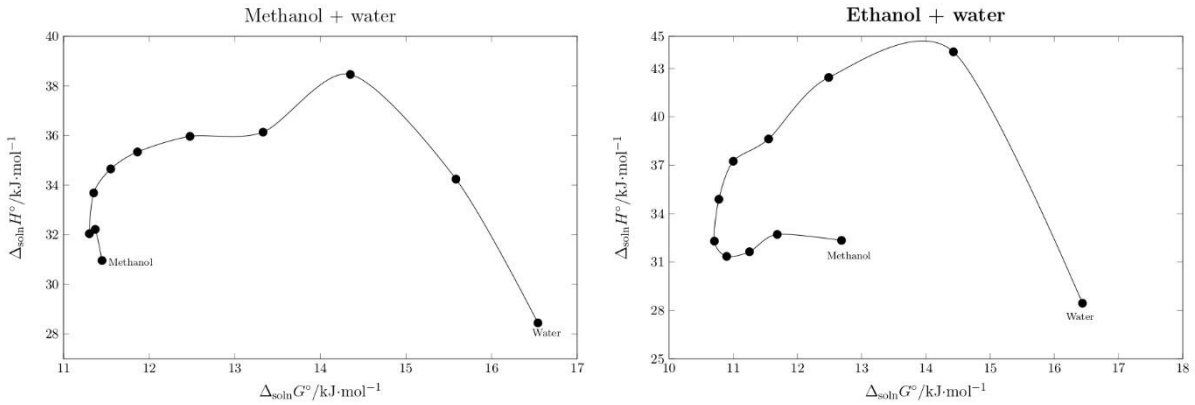

Figure 5. $\Delta_{soln}H°$ vs. $\Delta_{soln}G°$ enthalpy-entropy compensation plot for solubility of MET in {MeOH (1[a]) + W (2)} and {EtOH (1[b]) + W (2)} cosolvent mixtures.

3.4. Preferential solvation

Although the thermodynamic analyses provide information of considerable importance for understanding the dissolution process of the drug, they do not provide information about the actual molecular environment of the drug in cosolvent mixtures [64–66]. The inverse Kirkwood-Buff integral (IKBI) approach identifies the local composition around the MET molecules for each of the solvents making up the mixture (W + MeOH and W + EtOH) based on the solubility data and the excess Gibbs energy of the free cosolvent mixtures of MET. This can be attributed to the preferential solvation of MET in the {MeOH (1) + W (2)} and {EtOH (1) + W (2)} cosolvent mixture, which depends both on the molecular interactions of MET with W, MeOH and EtOH, and on the interactions between the solvents described by the excess Gibbs energy of the mixture (in the absence of MET), taking into account the competitive molecular interactions of the three components (MET, W, MeOH or EtOH) in the solution process.

The results of this study are expressed in terms of the preferential solvation parameters $\delta x_{1,3}$ for MET (3) by the components of the W (2), MeOH (1^a) and EtOH (1^b) mixture as [67,68]:

$$\delta x_{1,3} = x_{1,3}^{L} - x_1 = -\delta x_{2,3}, \tag{8}$$

where x_1 is the mole fraction of 1 in the original solute-free mixture and $x_{1,3}^{L}$ is the local mole fraction of 1 in the MET molecule environment. If $\delta x_{1,3} > 0$, MET will be surrounded in considerable proportion by MeOH or EtOH (solvated preferentially by the MeOH or EtOH) when compared with the proportion of MeOH or EtOH in the original mixture.

The mathematical expressions for the application of the IKBI model proposed by Professor Ben-Naim restructured by Professor Marcus are described as follows [69,70]:

$$\delta x_{1,3} = x_1 x_2 \left(G_{1,3} - G_{2,3}\right)\left(x_1 G_{1,3} + x_2 G_{2,3} + V_{cor}\right)^{-1} \tag{9}$$

$$G_{1,3} = RT\kappa_T - V_3 + x_2 V_2 D Q^{-1} \tag{10}$$

$$G_{2,3} = RT\kappa_T - V_3 + x_1 V_1 D Q^{-1} \tag{11}$$

$$V_{cor} = 2522.5\left[r_3 + 0.1363\sqrt[3]{x_1^L V_1 + (1 - x_1^L)V_2} - 0.085\right]^3 \tag{12}$$

$$D = \left(d\Delta_t G_{3,2\rightarrow 1+2}^{o}/dx_1\right)_{T,P} \tag{13}$$

$$Q = RT + x_1 x_2 \left(d^2 G_{1,2}^{E}/dx_2^2\right)_{T,P}, \tag{14}$$

where $G_{1,3}$ and $G_{2,3}$ denote the Kirkwood–Buff integrals ($cm^3\ mol^{-1}$), which are obtained from the thermodynamic data according to equations 10 and 11, and V_{cor} denotes the volume of correlation around MET within which preferential solvation occurs. κ_T is the isothermal compressibility of the mixtures (in GPa^{-1}) [71]. V_3 is the partial molar volume of the solute, and V_1 and V_2 are the volumes of the solvents ($cm^3\ mol^{-1}$). $G_{3,2\rightarrow 1+2}^{o}$ is the Gibbs energy of solute transfer from water to each cosolvent mixture, and $G_{1,2}^{E}$ is the excess Gibbs energy of the cosolvent MET-free {MeOH (1^a) + W (2)} and {EtOH (1^b) + W (2)} cosolvent mixtures. All the thermodynamic values required at 298.15 K for IKBI calculations in these mixtures were taken from the literature [72].

In Figure 6 and Table 6 the behaviour of $\delta x_{1,3}$ and other properties of preferential solvation in the function of the MeCN molar fraction at 298.15 K is denoted. The tendency of the solvation parameter is similar to that in other drugs, where the maximum solubility is reached in a cosolvent mixture and not in a pure solvent.

Figure 6 shows the Gibbs energy of transfer behaviour of MET from neat water to {MeOH (1^a) + W (2)} cosolvent mixtures at 307.3 K and from neat water to {EtOH (1^b) + W (2)} cosolvent mixtures at 310.0 K.

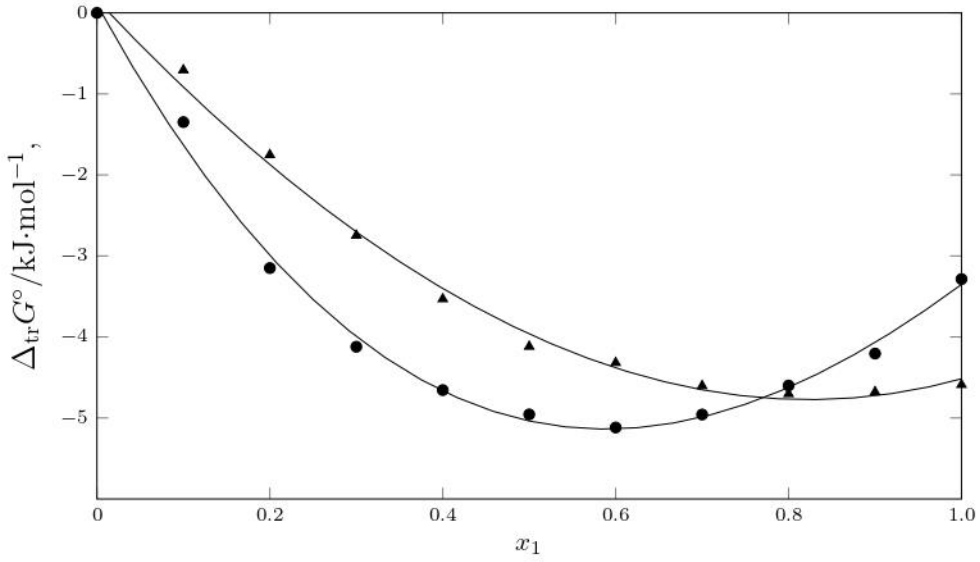

Figure 6. Gibbs energy of transfer of MET from neat water to {MeOH (1^a) + W (2)} cosolvent mixtures (▲) and from neat water to {ETOH (1^b) + W (2)} cosolvent mixtures (●).

The equations that represent the trend correspond to a second and third order polynomial (Equation 15 and 16).

$$\Delta_{tr}G^0_{3,2\rightarrow 1^a+2} = RT\ln\left(\frac{x_{3,2}}{x_{3,1^a+2}}\right) = 7.13x_{1a}^2 - 11.88x_{1a} + 0.19 \quad (15)$$

$$\Delta_{tr}G^0_{3,2\rightarrow 1^b+2} = RT\ln\left(\frac{x_{3,2}}{x_{3,1^b+2}}\right) = -4.63x_{1b}^3 + 20.60x_{1b}^2 - 19.42x_{1b} + 0.10 \quad (16)$$

According to equations 8 and 9, *D* is calculated as the derivative of Gibbs energy of transfer as a function of x_1 (Equation 17, 18).

$$D = \left(\frac{\partial \Delta_{tr}G^0_{(3,2\rightarrow 1a+2)}}{\partial x_{1a}}\right)_{T,p} = 14.26x_{1a} - 11.88 \quad (17)$$

$$D = \left(\frac{\partial \Delta_{tr}G^0_{(3,2\rightarrow 1b+2)}}{\partial x_{1b}}\right)_{T,p} = -13.89x_{1b}^2 + 41.2x_{1a} - 19.42 \quad (18)$$

In Figure 6 and Tables 6 and 7 the behaviour of $\delta x_{1,3}$ and other properties of preferential solvation in the function of the MeOH or EtOH molar fraction at 298.15 K is denoted. The tendency of the solvation parameter is like that in other drugs, where the maximum solubility is reached in a cosolvent mixture and not in a pure solvent.

Table 6. Some properties associated to preferential solvation of MET (3) in {MeOH (1[a]) + W (2)} mixtures at T_{hm} = 298.15 K.

x_1[a]	D/kJ.mol^{-1}	$G_{1,3}$/cm^3.mol^{-1}	$G_{2,3}$/cm^3.mol^{-1}	V_{cor}/cm^3.mol^{-1}	$100\delta x_{1,3}$
0.00	-11.89	-214.30	-127.67	672.61	0.00
0.10	-10.46	-196.38	-143.33	708.97	-0.85
0.20	-9.03	-184.40	-157.63	748.09	-0.73
0.30	-7.60	-173.87	-170.94	790.01	-0.10
0.40	-6.17	-162.58	-180.52	833.54	0.65
0.50	-4.74	-150.30	-181.68	876.63	1.10
0.60	-3.32	-139.02	-171.89	917.92	1.03
0.70	-1.89	-131.02	-154.00	958.08	0.59
0.80	-0.46	-126.75	-132.97	999.01	0.11
0.90	0.97	-125.30	-111.04	1041.91	-0.14
1.00	2.40	-125.71	-86.31	1085.19	0.00

[a] x_1 is the mole fraction of MeOH (1) in the {MeOH (1[a]) + W (2)} mixtures free of MET (3).

Table 7. Some properties associated to preferential solvation of MET (3) in {EtOH(1[b]) + W (2)} mixtures at T_{hm} = 298.15 K.

x_1[a]	D/kJ.mol^{-1}	$G_{1,3}$/cm^3.mol^{-1}	$G_{2,3}$/cm^3.mol^{-1}	V_{cor}/cm^3.mol^{-1}	$100\delta x_{1,3}$
0.00	-19.42	-269.27	-127.67	672.73	0.00
0.10	-15.44	-250.13	-168.68	734.90	-1.31
0.20	-11.74	-219.36	-199.40	811.84	-0.52
0.30	-8.31	-188.36	-212.38	891.74	0.73
0.40	-5.17	-162.48	-206.72	966.77	1.37
0.50	-2.30	-141.79	-179.11	1034.84	1.07
0.60	0.29	-124.80	-116.74	1095.71	-0.20
0.70	2.61	-112.35	-4.36	1151.58	-2.12
0.80	4.64	-110.64	110.98	1213.43	-3.09
0.90	6.40	-118.98	121.02	1289.80	-1.81
1.00	7.88	-125.94	60.59	1368.34	0.00

[a] x_1 is the mole fraction of EtOH (1) in the {EtOH (1[b]) + W (2)} mixtures free of MET (3).

The $\delta x_{1,3}$ values vary non-linearly in both cosolvent mixtures (Figure 7). With respect to mixture {MeOH (1[a]) + W (2)}, the addition of MeOH (1) to W (2) tends to make the $\delta x_{1,3}$ values of this drug negative from pure water (2) to the mixture of 0.30 in mole fraction of MeOH (1), reaching minimum values close to -0.010 in the mixture with 0.1 in mole fraction of methanol (1). Possibly the structuring of the water molecules around the non-polar groups of MET by hydrophobic hydration contributes to the reduction of the net $\delta x_{1,3}$ to negative values in these water-rich mixtures.

In mixtures with a composition of $0.30 < x_1 < 0.9$, the local mole fractions of methanol (1[a]) are higher than those of water (2). Thus, the effect of the cosolvent may be related to the disruption of the ordered structure of water by hydrogen bonding around the nonpolar moieties of the drug, as noted above. The highest preferential solvation by the co-solvent reaches a maximum value at x_1 = 0.50 ($\delta x_{1,3}$ close to 0.011 at 293.15 K).

MET in cosolvent mixtures {EtOH (1[b]) + W (2)} shows analogous behaviour, solvating in water- and ethanol-rich mixtures.

According to the preferential solvation results, it is assumed that in intermediate compositions MET (3) acts as a Lewis acid with methanol (1) and ethanol molecules, because these cosolvents are more basic than water, i.e. the Kamlet-Taft hydrogen bond acceptor parameters for MeOH and EtOH are $\beta = 0.62$ and $\beta = 0.77$, respectively, and 0.47 for water [73]. However, the specific solute-solvent interactions remain unclear despite the developed treatments.

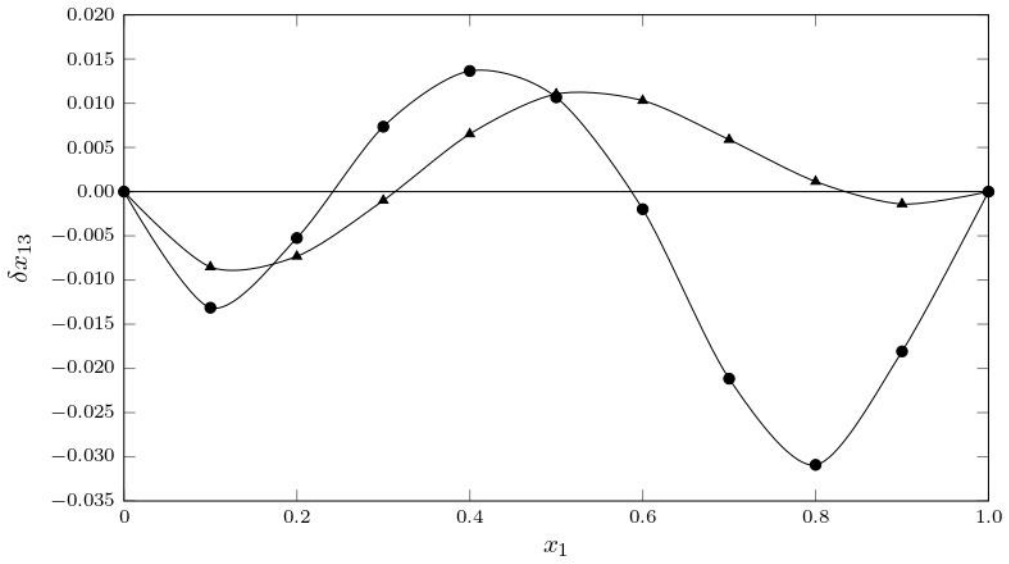

Figure 7. $\delta x_{1,3}$ values for MET (3) in {MeOH (1[a]) + W (2)}(▲) and {EtOH (1[b]) + W (2)}(●) co-solvent mixtures at 298.15 K.

4. CONCLUSIONS

The findings indicate that the dissolution process of metronidazole is endothermic and favoured by entropy. The addition of both methanol and ethanol demonstrated a positive cosolvent effect, particularly in intermediate and water-rich mixtures, enhancing the solubility of metronidazole. Regarding preferential solvation, the results were not entirely conclusive. While some preferential solvation by the cosolvents (methanol or ethanol) was observed, especially in intermediate mixtures where the local mole fractions of the alcohol were higher than water, the values of the preferential solvation parameter.

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CONFLICT OF INTEREST

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

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Artigo de pesquisa cientifica

Investigação de Enterobacterales resistentes ao meropenem e produtoras de carbapenemases em uma estação de tratamento de esgoto de Minas Gerais, Brasil

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RESUMO

Introdução: Bactérias gram-negativas, apesar de ubíquas na natureza, podem causar infecções, desenvolver e disseminar mecanismos de resistência aos antimicrobianos. Destaca-se a resistência antimicrobiana (RAM) a fármacos de relevância clínica como meropenem, para o qual o estudo da resistência em ambientes aquáticos é pouco explorado. **Objetivo:** Foi investigada a presença de bactérias gram-negativas resistentes ao meropenem (BRM) em uma estação de tratamento de esgoto (ETE) de Minas Gerais, Brasil. Dois litros de esgoto bruto (EB) e de efluente (EF) foram coletados. 100μL de cada amostra foram submetidos à cultura sem e com meropenem (8 μg/mL). Posteriormente, foi realizada diluição seriada e 100 μL de cada diluição inoculados em ágares para a recuperação de Gram-negativas. Os morfotipos foram identificadas utilizando ágar cromogênico e submetidos ao teste de inativação de carbapenêmico na ausência e presença de EDTA (EUCAST, 2017; CLSI, 2022) para a investigação da produção de carbapenemases. **Resultados:** Um total de 34 morfotipos bacterianos, todos pertenceram à ordem *Enterobacteriales* (26 *Escherichia coli* e quatro *Enterobacter sp*. em EB e quatro *E. coli* em EF) foram recuperados. Todas as BRM foram produtoras de carbapenemase, a maioria (88,6%) do tipo serinocarbapenemase, enquanto produtora de metalobetalactamase foi exclusivamente encontrada em EB. **Conclusão:** Apesar da redução dos morfotipos em EF, sua descarga em cursos d'água deve ser considerada um importante meio de veiculação de BRM no ambiente. Nossos dados apontam para a necessidade de mais estudos afim de conhecer e estabelecer estratégias de contenção de disseminação de RAM, incluindo a implementação de mais ETEs.

Palavras-chave: Bactérias gram-negativas; Meropenem; Estação de tratamento de esgoto; Farmacorresistência bacteriana; Ambiente aquático.

SUMMARY

Investigation of meropenem-resistant and carbapenemase-producing Enterobacterales in a sewage treatment plant in Minas Gerais, Brazil

Introduction: Gram-negative bacteria, despite being ubiquitous in nature, can cause infections and develop and disseminate mechanisms of resistance to antimicrobials. Antimicrobial resistance (AMR) to drugs of clinical relevance such as meropenem stands out, for which the study of resistance in aquatic environments is little explored. **Objective:** The presence of gram-negative bacteria resistant to meropenem (BRM) was investigated in a sewage treatment plant (WWTP) in Minas Gerais, Brazil. Two liters of raw sewage (RS) and effluent (EF) were collected. 100μL of each sample was subjected to culture without and with meropenem (8 μg/mL). Subsequently, serial dilution was performed and 100 μL of each dilution was inoculated into agars for the recovery of Gram-negatives. The morphotypes were identified using chromogenic agar and subjected to the carbapenem inactivation test in the absence and presence of EDTA (EUCAST, 2017; CLSI, 2022) to investigate the production of carbapenemases. **Results:** A total of 34 bacterial morphotypes, all belonging to the order Enterobacteriales (26 *Escherichia coli* and four *Enterobacter* sp. in RS and four *E. coli* in EF) were recovered. All BRM were carbapenemase producers, the majority (88.6%) of the serinecarbapenemase type, while metallobetalactamase producers were exclusively found in RS. **Conclusion:** Despite the reduction of morphotypes in RS, its discharge into waterways must be considered an important means of transporting BRM in the environment. Our data point to the need for more studies to understand and establish strategies to contain the spread of AMR, including the implementation of more WWTPs.

Keywords: Gram-negative bacteria; Meropenem; Sewage treatment station; Bacterial pharmacoresistance; Aquatic environment.

RESUMEN

Investigación de Enterobacteriales resistentes a meropenem y productores de carbapenemasas en una planta de tratamiento de aguas residuales en Minas Gerais, Brasil

Introducción: Las bacterias gramnegativas, a pesar de ser ubicuas en la naturaleza, pueden causar infecciones y desarrollar y difundir mecanismos de resistencia a los antimicrobianos. Destaca la resistencia antimicrobiana (RAM) a fármacos de relevancia clínica como meropenem, por lo que el estudio de resistencia en ambientes acuáticos está poco explorado. **Objetivo:** Se investigó la presencia de bacterias gramnegativas resistentes a meropenem (BRM) en una planta de tratamiento de aguas residuales (PTAR) en Minas Gerais, Brasil. Se recogieron dos litros de aguas residuales (AR) y efluentes (EF). Se sometieron a cultivo 100 μL de cada muestra con y sin meropenem (8 μg/mL). Posteriormente se realizaron diluciones seriadas y se inocularon 100 μL de cada dilución en agares para la recuperación de Gram negativos. Los morfotipos se identificaron utilizando agar cromogénico y se sometieron a la prueba de inactivación de carbapenems en ausencia y presencia de EDTA (EUCAST, 2017; CLSI, 2022) para investigar la producción de carbapenemasas. **Resultados:** Se recuperaron un total de 34 morfotipos bacterianos, todos pertenecientes al orden Enterobacteriales (26 *Escherichia coli* y cuatro *Enterobacter* sp. en AR y cuatro *E. coli* en EF). Todos los BRM fueron productores de carbapenemasas, la mayoría (88,6%) del tipo serinacarbapenemasas, mientras que los productores de metalobetalactamasas se encontraron exclusivamente en AR. **Conclusión:** A pesar de la reducción de morfotipos en AR, su descarga a vías fluviales debe considerarse un medio importante de transporte de BRM en el medio ambiente. Nuestros datos apuntan a la necesidad de realizar más estudios para comprender y establecer estrategias para contener la propagación de la resistencia a los antimicrobianos, incluida la implementación de más PTAR.

Palabras clave: Bacterias Gram negativas; Meropenem; Planta de tratamiento de aguas residuales; Farmacorresistencia bacteriana; Ambiente acuático.

1. INTRODUÇÃO

Resistência antimicrobiana (RAM), apesar de ser um evento evolutivo, é um grande desafio à humanidade, com impacto direto na sobrevida de pacientes com infecções por bactérias resistentes, para os quais as opções terapêuticas são reduzidas [1, 2].

Antimicrobianos são usados na profilaxia e terapêutica de infecções em seres humanos e animais, além disso, como promotores de crescimento na criação de animais para o consumo humano. Ademais, seus resíduos são considerados micropoluentes e têm sido encontrados em diversos ambientes [3]. Por exemplo, no ambiente aquático, chegam por diversas rotas, tais como esgotos e efluentes das estações de tratamento de esgotos (ETEs), interferindo na ecologia microbiana, uma vez que exercem pressão seletiva e assim favorecem a RAM e as variabilidades genética e fenotípica [4].

A água é um recurso indispensável para a vida e grandes esforços têm sido feitos para mantê-la disponível no planeta. Assim, as ETEs estão cada vez mais sendo implementadas visando a reutilização da água. O processo de tratamento do esgoto instituído em uma ETE geralmente acontece em estágios, porém nenhum deles contempla a remoção de resíduos de antimicrobianos [5]. Dessa forma, a população bacteriana das ETEs, altamente complexa, considerando os micropoluentes aos quais está exposta, frequentemente apresenta fenótipo de resistência a antimicrobianos [6].

Destacam-se neste contexto bactérias gram-negativas da ordem Enterobacteriales, sobretudo *Escherichia coli, Proteus* spp., *Enterobacter* spp., *Pantoea* spp. e *Morganella morganii* que fazem parte da microbiota do trato intestinal de humanos e animais [7]. Estas bactérias são também agentes causadores de infecções relacionadas à assistência à saúde (IRAs) e da comunidade, incluindo septicemias, infecções entéricas, infecções urinárias e infecções cutâneas [8].

No tratamento de infecções por Enterobacteriales estão disponíveis várias classes de antimicrobianos e a escolha clínica é baseada no local de instalação da infecção e do perfil de susceptibilidade bacteriana, podendo ser destacado o uso de betalactâmicos, quinolonas e aminoglicosídeos, porém a ampla utilização desses antimicrobianos tem resultado em aumento da RAM [9].

Para betalactâmicos, uma classe que inclui o maior número de compostos (penicilinas, cefalosporinas, carbapenêmicos) e que agem inibindo a síntese da parede celular bacteriana, vários mecanismos de resistência estão descritos, envolvendo modificações estruturais das proteínas ligadoras de penicilina (PLP), alterações nos canais de porina e produção de enzimas betalactamases codificadas pelo gene *bla*, capazes de hidrolisar o anel betalactâmico inativando o antimicrobiano [10].

Os carbapenêmicos (meropenem, imipenem, ertapenem e doripenem) são considerados de última geração e permanecem estáveis diante dos mecanismos enzimáticos primários de resistência aos betalactâmicos [11]. Contudo, devido a sua maior utilização em função do aumento de bactérias multirresistentes (MDR), bactérias resistentes aos carbapenêmicos (BRC) têm sido frequentemente relatadas no cenário clínico imprimindo grande desafio no manejo dessas infecções [12].

Embora exista grande variedade de mecanismos de resistência aos antimicrobianos betalactâmicos, especificamente para os carbapenêmicos, a produção de carbapenemasescujo os genes apresentam alto potencial de disseminação via transferência horizontal, são de maior preocupação [13]. Essas enzimas codificadas podem hidrolisar também cefalosporinas, peni-

cilinas e monobactam [14] e são distribuídas entre as classes A, C e D de Ambler (serino-carbapenemases) que apresentam um grupo serina em seu sítio ativo e podem ser inativadas por inibidores de betalactamases e B (metalobetalactamases - MBL) que possuem íon zinco no seu sítio ativo e são inibidas por compostos quelantes como o ácido etilenodiamino tetra-acético (EDTA) [15, 16].

Destacam-se, devido a maior disseminação entre as BRC, as serinocarbapenemases do tipo KPC (*Klebsiella pneumoniae Carbapenemase*), IMI (*imipenem hydrolyzing carbapenemase*) e SME (*Serratia marcescens enzyme*) e as MBL SPM (São Paulo metalobetalactamase), GIM (*German mipenemase*), VIM (Verona imipenemase), IMP (Imipenemase) e NDM (*New Delhi metalobetalactamases*). Ainda, na classe D estão incluídas as oxacilinases que hidrolisam as oxacilinas, oximinocefalosporinas e carbapenêmicos e abrangem as carbapenemases do subtipo OXA [17-19].

Ao contrário do relatado na clínica, a presença BRC ainda é escassa quando se trata do cenário ambiental. Entretanto, alguns estudos mostram que BRC, mais frequentemente *E. coli*, estão distribuídas em águas de esgoto doméstico, hospitalar e rios no Japão [20] e Índia [21]. No Brasil, de acordo com Abrantes [22], Enterobacteriales com esse fenótipo foram detectadas em amostras de ETE do Rio de Janeiro, o que aponta para um cenário crítico, que pode inviabilizar o uso dos carbapenêmicos. Deve ser destacada a possibilidade de intercâmbio dessas bactérias entre os diversos ambientes de acordo com a perspectiva *One Health* e também que ambientes como o aquático são considerados reservatórios de RAM, o que pode ser um risco iminente à saúde pública [23, 24].

Diante do exposto, este trabalho teve como objetivo investigar a presença de bactérias gram-negativas resistentes ao meropenem (BRM), um importante carbapenêmico amplamente utilizado na clínica, em amostras de águas de uma ETE bem como investigar a produção de mecanismos de resistência enzimático aos carbapenêmicos circulando entre essas bactérias. Nossos dados poderão contribuir para aumentar o conhecimento sobre a presença de BRC em ambientes aquáticos e alertar para a necessidade de monitoramento da sua disseminação.

2. MATERIAL E MÉTODOS

2.1. Relação de reagentes e materiais utilizados nos experimentos

Brain Heart infusion (Difco, USA); Meropenem pó, pureza ≥98% (HPLC) (Sigma-Aldrich, Alemanha); Ágar MacConkey (Isofar, Brasil); Ágar Cetrimide (TM Media, India); Ágar Cromogênico (Probac, Brasil); Glicose P.A. (Synth, Brasil); Lactose P.A. (Synth, Brasil); Sacarose P.A. (Synth, Brasil); Ágar SIM (Kasvi, Brasil); Caldo lisina (Synth, Brasil); Ágar citrato Simmons (Kasvi, Brasil); Caldo malonato (HIMEDIA, India); Caldo urease (Synth, Brasil); Enzima citocromo oxidase (Laborclin, Brasil); Glicerol, pureza ≥99.5% (Sigma-Aldrich, Alemanha); EDTA (Synth, Brasil); Meropenem disco10 mcg (SensibioDisc – CECON, Brasil); Ágar Muller Hinton (Isofar, Brasil).

2.2. Amostras de água e recuperação de BRM

Foram coletados dois litros de água (um do esgoto bruto - EB e um do efluente - EF) da Estação de Tratamento de Esgotos Várzea das Flores, na cidade de Carmópolis de Minas - MG (-20º53’66.40” S -44º62’72.97” W) no dia 27 de março de 2023.

As amostras foram coletadas em frascos de polipropileno com tampa de rosca previamente esterilizados, armazenados em gelo até a análise no Laboratório de Diagnóstico Laboratorial e Microbiologia Clínica da Universidade Federal de São João del-Rei, Campus Centro-Oeste Dona Lindu, Divinópolis-MG.

Para a verificação do possível crescimento de BRM foi realizada cultura enriquecida, de acordo com Paiva e colaboradores [25], com modificações. Um volume de 100μL de cada amostra foi inoculada em frascos contendo: i) apenas 100 mL de caldo *Brain Heart infusion* (BHI, Difco) para controle de viabilidade e crescimento bacteriano e ii) 100 mL de caldo BHI acrescido de meropenem (MEM, Sigma-Aldrich) na concentração final de 8 μg/mL, que foi determinada de acordo com os *breakpoints* do *Brazilian Committee on Antimicrobial Susceptibility Testing* [26], o qual classifica como "resistente" para Enterobacterales e *Pseudomonas aeruginosa* crescendo a partir desta concentração. Todos os frascos foram incubados a 35±2 °C por 24 horas.

2.3. Isolamento e identificação das colônias de BRM

Após o período de incubação, os frascos foram observados para a inspeção macroscópica da turbidez, foram realizadas diluições seriadas (10^{-1} a 10^{-7}) dos frascos de cultura e 100 μL de cada diluição foram transferidos para placas de ágar MacConkey (Isofar, Brasil) e ágar Cetrimide (TM Media, Indian), distribuídos na superfície do ágar utilizando a técnica de *spread plate*, em duplicata.

As placas foram incubadas a 35±2 °C por até 48 horas. Na pesquisa de espécies de Enterobacterales, após o período de incubação, as placas de ágar MacConkey foram analisadas observando o crescimento de colônias fermentadoras ou não de lactose. Todas as colônias foram submetidas à coloração de Gram para confirmação da morfologia e foram identificadas utilizando ágar Cromogênico (Probac, Brasil) de acordo com a orientação do fabricante. Além disso, algumas colônias foram submetidas a provas de identificação bioquímicas-fisiológicas clássicas, incluindo provas fermentação de carboidratos (glicose, lactose e sacarose), da produção de pigmentos, da motilidade, da descarboxilação de lisina, da produção de indol, da produção de H_2S, da utilização de citrato e malonato como fonte de carbono e da produção uréase [27].

O ágar cetrimide, o qual é específico para a recuperação de *P.* aeruginosa, foi analisado observando a presença de colônias grandes, apresentando pigmento verde difundido no meio de cultura [28] e para a confirmação da identificação da espécie foram realizados os testes da produção da enzima citocromo oxidase e capacidade de crescimento em temperatura de 42 °C [29].

Posteriormente, todas as BRM foram inoculadas em ágar MacConckey para verificação da pureza da cultura por avaliação da morfologia das colônias. Desta cultura, três a cinco colônias foram suspensas em caldo BHI e incubadas à temperatura de 35±2 °C por 18-24 horas. Após este período de incubação, 500 μL da cultura foram acrescidos de glicerol 15% v/v e estocado a –20 °C e as BRM foram catalogadas e incorporadas à bacterioteca do Laboratório de Microscopia, Diagnóstico Laboratorial e Microbiologia Clínica da Universidade Federal de São João del-Rei, *Campus* Centro-Oeste Dona Lindu da UFSJ para realização dos experimentos.

2.4. Investigação da produção de carbapenemases pelos isolados recuperados do EB e EF

Todas as BRM foram submetidas ao teste de inativação de carbapenêmico na ausência (mCIM) e presença (eCIM) de EDTA [30, 31] (EUCAST, 2017; CLSI, 2022) para a investigação da produção de carbapenemases. Para a realização deste teste, três a cinco colônias de cada isolado

crescido a partir de ágar nutriente foram inoculadas em 2 mL de caldo BHI (Isofar, Brasil). Simultaneamente, um disco de meropenem 10 mcg (SensibioDisc – CECON) foi colocado em cada tubo de ensaio e incubados a 35±2 °C por 4 horas.

Após a incubação, os discos de meropenem foram removidos dos tubos e colocados em uma placa de ágar Muller Hinton (Isofar, Brasil) previamente inoculada com a cepa indicadora *E. coli* ATCC® 25922, cujo padrão de turvação da suspensão foi compatível com a escala 0,5 McFarland. As placas foram incubadas a 35±2 °C e a leitura e interpretação foram realizadas segundo as orientações do EUCAST [30] e CLSI [31]. De acordo com esses protocolos, a observação de um halo de inibição específico (tamanho em mm interpretado de acordo com os protocolos citados) para o meropenem é indicativo de que o isolado produz uma carbapenemase.

Simultaneamente ao teste mCIM, foi realizado o teste eCIM o qual apresenta como modificação a utilização de um tubo de caldo BHI de cada BRM contendo 20 µL de EDTA 0,5 M (pH 7,8 – 8,0). De acordo com o CLSI [31], este procedimento permite a diferenciação das carbapenemases metalobetalactamase e serinocarbapenemase, considerando que as primeiras têm sua atividade dependente do zinco, que no teste será quelado pelo EDTA. Nesta pesquisa, quando há um aumento de ≥ 5mm no halo de inibição do eCIM em relação ao mCIM é considerado a detecção positiva de metalobetactamase no isolado, enquanto aumento ≤ 4 mm correlaciona com produção de serinocarbapenemase.

2.5. Análise estatística

Foi utilizado o programa estatístico SigmaXL. O teste não paramétrico de Fischer foi utilizado para comparações dos dados referentes ao número de morfotipos recuperados em EB e EF. Os valores comparados foram considerados estatisticamente significativos quando os valores de *p* foram menores que 0,05.

3. RESULTADOS E DISCUSSÃO

É conhecido que grandes quantidades de antimicrobianos e seus resíduos são liberadas nas águas residuais em função do metabolismo incompleto em humanos e animais, descarte inadequado de fármacos e como resíduo de indústrias farmacêuticas [32]. Além de favorecer a seleção de bactérias resistentes, o lançamento de antimicrobianos nos recursos hídricos receptores pode impactar a microbiota, a flora e a fauna residente. Dessa forma, há uma necessidade de gerenciamento desses micropoluentes recalcitrantes para diminuir o impacto ecológico [33, 34].

As amostras de água incluídas neste estudo são provenientes de uma ETE que utiliza para o tratamento um reator anaeróbio (UASB) e uma lagoa facultativa, onde o esgoto é estabilizado por processos aeróbios e anaeróbios por meio de bactérias dispersas no líquido.

A partir da cultura enriquecida com meropenem, foi observado um crescimento abundante de bactérias gram-negativas no ágar MacConkey tanto do EB quanto do EF, porém, em baixa diversidade, com colônias fermentadoras e não fermentadoras de lactose. Considerando o uso do ágar cetrimide, específico para recuperação de *P. aeruginosa*, nenhum crescimento foi observado o que é corroborado com estudos realizados em águas residuais no Brasil [35-37]. Esse é um fato curioso uma vez que essa espécie tem a habilidade de persistir por longos períodos em ambientes adversos [38], possivelmente a disponibilidade reduzida de oxigênio pode ter alterado seu metabolismo inviabilizando sua recuperação *in vitro* [39].

Assim, a partir do ágar MacConkey, um total de 34 morfotipos de BRM (30 em EB e quatro em EF) foi obtido, mostrando uma redução de 76,5% dos morfotipos bacterianos no EF da ETE.

Porém, essa diferença não é significativa (p=1). A redução bacteriana observada pode estar relacionada com a implementação do tratamento misto do esgoto (UASB e lagoa algal aeróbia) que, de acordo com Massé [40], promove a oxidação carbonácea, a nitrificação, a desnitrificação e a remoção biológica do fósforo, este último junto com o nitrogênio e outros nutrientes importantes fontes de energia para as bactérias heterotróficas, tais como a maioria dos microrganismos do trato intestinal humano [41, 42]. Dessa forma, é aumentada a eficiência global do tratamento do esgoto com maior depuração bacteriana e melhoria da qualidade do efluente no que se refere a possibilidade de veiculação de potenciais patógenos [43-45].

Todos os morfotipos foram identificados como pertencentes a ordem Enterobacterales (26 *E. coli* e quatro *Enterobacter spp*. em EB e quatro *E. coli* em EF, Quadro 1). É imprescindível destacar a presença da ordem Enterobacterales na maioria dos estudos feitos em águas residuais [46, 47], fato relacionado ao microbioma deste ambiente, majoritariamente composto por microrganismos da microbiota intestinal que inclui uma diversidade de Enterobacteriales. Porém, é de particular preocupação o potencial destas bactérias para causar infecções em seres humanos e também de adquirir e disseminar RAM via transferência horizontal de genes [48, 49].

Quadro 1. Enterobacterales recuperadas das amostras de esgoto bruto (EB) e efluente (EF) da Estação de Tratamento de Esgotos (ETE) Várzea das Flores, Carmópolis de Minas-MG e carbapenemases detectadas.

Espécies bacterianas (n=34)	**Carbapenemase detectada**
B1_*Escherichia coli*	Serinocarbapenemase
B2_*Enterobacter* sp.	Serinocarbapenemase
B3_*Enterobacter* sp.	Serinocarbapenemase
B4_*Escherichia coli*	Serinocarbapenemase
B5_*Escherichia coli*	Serinocarbapenemase
B6_*Enterobacter* sp.	Serinocarbapenemase
B7_*Escherichia coli*	Serinocarbapenemase
B8_*Escherichia coli*	Serinocarbapenemase
B9_*Escherichia coli*	Serinocarbapenemase
B10_*Enterobacter* sp.	Serinocarbapenemase
B11_*Escherichia coli*	Metalobetalactamase
B12_*Escherichia coli*	Serinocarbapenemase
B13_*Escherichia coli*	Serinocarbapenemase
B14_*Escherichia coli*	Serinocarbapenemase
B15_*Escherichia coli*	Metalobetalactamase
B16_*Escherichia coli*	Serinocarbapenemase
B17_*Escherichia coli*	Serinocarbapenemase
B18_*Escherichia coli*	Serinocarbapenemase
B19_*Escherichia coli*	Serinocarbapenemase
B20_*Escherichia coli*	Serinocarbapenemase
B21_*Escherichia coli*	Serinocarbapenemase
B22_*Escherichia coli*	Serinocarbapenemase
B23_*Escherichia coli*	Serinocarbapenemase
B24_*Escherichia coli*	Serinocarbapenemase
B25_*Escherichia coli*	Serinocarbapenemase
B26_*Escherichia coli*	Serinocarbapenemase
B27_*Escherichia coli*	Serinocarbapenemase

B28_*Escherichia coli*	Serinocarbapenemase
B29_*Escherichia coli*	Metalobetalactamase
B30_*Escherichia coli*	Metalobetalactamase
E1_*Escherichia coli*	Serinocarbapenemase
E2_*Escherichia coli*	Serinocarbapenemase
E3_*Escherichia coli*	Serinocarbapenemase
E4_*Escherichia coli*	Serinocarbapenemase

B: bactéria recuperada do esgoto bruto, E: bactéria recuperada do efluente.

E. coli foi a espécie mais recuperada em EB e a única em EF, certamente porque é um componente da microbiota do trato gastrointestinal de humanos e animais [50] e chega às ETEs com o aporte de esgoto doméstico [36, 51]. Ratificam esse achado os estudos de Anastasi e colaboladores [52] e Paiva e colaboradores [35] realizados em ETEs de Queensland na Austrália e Belo Horizonte, Brasil respectivamente.

Por outro lado, o gênero *Enterobacter* de fácil adaptação e patógeno oportunista [53], encontrado em menor quantidade apenas em EB (4/30, 13,3%), parece mesmo não ser tão frequente em amostras de esgoto, como também relatado por Resende [54] em uma ETE de Goiânia, Goiás. Ao contrário, Hooban e colaboradores [55] relataram o achado de 83,0% de *Enterobacter* spp. em amostras de água na Irlanda, porém foram incluídas amostras de esgoto e rios, o que pode justificar essa diferença pois, como observado por Simião e colaboradores [56], rio ao longo do seu percurso pode abrigar *Enterobacter* spp.

Enzimas carbapenamases são relatadas como importante mecanismo de resistência a antimicrobianos betalactâmicos, incluindo carbapenêmicos e junto com outros tipos de betalactamases têm sido recorrentes em isolados bacterianos de ambientes aquáticos, como águas residuais, rios, lagos ou mar [57].

Um fato alarmante aqui observado foi que todos os isolados, tanto de EB quanto de EF em drenagem espontânea para cursos d'água foram produtoras de carbapenemases (Quadro 1), ou seja, BRM é absolutamente integrante da comunidade bacteriana da ETE estudada. A maioria das BRM (88,6%, 26 de EB e 4 de EF) foi produtora de serinocarbapenemase. De fato, BRC produtores de serinocarbapenemase, especialmente do tipo KPC, estão amplamente disseminadas em várias partes do mundo e são consideradas endêmicas no Brasil desde 2009 [58, 59], tanto no cenário clínico quanto ambiental [60, 61]. Confirmando a disseminação desse mecanismo de resistência, vários estudos têm relatado o achado do *gene bla*$_{KPC}$ não apenas em *K. pneumoniae*, mas também em *E. coli* e *Enterobacter* spp. [22, 58, 60, 62].

Da mesma forma, Enterobacteriales produtoras de metalobetalactamase, apesar de terem sido descritas mais recentemente [63, 64], têm sido frequentemente encontradas em esgotos e efluentes hospitalares [65]. Possivelmente esse achado está relacionado com a elevada quantidade de antimicrobianos utilizados e descartados no ambiente aquático, tanto de origem doméstica quanto hospitalar, além da persistência e disseminação dessas cepas neste ambiente [66]. Vale ressaltar que a ETE aqui estudada recebe também as águas residuais da Santa Casa de Misericórdia, principal hospital da cidade de Carmópolis de Minas – MG. Embora não seja possível afirmar que tais cepas são advindas de ambiente hospitalar, é certo que há interferência na resistência antimicrobiana em função da possibilidade de intercâmbio de mecanismos de resistência. Rafraf e colaboradores [67] relatam que efluentes hospitalares podem ser considerados fontes urbanas de contaminação ambiental com real impacto no desenvolvimento e disseminação de RAM.

A indissociabilidade da qualidade e quantidade da água com o estado de saúde humana, saúde pública, meio produtivo, matéria prima, condição essencial para a vida, manutenção de diversos tipos de biomas, torna evidente a sua importância para a humanidade e reforça a perspectiva defendida pelo conceito *One Health* [68]. Dessa forma, é de extrema importância o monitoramento e o controle de qualidade dos tratamentos de esgoto e consequentemente do efluente que é despejado nos rios, uma vez que isso afeta diretamente a qualidade da água utilizada pela população.

Por fim, esse estudo apresenta limitações, tais como número amostral e a não utilização de metodologia molecular, porém, esses fatos não diminuem a relevância do estudo no sentido de ampliar o conhecimento da distribuição de BRC fora do ambiente clínico bem como dos mecanismos de resistência circulantes, ainda pouco contemplado nas pesquisas.

4. CONCLUSÃO

Bactérias da ordem Enterobacterales, sobretudo *E. coli*, são as mais prevalentes em amostras de ETEs, especialmente de esgoto bruto. Sistemas de tratamento de esgoto mistos parecem ter um papel importante e maior eficiência na redução de bactérias do efluente e, portanto, contribuem para devolver ao ambiente uma água que traz menor risco à saúde da população.

BRM circulam em amostras de água de ETEs, sendo que mecanismos enzimáticos de transferência horizontal parecem estar mais disseminados. Os dados obtidos poderão evidenciar a necessidade de tomada de ação frente a descarga e tratamento do esgoto doméstico no sentido de conter a disseminação da RAM, especialmente aos carbapenêmicos e, consequentemente, reduzir os riscos à saúde da população do entorno dos cursos d´água que recebem os efluentes das ETEs.

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Todos os autores relatam não ter conflitos de interesse.

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Scientific research article

Toxicity of flavone and its hydroxylated derivatives: a Study *in silico*, *in vitro* and *in vivo*

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SUMMARY

Purpose: The present study investigated the toxicity and theoretical pharmacokinetics, cytotoxicity, and genotoxicity of flavone and its hydroxylated derivatives: 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone. **Methods**: The *in silico* study was conducted using OSIRISsoftware; cytotoxicity was determined through models of hemolysis and Erythrocyte Osmotic Fragility (EOF) using a 0.5 % solution of human erythrocytes with blood types A, B and O, having positive (+) and negative (-) Rh factors; and genotoxicity was evaluated using the micronucleus test. **Results**: Flavone and its hydroxylated derivatives do not present significant toxicity risks (theoretically), yet they do have good oral bioavailability. Flavone, 5-hydroxyflavone and 6-hydroxyflavone showed moderate hemolytic potential at higher concentrations. The hydroxylated flavones protected cells types A and O from osmotic stress at a minimum concentration of 500 μg/mL, in blood types A and O. In the genotoxicity evaluation, orally administered flavone did not show genotoxicity compared to cyclophosphamide, a proven genotoxic agent. **Conclusion**: It is concluded that the flavones have low theoretical toxicity, good bioavailability, moderate cytotoxicity in higher concentrations and nogenotoxicity, suggesting a considerable margin of safety for future pharmacological use, and showing the importance of including computational chemistry techniques to guide biological protocols.

Keywords: Flavonoids; cytotoxicity; hemolysis; erythrocyte osmotic fragility; micronucleus test.

RESUMEN

Toxicidad de la flavona y sus derivados hidroxilados: un estudio *in silico*, *in vitro* e *in vivo*

Propósito: El presente estudio investigó la toxicidad y farmacocinética teórica, citotoxicidad y genotoxicidad de la flavona y sus derivados hidroxilados: 3-hidroxiflavona, 5-hidroxiflavona y 6-hidroxiflavona. **Métodos**: El estudio in silico se llevó a cabo utilizando el software OSIRIS; la citotoxicidad se determinó mediante modelos de hemólisis y fragilidad osmótica de los eritrocitos (EOF) utilizando una solución al 0,5% de eritrocitos humanos de los tipos sanguíneos A, B y O, con factores Rh positivos (+) y negativos (-); y la genotoxicidad se evaluó mediante el test de micronúcleos. **Resultados**: La flavona y sus derivados hidroxilados no presentan riesgos significativos de toxicidad (teóricamente), pero tienen buena biodisponibilidad oral. La flavona, 5-hidroxiflavona y 6-hidroxiflavona mostraron potencial hemolítico moderado en concentraciones más altas. Los flavonoides hidroxilados protegieron a los tipos celulares A y O del estrés osmótico a una concentración mínima de 500 μg/mL, en los tipos sanguíneos A y O. En la evaluación de genotoxicidad, la flavona administrada oralmente no mostró genotoxicidad en comparación con la ciclofosfamida, un agente genotóxico comprobado. **Conclusión**: Se concluye que las flavonas tienen baja toxicidad teórica, buena biodisponibilidad, citotoxicidad moderada en concentraciones más altas y ninguna genotoxicidad, sugiriendo un margen de seguridad considerable para su uso farmacológico futuro, y destacando la importancia de incluir técnicas de química computacional para orientar protocolos biológicos.

Palabras clave: Flavonoides; citotoxicidad; hemólises; fragilidad osmótica eritrocitária; test de micronúcleos.

RESUMO

Toxicidade da flavona e seus derivados hidroxilados: um estudo *in silico*, *in vitro* e *in vivo*

Propósito: O presente estudo investigou a toxicidade e farmacocinética teórica, citotoxicidade e genotoxicidade da flavona e seus derivados hidroxilados: 3-hidroxiflavona, 5-hidroxiflavona e 6-hidroxiflavona. **Métodos**: O estudo *in silico* foi conduzido utilizando o *software* OSIRIS; a citotoxicidade foi determinada através de modelos de hemólise e fragilidade osmótica dos eritrócitos (EOF) usando uma solução a 0,5% de eritrócitos humanos dos tipos sanguíneos A, B e O, com fatores Rh positivos (+) e negativos (-); e a genotoxicidade foi avaliada pelo teste do micronúcleo. **Resultados**: A flavona e seus derivados hidroxilados não apresentam riscos significativos de toxicidade (teoricamente), mas têm boa biodisponibilidade oral. A flavona, 5-hidroxiflavona e 6-hidroxiflavona mostraram potencial hemolítico moderado em concentrações mais elevadas. Os flavonóides hidroxilados protegeram os tipos celulares A e O do estresse osmótico a uma concentração mínima de 500 μg/mL, nos tipos sanguíneos A e O. Na avaliação de genotoxicidade, a flavona administrada oralmente não mostrou genotoxicidade em comparação com a ciclofosfamida, um agente genotóxico comprovado. **Conclusão**: Conclui-se que as flavonas têm baixa toxicidade teórica, boa biodisponibilidade, citotoxicidade moderada em concentrações mais elevadas e nenhuma genotoxicidade, sugerindo uma margem de segurança considerável para uso farmacológico futuro, e destacando a importância da inclusão de técnicas de química computacional para orientar protocolos biológicos.

Palavras-chave: Flavonoides; citotoxicidade; hemólise; fragilidade osmótica eritrocitária; teste do micronúcleo.

1. INTRODUCTION

Plants and herbs have played an important role in healthcare since ancient times; today it is known that 80% of the world's population depends on plant-derived medicines as the first choice of treatment in primary healthcare. In the last century alone, traditional knowledge obtained from many sources has made the formulation of roughly 121 pharmaceutical products possible. During the 1950s until the 1970s, approximately 100 new drugs based on plants were

commercialized. It is also well known that approximately 80% of cardiovascular, antimicrobial, immunosuppressive, and antineoplastic drugs have their origins in plants. It is also accepted that most drug ingredients are either derived or developed from natural products and compounds. In United States, roughly 25% of all pharmaceutical prescriptions contain at least one plant-derived ingredient. Speaking in broader terms, about 50% of pharmaceuticals are developed from active ingredients first identified or isolated from herbs, plants, animals, or insects [1, 2].

In this scenario, for future medicines, flavonoids stand out. Phenolic compounds are one of largest groups of secondary plant constituents, and flavonoids figure importantly in this group. Flavonoids are found in in numerous specimens of the plant kingdom, mainly concentrated in fruit skin, in vacuoles of plant cells, and in epidermic pigments. Flavonoids are classified into six major subgroups: chalcones, flavones (e.g., flavone, apigenin, and luteolin), flavonols (e.g. quercetin, kaempferol, myricetin, and fisetin), flavandiols, anthocyanins, and proanthocyanidins (condensed tannins). These substances are important to plants because they are involved in processes like UV (Ultra Violet) protection, pigmentation, stimulation of nitrogen-fixing nodules, and plant disease resistance. Their popularity is due to the fact they are indeed bioactive, and can provide health benefits to human body such as their anti-allergic, anti-cancer, antioxidant, anti-inflammatory, anti-viral, nutritional and functional activities [3].

Among flavonoids, natural flavones and their synthetic derivatives have demonstrated several biological activities, like neuroprotective, cardioprotective and antimicrobial, including activities mentioned above, antioxidant, anti-inflammatory, antitumor and anti-allergic [4]. Flavones are natural products of the benzopyran class constituting an important group of oxygen heterocycles, they exhibit biological activities which are dependent on the nature and position of the substituents on the flavone skeleton. The flavones have a unique ability to modulate various enzyme systems, these compounds are active against metabolic and infectious diseases and also demonstrate anti-estrogenic and cytotoxic activities [5].

It is prudent, before any investigation into the mechanism of action of a molecule, to realize toxicity tests. There are different cytotoxicity assays, and the Red Blood Cell (RBC) assay is a simple, fast and effective way to observe injury that a xenobiotic substance might produce. The method is based on measurement of hemoglobin efflux from erythrocytes exposed to a toxic substance [6, 7]. *In vivo* genotoxicity assays investigate substances toxic to DNA and the carcinogenetic potential of chemical or physical agents. Such data can be evaluated by methods such as comet assay, micronuclei, apoptosis, and phagocytosis counts. The micronucleus test seeks to quantify small individual nuclei called micronuclei (MN), these nuclei appear when chromosome fragments or whole chromosomes are not coupled to chromosome groupings in a cell [8].

Computational models of prediction, also called *in silico* predictive tools, play a major role in the repertoire of methodological alternatives to guide pharmaceutical research, there are of course *in vitro* and *in vivo* models. These tools are used to study both existing and hypothetical substances and deliver fast results [9].

Software like Osiris has been developed and used to provide theoretical toxicological risk assessments according to the molecular structure of the substance, giving predictions regarding mutagenicity, tumorigenicity, influence on the reproductive system, and data such as: cLog*P* value (partition coefficient between n-octanol and water), druglikeness (similarity to other molecules available in the market), and "drug-score" (combination of druglikeness, clog*P*, molecular mass, and toxicological risk results) [9].

Given these assumptions, the present study set out to investigate the theoretical toxicological characteristics of flavone (Figure 1) and its hydroxylated derivatives 3-hydroxyflavone (Figure 2), 5-hydroxyflavone (Figure 3) and 6-hydroxyflavone (Figure 4) to test their cytotoxicity and genotoxicity. While seeking toxicological safety evidence for their use, we hope to contribute to the expanding range of effective alternatives for pharmacological evaluation, and disease treatment.

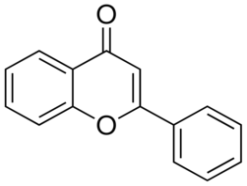


Figure 1. Flavone

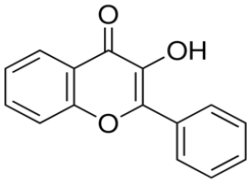


Figure 2. 3-hydroxyflavone

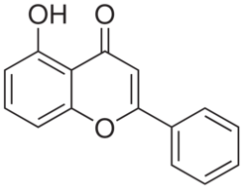


Figure 3. 5-hydroxyflavone

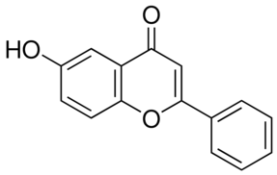


Figure 4. 6-hydroxyflavone

2. MATERIALS AND METHODS

2.1. Flavones

The flavones investigated were kindly provided by Prof. Dr. José Maria Barbosa Filho from the Phytochemical Laboratory of Natural Products of the Postgraduate Program in Natural and Synthetic Bioactive, Center of Biological Sciences and Health, Department of Pharmacy, State University of Paraiba Products– CCS/UFPB, having been purchased from Sigma, USA. These included: flavone, 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone.

To perform the experimental protocol for determination of antibacterial activity, solutions with a concentration of 10 mg/mL of flavonoid, using DMSO as a vehicle were prepared.

2.2. Human erythrocytes

Human erythrocytes of blood types A, B, and O, with positive and negative Rh factors were obtained from bags containing erythrocyte concentrate (that would no longer be used for transfusion), from the Transfusion Unit of the University Hospital Lauro Wanderley/UFPB.

Manipulation and disposal of the erythrocytes followed the Safety Standards standardized by said Unit.

The project was approved by the Research Ethics Committee of the Health Sciences Center, Federal University of Paraíba, Protocol: 0184/14 and CAEE: 30652314.0.0000.5188.

2.3. Animals

To perform the micronucleus assay, *Mus musculus* mice of the Swiss strain from the Prof. Dr. Thomas George *Vivarium,* and weighing between 20-30 g were used. The mice were kept at a temperature of 21 ± 1 °C, light and dark cycle of 12 h, and fed with pellet-type feed and water *ad libitum*.

This experimental protocol was approved by the Committee on Ethics in Animal Use (CEUA), of the Biotechnology Center (CBiotec) of UFPB, with registration N°. 3306/13.

2.4. *In silico* assay

OSIRIS

The chemical structures were submitted to *in silico* study for theoretical analysis of flavone toxicity and pharmacokinetic parameters. These parameters were ADME (Absorption, Distribution, Metabolism, and Excretion) and toxicity, using the Osiris Property Explorer program.

In this analysis we determined the potential druglikeness and drug-score that are related to topological descriptors, and other properties such as cLog*P* and molecular mass [10]. *In silico* evaluation of molecular toxicity also included theoretical analysis of mutagenic effect, tumorigenicity, irritant properties, and effects on reproduction as described by Abreu [11].

Finally, since the flavones were to be administered orally, requiring good absorption in the gastrointestinal tract, we used the Lipinski "Rule of Five".

2.5. Cytotoxicity assays

2.5.1. Hemolysis assay

A human blood sample was mixed with 0.9% NaCl at a ratio of 1:30 and centrifuged at 2500 rpm for 5 minutes, the procedure was repeated twice more and 500 μL of the pellet of the last centrifugation was resuspended in 100 ml of 0.9% NaCl, resulting in a 0.5% solution. Samples of the flavones at concentrations of 1, 10, 100, 500 and 1000 μg/mL were added to 2 mL of the erythrocyte solution. Tubes containing the erythrocyte solution alone formed the negative control group (0% hemolysis), and erythrocyte solution plus 1% Triton X-100 (as a hemolyzing agent) was used as the positive control (100% hemolysis). The samples were incubated for 1 hour at 25 ± 2°C under slow and constant (100 rpm) shaking. After this, centrifugation was performed at 2500 rpm for 5 minutes, and hemolysis was quantified by reading an aliquot of the supernatant using spectrophotometry at a wavelength of 540 nm [12].

The experiments were performed in triplicate and the results expressed in % hemolysis as compared to the negative control group.

2.5.2. Evaluation of osmotic fragility in human erythrocytes in the presence of flavones

A solution of each flavone and its derivates at concentrations of 1, 10 and 100 μg/ml was incubated in tubes containing 2 ml of a 0.5% solution of erythrocytes for 1 hour at 25 ± 2 °C. After this, the preparations were centrifuged at 2500 rpm for 5 minutes and the supernatant discarded. The erythrocytes were then resuspended in a hypotonic sodium chloride solution (0.24%) solution, and shaken at 100 rpm for 20 minutes at 25 ± 2 °C. After this period, the

samples were centrifuged at 2500 rpm for 5 minutes and hemolysis was quantified by means of an aliquot of the supernatant read using spectrophotometry at a wavelength of 540 nm [13].

A solution of erythrocytes was used as a negative control (0% hemolysis) and a solution of erythrocytes in the presence of a 0.24% sodium chloride solution was used as a positive control (100% hemolysis).

The experiments were performed in triplicate and the results expressed as % hemolysis as compared to the positive control group.

2.6. Genotoxicity assays

2.6.1. Investigation of Flavonoid clastogenic and aneugenic potential in mouse erythrocytes

The experimental procedures were performed according to Resolution No. 90, of the National Agency of Sanitary Surveillance - ANVISA (RE 90/2004) [14]. Swiss mice were divided into groups composed of three males and three females who were given oral flavone solution doses at 200 mg/kg. Another group, the positive control, was treated with cyclophosphamide, a proven mutagenic agent, at a dose of 50 mg/kg. After 24 hours, the animals were euthanized and a blood sample was collected for the preparation of slides, which were stained with panopticus and observed under an optical microscope at 1000x magnification to count micronuclei. Slides were analyzed for presence or absence of micronuclei in the erythrocytes of each animal. About 2000 erythrocytes per animal were counted [15].

Results were expressed as the percentage of mean ± standard deviation compared to the positive control group.

2.7. Statistical analysis

The results obtained in the experiments were analyzed using GraphPad Prism 5.0® software, San Diego, CA, USA, using the ANOVA test followed by Dunnett's post-test or the unpaired Student t-test for two-column analysis. The values were expressed as mean ± standard error of the mean (SEM) or standard deviation of the mean (SD) and considered significant when $p < 0.05$.

3. RESULTS

3.1. *In silico* assay

The OSIRIS analyses indicated low theoretical risk of toxicity for the flavonoids, except one flavone exhibiting mutagenic potential (chart 1).

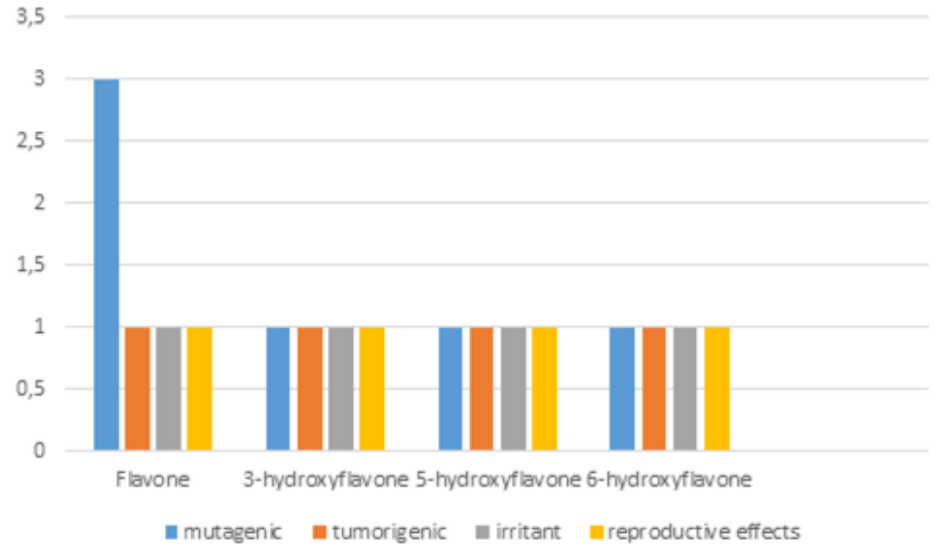


Chart 1. Theoretical risk of toxicity for Flavone and its derivatives calculated using the OSIRIS Property Explorer software.

In addition, OSIRIS presented considerable values for "druglikeness" and "drug-score" (drug-score = correlation between "druglikeness", cLog*P*, molecular weight, and risk of toxicity), generating important data, and revealing possible future medicinal use for the tested substances (table 1).

Table 1. Evaluation of theoretical toxicity risks.

Parameters / **Flavones**	**cLog*P*** **(2 – 5)**	**"Druglikeness"** **(≤1)**	**"Drug-score"** **(≤1)**
Flavone	3.74	1.85	0.45
3-hydroxyflavone	3.45	1.62	0.76
5-hydroxyflavone	3.47	1.33	0.75
6-hydroxyflavone	3.23	1.35	0.75

cLog*P* = Partition coefficient octanol: water - confers solubility/Druglikeness = similarity with other structures available on the market/Drug-score = combination of results of "druglikeness", cLog*P*, molecular mass and toxicological risks.

As for theoretical bioavailability with oral administration, all of the flavonoids assessed met the "rule of five" Lipinski standards in which each substance must possess at least three of the four requirements listed (table 2). In this way, the result obtained demonstrated that flavone, 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone will be bioavailable after oral administration.

Table 2. Theoretical analysis of the physicochemical properties involved in the oral bioavailability of flavones according to Lipinski's "Rule of Five".

Parameters / Substance	nDLH (≤ 5)	nALH (≤ 10)	Da (≤ 500)	cLog*P* (≤ 5)
Flavone	0	2	222	3.74
3-hydroxyflavone	1	3	238	3.45
5-hydroxyflavone	1	3	238	3.47
6-hydroxyflavone	1	3	238	3.23

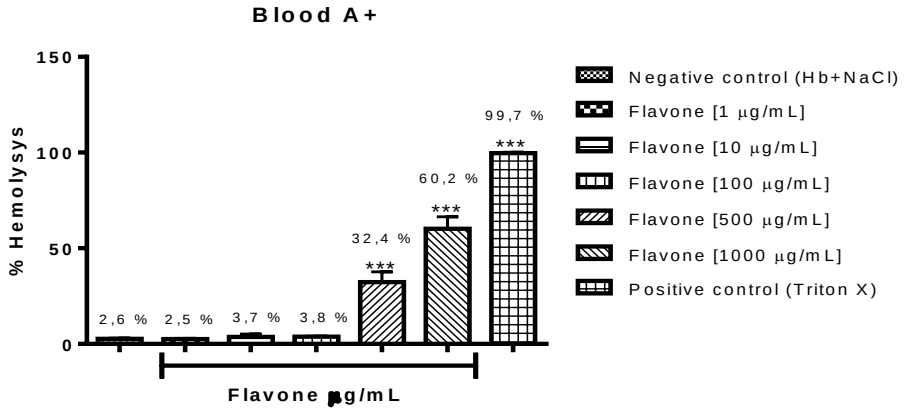
*nDLH: Number of hydrogen bond donors; *NALH: number of hydrogen bonding acceptors; *Da: molecular weight; *cLog*P*: partition coefficient octanol: water.

3.2. Cytotoxicity assays

3.2.1. Evaluation of Hemolytic potential in human erythrocytes

In this assay it was found that flavone induced hemolysis only at concentrations of 500 μg/mL and 1000 μg/mL as compared to the negative control group, and as expressed in charts 2a to 4b.

a)



b)

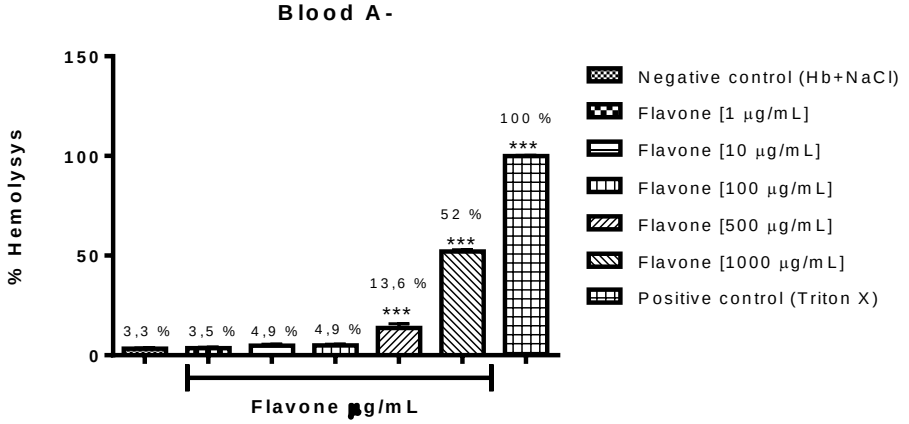
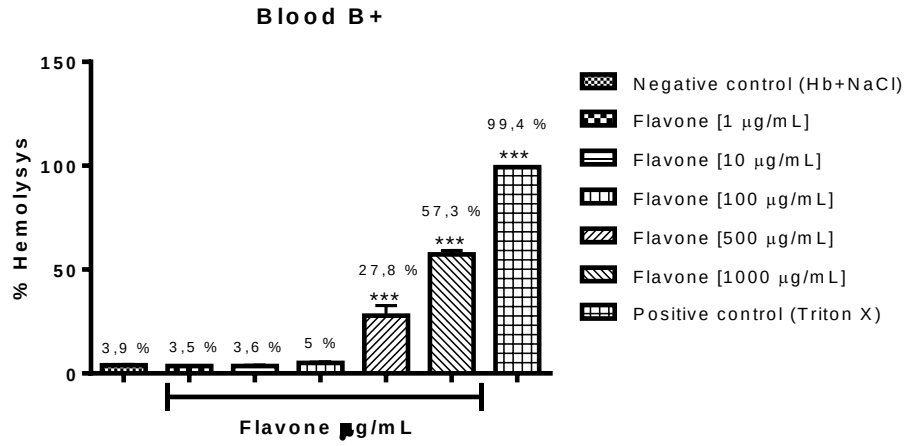


Chart 2. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p < 0.001$ ($n = 3$).

a)



b)

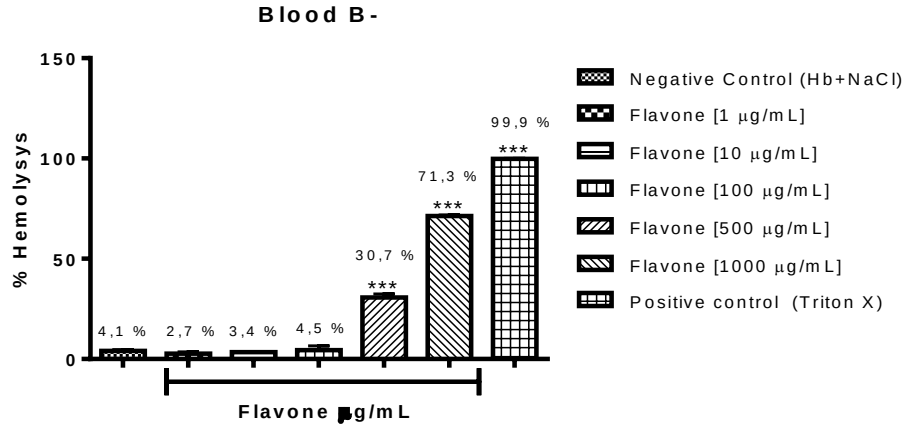
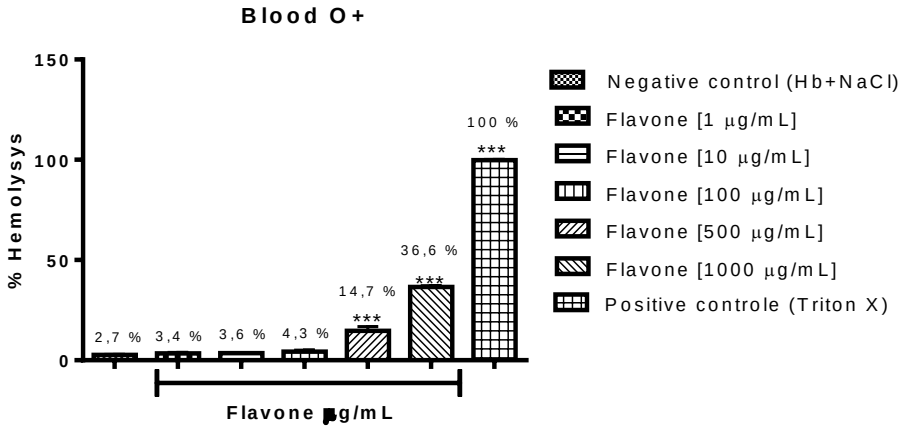


Chart 3. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p < 0.001$ ($n = 3$).

a)



b)

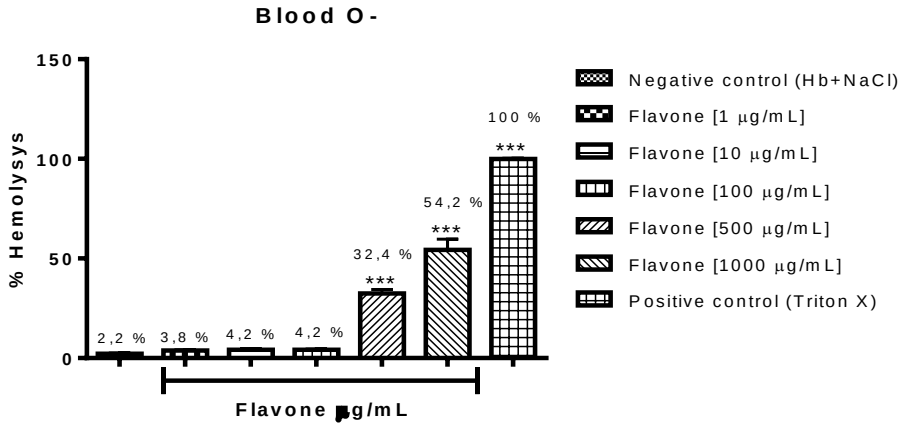
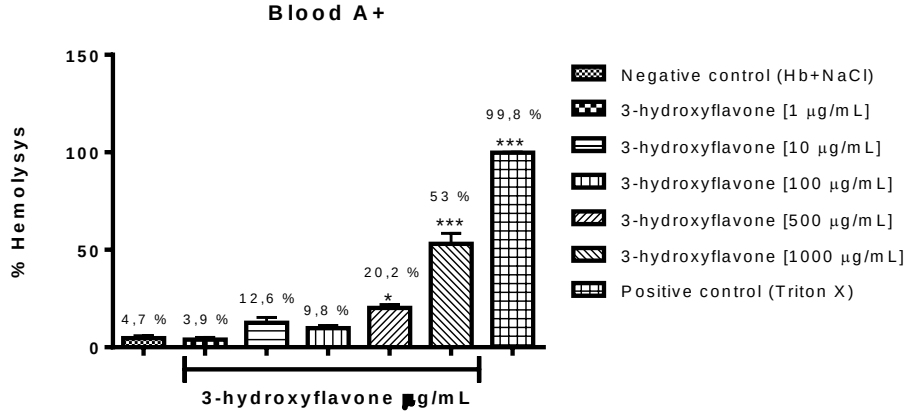


Chart 4. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by flavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p < 0.001 (n = 3).

3-hydroxyflavone promoted cytotoxicity at concentrations of 500 µg/mL and 1000 µg/mL in erythrocytes type A+ and A-, B- and O+. Only B- cells showed greater sensitivity to 3-hydroxyflavone, since lysis occurred upon incubation from the concentration of 100 µg/mL, as compared to the negative control group (charts 5a to 7b).

a)



b)

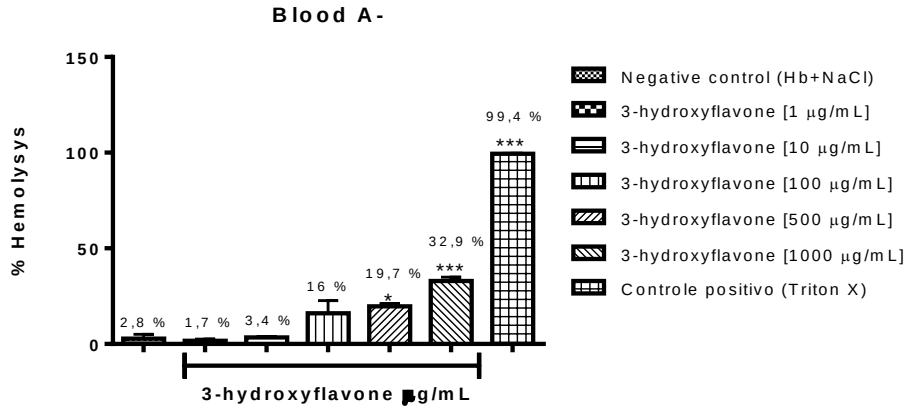
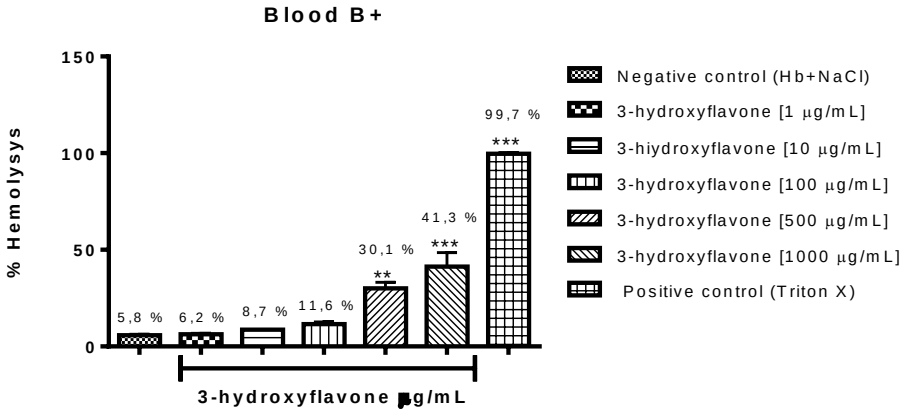


Chart 5. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. *$p < 0.05$, ***$p<0.001$ (n=3).

a)



b)

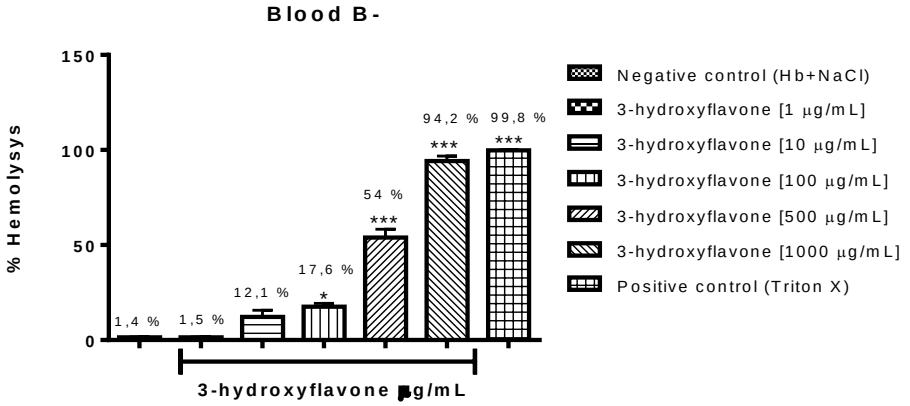
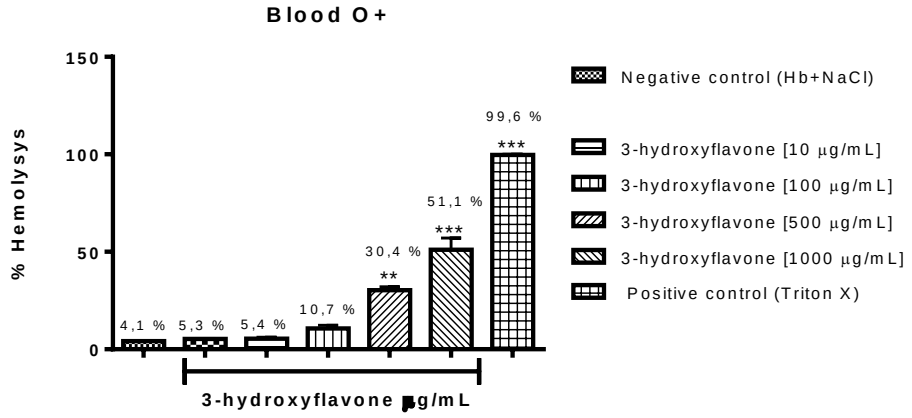


Chart 6. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. *$p < 0.05$, ***$p<0.001$ (n=3).

a)



b)

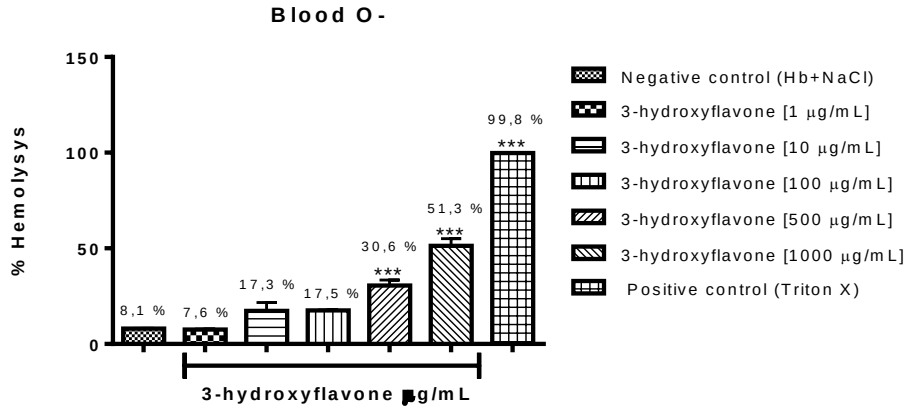
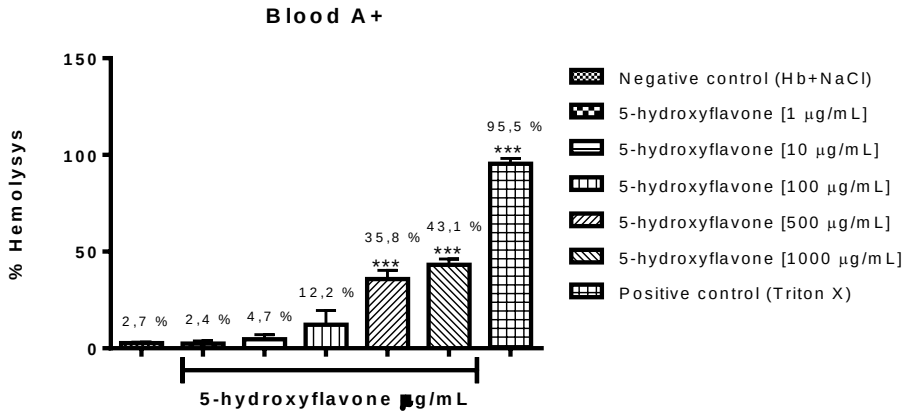


Chart 7. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 3-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

The flavone 5-hydroxyflavone, at concentrations of 500 µg/mL and 1000 µg/mL was toxic for all erythrocyte types tested, as compared to the negative control, as can be seen in charts 8a to 10b.

a)



b)

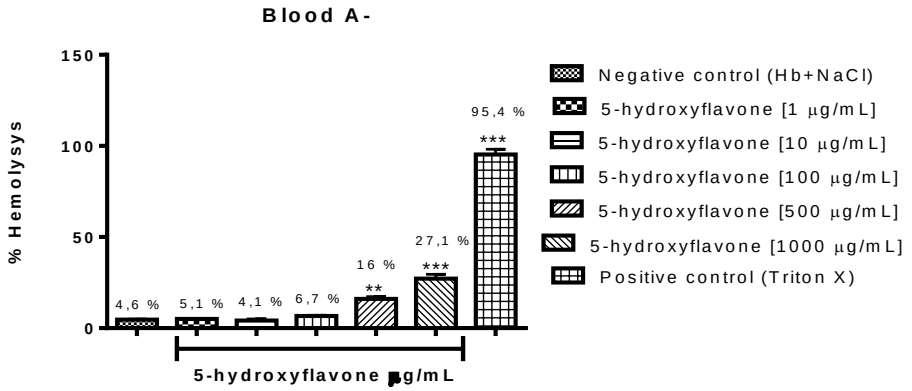
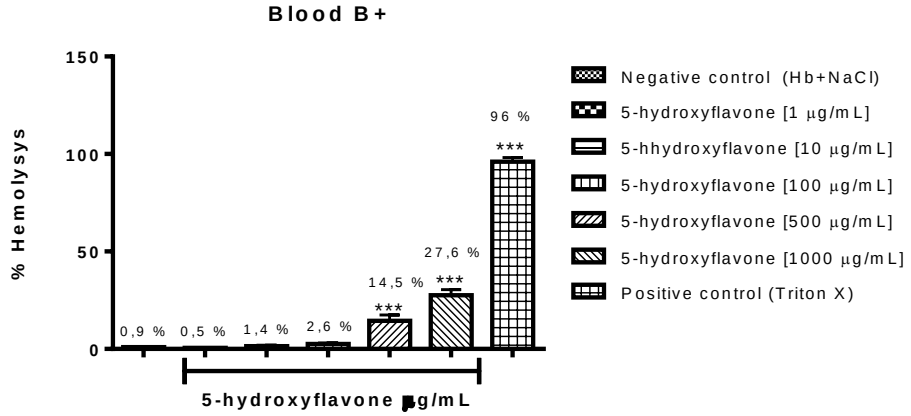


Chart 8. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

a)



b)

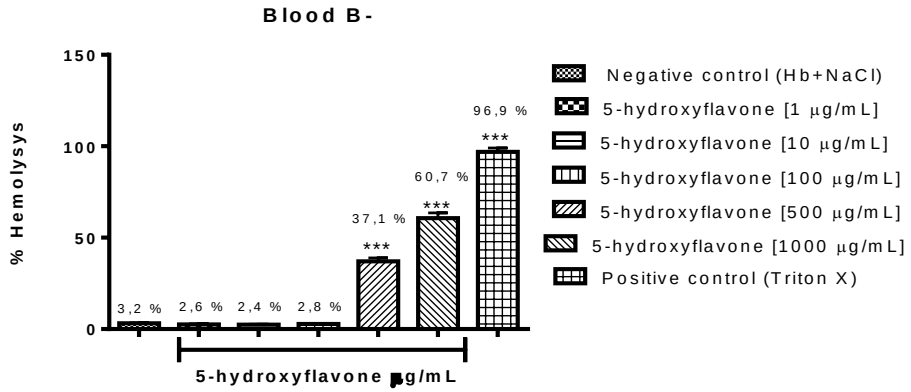
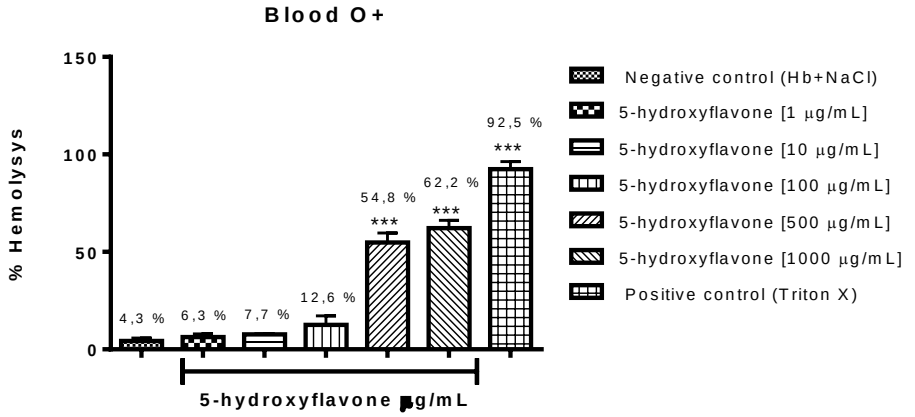


Chart 9. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p<0.001 (n=3).

a)



b)

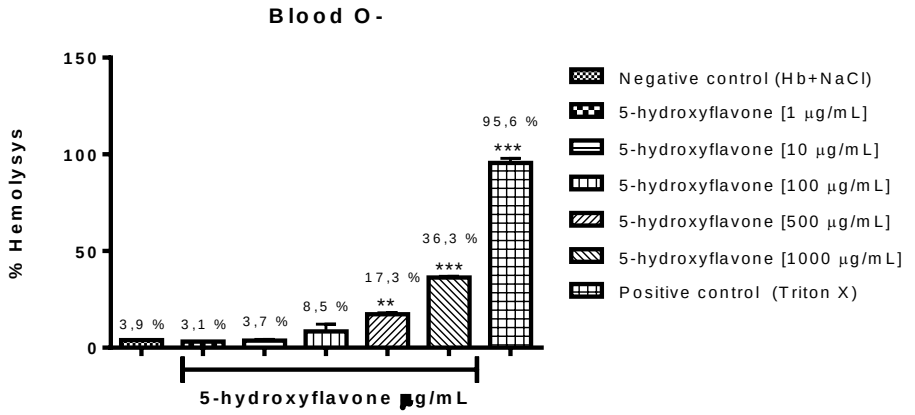
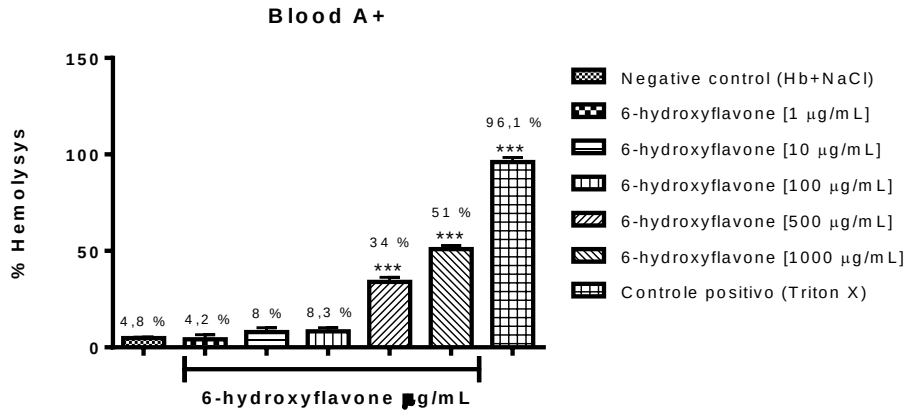


Chart 10. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 5-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p < 0.01, ***p<0.001 (n=3).

Finally, 6-hydroxyflavone, similar to flavone and 5-hydroxyflavone, was shown to have potential to induce hemolysis at concentrations of 500 µg/mL and 1000 µg/mL in types A, B, and O; both Rh+ and Rh-, when compared to the negative control. These results are shown in charts 11a and 11b, 12a and 12b and 13a and 13b.

a)



b)

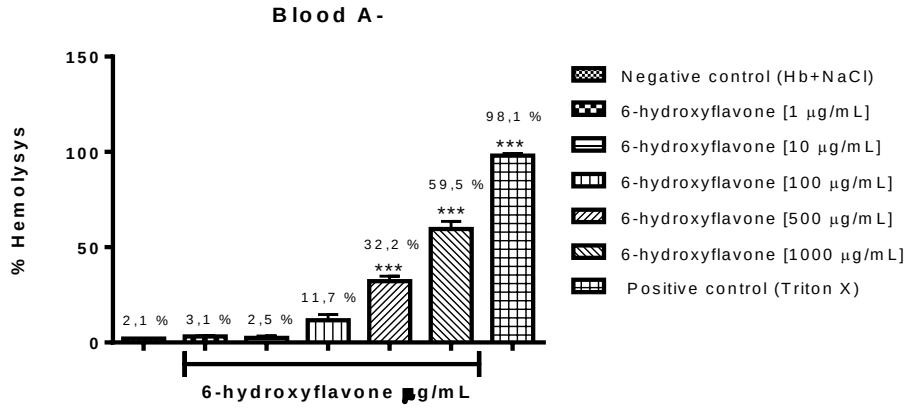
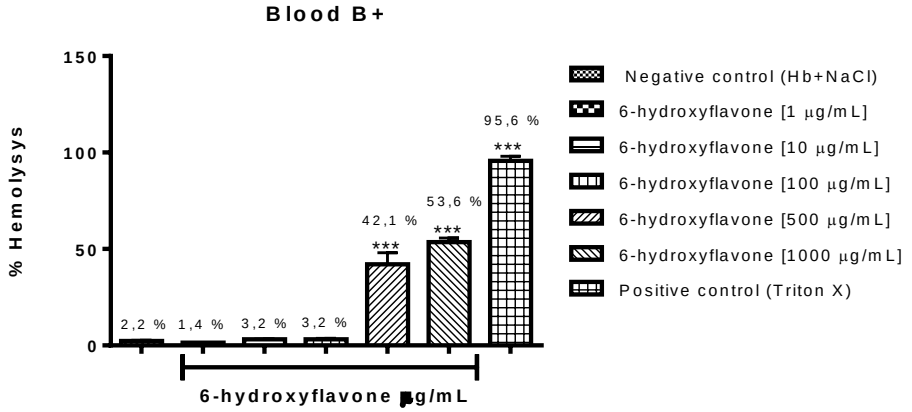


Chart 11. Evaluation of cytotoxicity in erythrocytes type A, Rh+ (a) and A, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***$p<0.001$ (n=3).

a)



b)

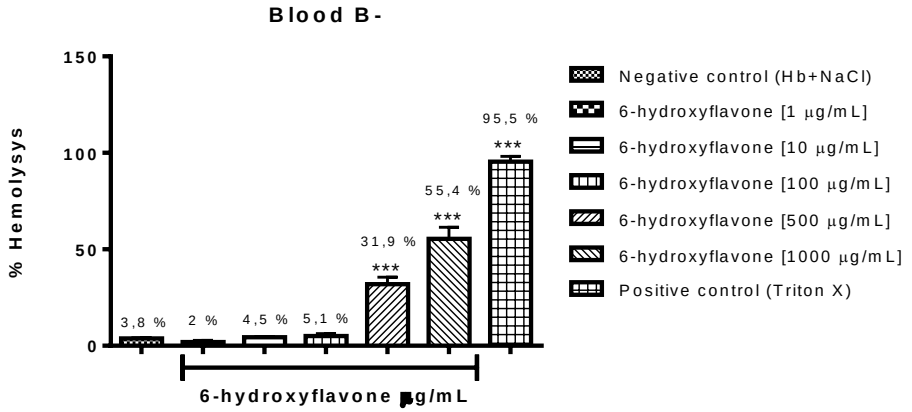
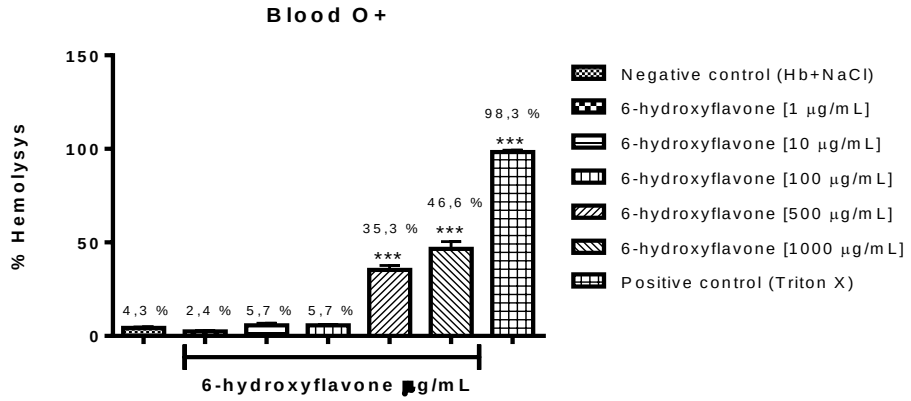


Chart 12. Evaluation of cytotoxicity in erythrocytes type B, Rh+ (a) and B, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p<0.001 (n=3).

a)



b)

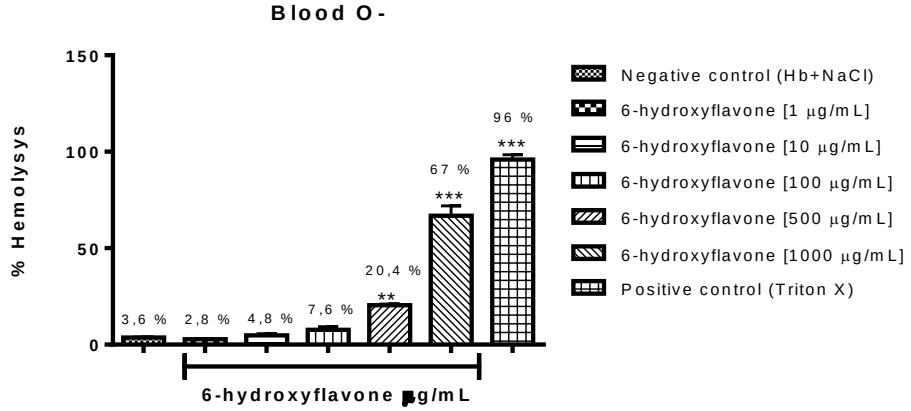


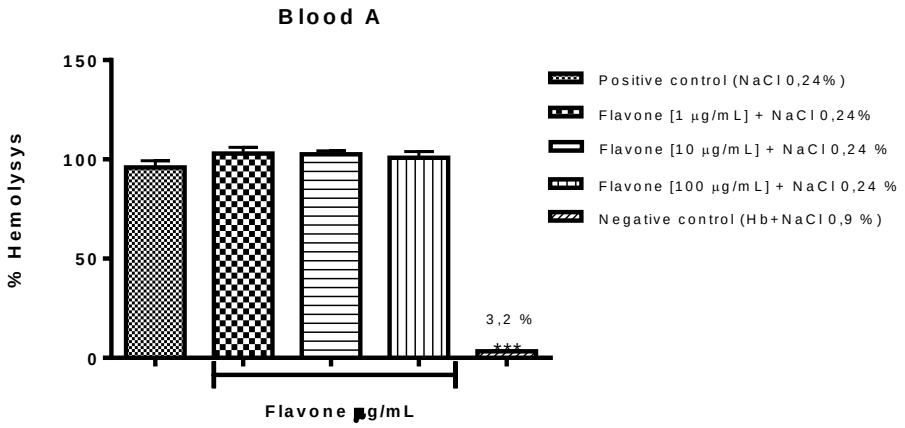
Chart 13. Evaluation of cytotoxicity in erythrocytes type O, Rh+ (a) and O, Rh- (b) induced by 6-hydroxyflavone. The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

The results present moderate flavonoid cytotoxicity in higher concentrations (500 and 1000 µg/mL) in all blood types A, and B; without Rh involvement.

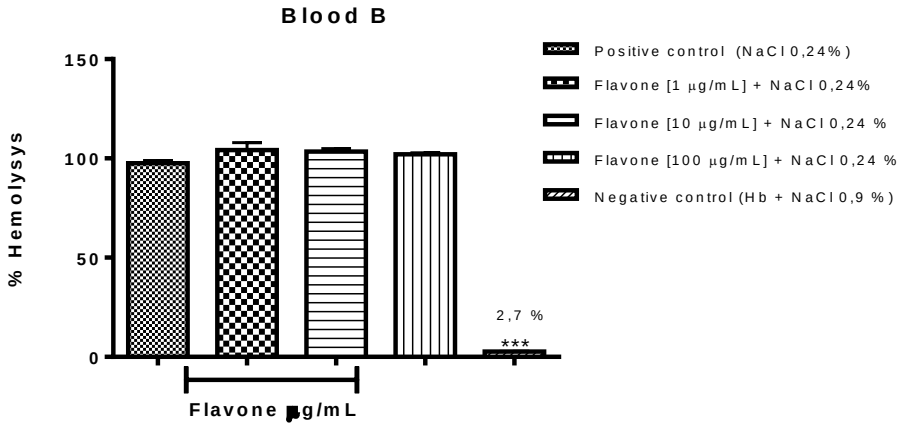
3.2.2. Evaluation of osmotic fragility in human erythrocytes in the presence of Flavones

When the degree of hemolysis in hypotonic medium (0.24% NaCl solution) was analyzed, flavone did not promote reductions for any of the tested concentrations (1, 10 and 100 µg/mL); in erythrocytes types A, B, or O; this, as compared to the positive control group (NaCl solution 0.24%), in which maximum membrane lysis was observed (chart 14).

a)



b)



c)

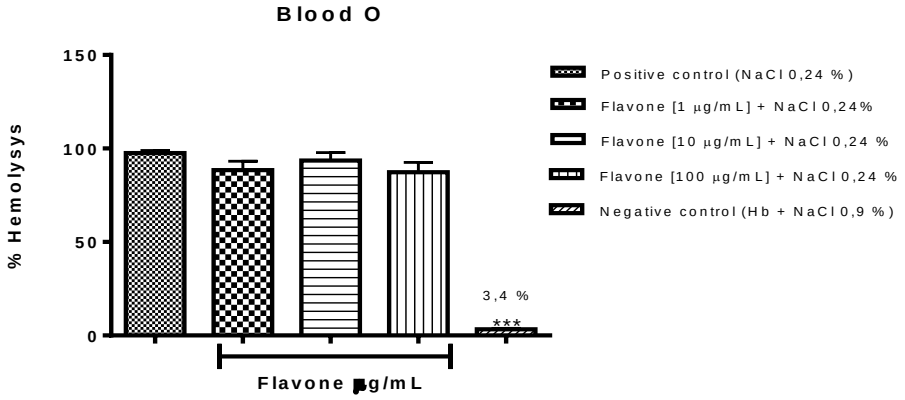
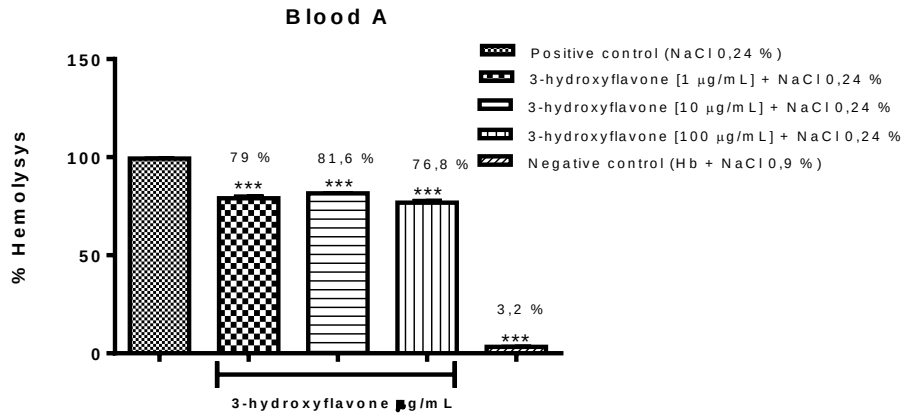


Chart 14. Antihemolytic activity of flavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

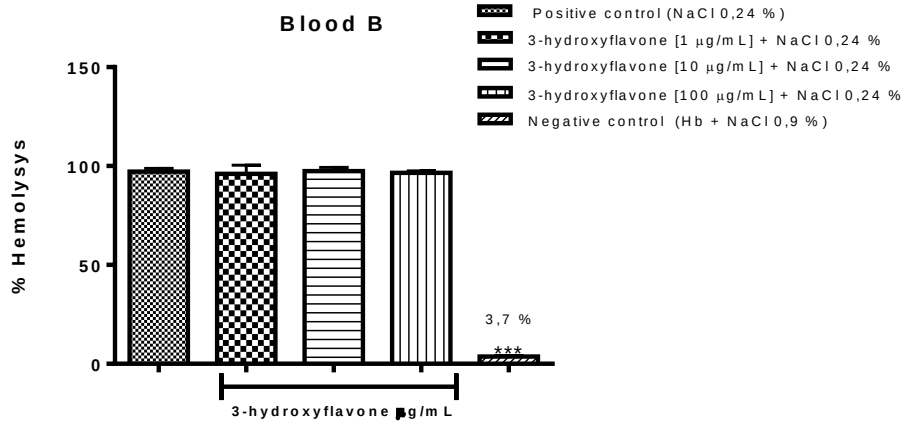
The flavonoid 3-hydroxyflavone, when incubated with blood type A, protected the erythrocytes from osmotic stress at concentrations of 1, 10 and 100 µg/mL, as compared to the positive control group (NaCl 0.24%), thus reducing the respective hemolysis percentages to 79%,

81.6% and 76.8%. This result differed from that observed for blood types B and O, where none of the concentrations used offered protection against hemolysis (chart 15).

a)



b)



c)

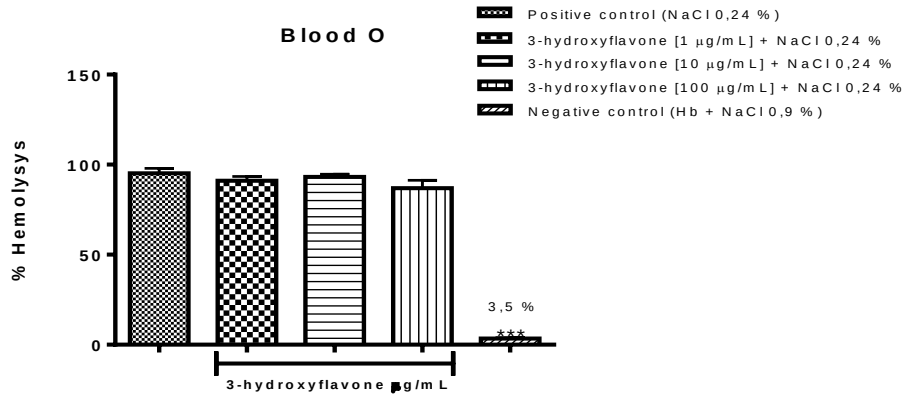
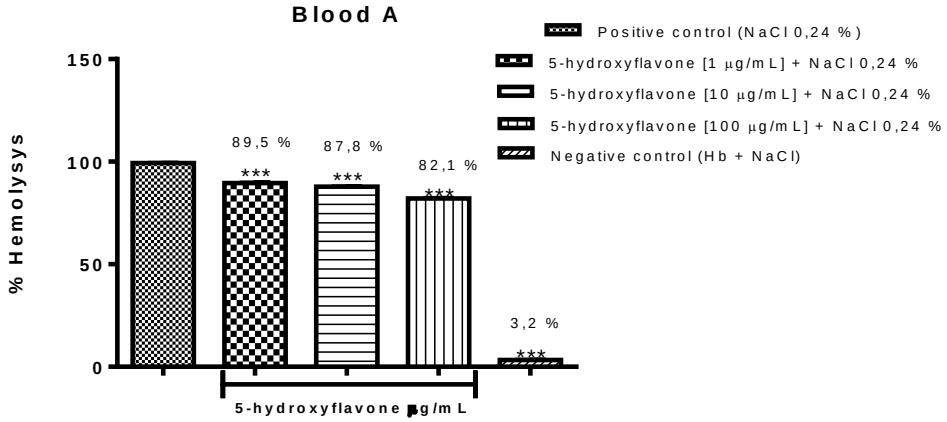


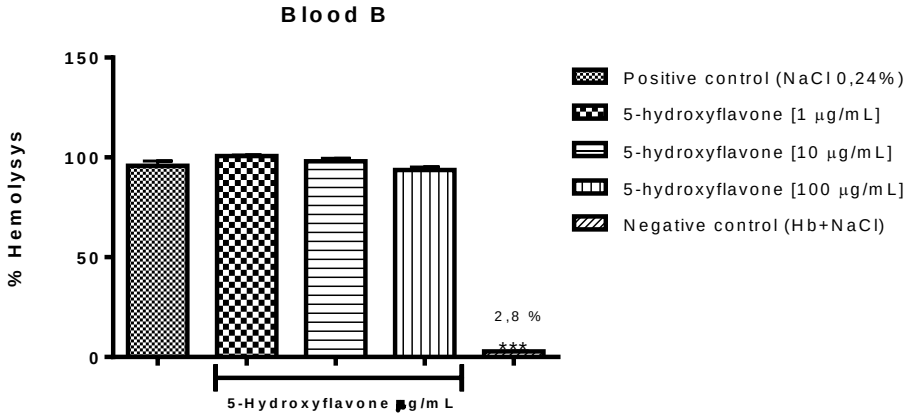
Chart 15. Antihemolytic activity of 3-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%) The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. ***p< 0.001 (n=3).

The concentrations of 1, 10 and 100 μg/mL of 5-hydroxyflavone protected types A and O erythrocytes, reducing respective hemolysis to 89.5%, 87.8% and 82.1% (blood A), and 86%, 92.7% and 86.3% (blood O), as compared to the NaCl control group 0.24% (99.3% hemolysis), and as observed in chart16.

a)



b)



c)

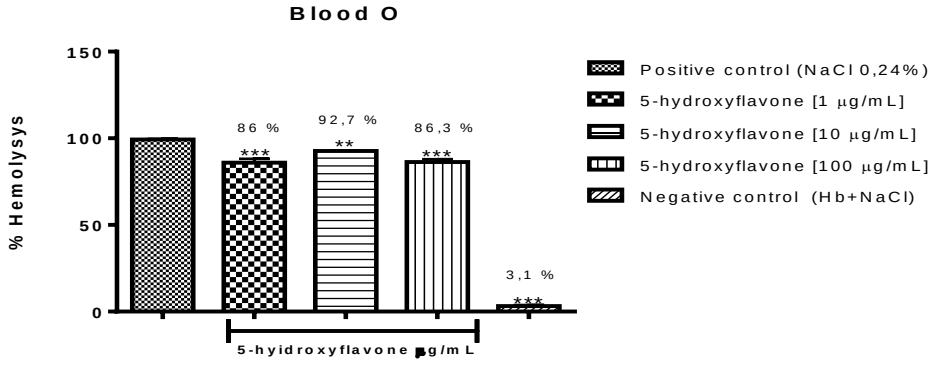
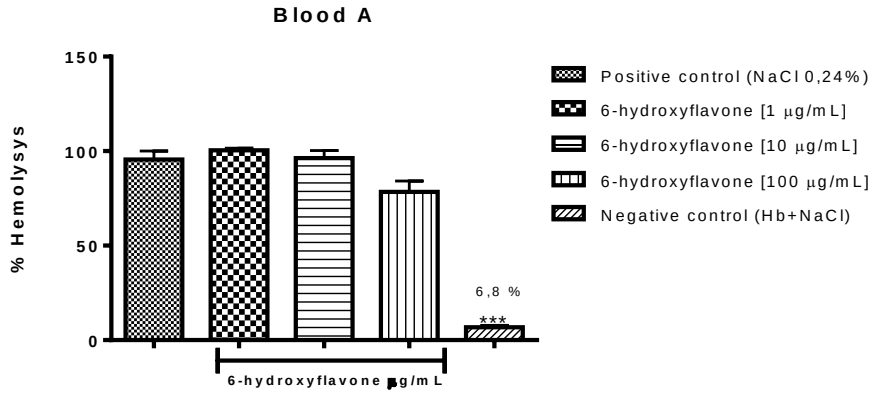


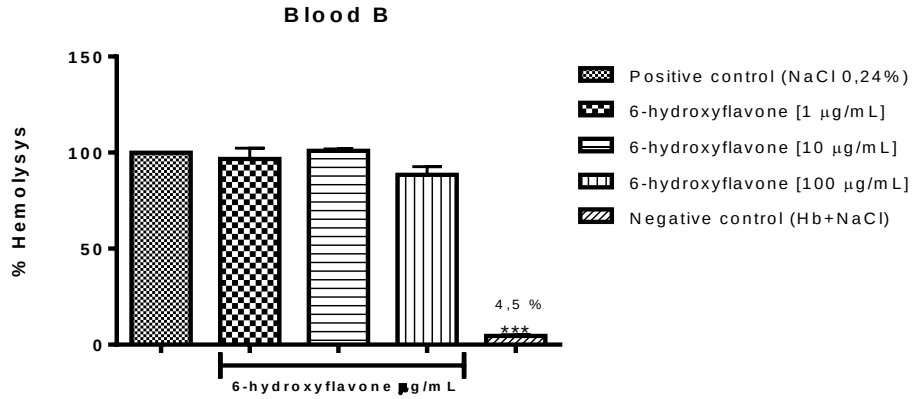
Chart 16. Antihemolytic activity of 5-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

The flavonoid 6-hydroxyflavone was effective against the osmotic stress caused by the hypotonic solution in blood type O, at which concentrations of 1, 10 and 100 μg/mL significantly reduced hemolysis to 75.6%, 74.7%, and 68 %, Compared to the positive control group (NaCl 0.24%), as we can see in chart 17.

a)



b)



c)

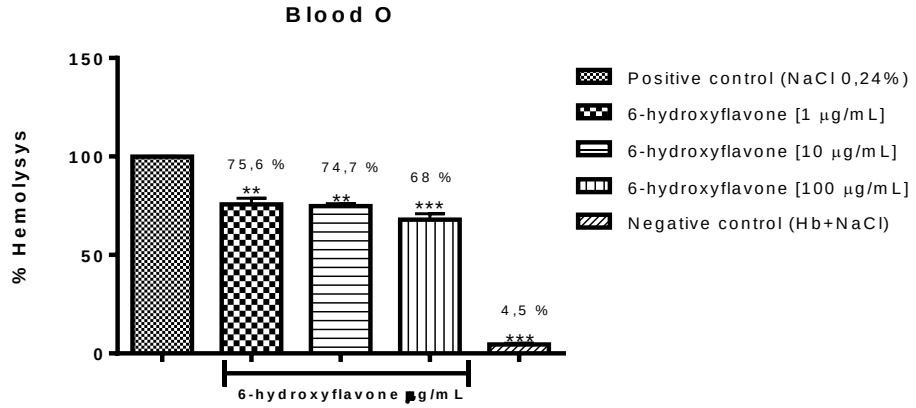


Chart 17. Anti-hemolytic activity of 6-hydroxyflavone on blood types A (a), B (b) and O (c) when in hypotonic solution (NaCl 0.24%). The results are expressed as mean ± SEM. Analysis by ANOVA followed by Dunnett post-test. **p<0.01, ***p< 0.001 (n=3).

From these results, antihemolytic activity can be conferred to the flavonoids 3-hydroxyflavone with blood type A, 5-hydroxyflavone with blood type A and O, and 6-hydroxyflavone with blood type O in the presence of osmotic stress caused by the hypotonic solution (NaCl 0.24%).

3.3. Genotoxicity assay

3.3.1. Investigation of the clastogenic and aneugenic potential of Flavone in mouse erythrocytes

To analyze the genotoxicity of flavone, the micronucleus protocol was performed in order to detect the frequency of formation of these structures in peripheral blood red cells of mice. As shown in Table 3 and figure 5, flavone at a dose of 200 mg/kg administered orally significantly reduced (1.0 ± 0.6 %) the frequency of micronucleus formation compared to the positive control group (9.8 ± 1.5%) represented by the drug cyclophosphamide at 50 mg/kg.

Table 3. Frequency of micronucleated erythrocytes in peripheral blood of male and female Swiss mice.

Tested substances	**Micronucleated erythrocytes (%) (average ± SD)**
Cyclophosphamide (50 mg/kg)	9.8 ± 1.5
Flavone (200 mg/kg)	1.0 ± 0.6***

Results are expressed as a percentage of the mean compared to the positive control group (Cyclophosphamide 50 mg/kg). Test analysis t *** p <0.001 (n = 6). SD = Standard Deviation.

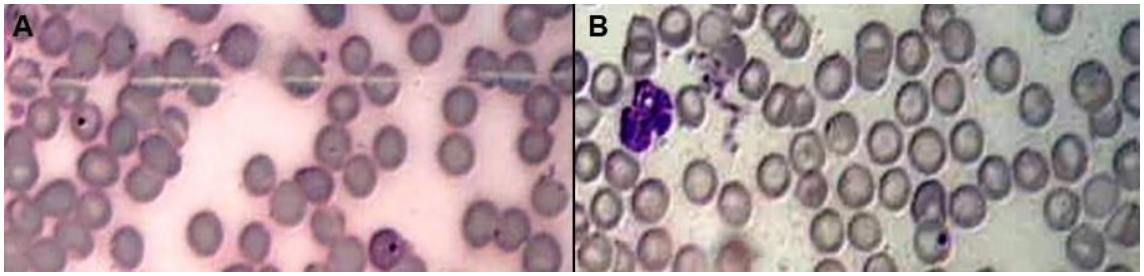


Figure 5. Erythrocytes in mice treated with cyclophosphamide 50 mg/kg (positive control) (A) and flavone 200 mg/kg (B).

The results show that flavone exhibits low genotoxicity at dose of 200 mg/kg administered orally.

4. DISCUSSION

The development of new compounds can be influenced by computational method predictions of ADME/Tox properties; either to avoid potential related problems or to warn that these same problems may well appear experimentally [16].

In silico study of the metabolism, through the application of molecular modeling techniques, is on the rise for developing new drugs [17, 18] and is recommended by various regulatory agencies for technological development studies in order to verify theoretical toxicities of pharmaceuticals in the mammalian metabolic environment [19]. *In silico* toxicological eval-

uations bring a new paradigm to assessing toxicity in which predictions are made using computational tools such as: QSAR (Quantitative Structure-Activity Relationship), REA models (Structure-activity), statistical models, and others [20].

The flavonoid molecular structure analyses performed using the Osiris software made it possible to theoretically characterize the flavone derivates (excluding flavone) as low toxicity risks. Flavone presented considerable mutagenicity and which may be associated to the lower value "Drug-score" (a combination of druglikeness, clog*P*, molecular weight and risk of toxicity) (Chart 1, and Table 1).

The results obtained from the Lipinski "Rule of five" tests demonstrate that all four flavonoids will likely be biologically available on oral administration having met all of the requirements imposed by this rule (table 2). The rule recommends that most "drug-like" molecules have a log*P* value of≤5, a molecular weight of≤ 500u, a hydrogen bond acceptor number of≤10, and a hydrogen bond donor number of ≤5. Molecules violating more than one of these parameters may have problems with bioavailability [21].

As for the *in vitro* toxicological flavonoid analyses, preliminary toxicity tests are an excellent tool to help reduce the use of animals of concern to animal ethics committees [22]. Mammalian erythrocytes represent a good model for evaluating organic and inorganic, and natural or synthetic molecular cytotoxicity, using cellular damage as a measure. Hemolysis, consequent loss of hemoglobin, is the principal signal of blood cell membrane instability [7].

Flavone, 5-hydroxyflavone and 6-hydroxyflavone presented hemolytic potential in types A, B, and O erythrocytes with Rh factor positive (+) or negative (-) in concentrations of 500 μg/mL and 1000 μg/mL as compared to the negative control group (Hb + NaCl 0.9%). The flavonoid 3-hydroxyflavone presented a similar profile, that is, concentrations of 500 μg/mL and 1000 μg/mL induced hemolysis in erythrocytes of types A+, A-, B+, O+ and O- excepting blood type B-, which in a concentration of 100 μg/mL, caused cell lysis as compared to the negative control (charts 2 to 13).

In general, from the results obtained, low to moderate hemolytic activity may be attributed to these flavonoids, since according to Rangel et al. [12] a percentage of hemolysis between 0 and 40% can be considered as low, from 40 to 80% as moderate, and as high, greater than 80%.

The next step was to verify if in a hypotonic solution (0.24% NaCl) flavones would be able to protect erythrocytes from hemolysis in the osmotic fragility test, thus verifying their role in the physiology of erythrocyte membrane function. It is important to point out that in this experiment, concentrations of 500 and 1000 μg/mL are not considered, having led to hemolysis.

The results expressed in charts 14, 15, 16 and 17 confer antihemolytic activity to 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone in blood types A and O, this in view of the osmotic stress caused by the hypotonic medium used. 3-hydroxyflavone at concentrations of 1, 10 and 100 μg/mL demonstrated this potential for type A blood alone; 5-hydroxyflavone protected erythrocytes of blood types A and O; and 6-hydroxyflavone was effective for type O blood cells. By associating the reduced hemolysis data (up to the concentration of 100 μg/mL), low cytotoxicity can be attributed to said flavonoids, suggesting a considerable safety margin for future pharmacological use.

Evidence of low *in vitro* cytotoxicity corroborates the *in silico* results that predicted a possible flavonoid action as a membrane integrity agonist, this was verified by the hemolysis protocol. This applies as well to potential membrane permeability inhibition as detected for 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone in blood types A and O; which act on the functionality of the lipid bilayer. The difference in results can be justified by secondary

metabolite structural diversity which evidences influence of both the presence and hydroxyl-C-ring position variations on the flavonoids, this, besides respective antigen variations (type A), or absence (type O) in the erythrocyte membranes.

Genotoxicity assays to predict possible genotoxic or carcinogenic activity are also important to the development of new drugs, these tests carried out in the early stages; can provide information that may lead to less toxic structures. Genotoxic analysis detects irregularities in eukaryotic organisms, applied to the detection of agents that might cause disorders in the process of chromosome to spindle microfibrils binding, and might also induce chromosomal breaks. An increase in the number of micronucleated cells indicates chromosome damage [8, 23].

Analysis of flavone by micronuclei method indicated significant reduction (1.0 ± 0.6 %) in the frequency micronuclei observed for the oral dose of 200 mg/kg, when compared to the positive control (9.8 ± 1.5%), seen in table 3.

In a study conducted by Resende [24], the findings regarding flavone were similar to those obtained in this work, flavone promoted reductions in micronucleos formation. Resende's study tested the flavonoids quercetin, kaempferol, luteolin, fisetin, chrysin, galangin, flavone, 3-hydroxyflavone, 5-hydroxyflavone and 7-hydroxyflavone for antimutagenic activity using the Ames test, in bacterial strains TA98, TA100 and TA102 in combination with direct and indirect mutagens. The difference between their structures reflects the number and position of hydroxyl groups present in the molecule. All of the compounds showed a protective effect against both direct and indirect action mutagenic agents, and antimutagenic potential in more than one bacterial strain.

In a study by Souza *et al.* [8] the data demonstrated that flavone metabolites are also non-mutagenic and the same data did not demonstrate dose-response correlation, since the increase in flavone dosage did not alter the micronuclei frequency of groups treated with flavone.

In accordance with the *in silico* assays, all of the evaluated flavones met the requirements of Lipinski's Rule of Five, thus presenting good bioavailability with oral administration. Low theoretical toxicity can be attributed to 3-hydroxyflavone, 5-hydroxyflavone and 6-hydroxyflavone, excepting flavone which showed mutagenic potential. Flavonoids presented ideal values of "druglikeness" and "drug-score", which makes them candidates for future medications.

In the *in vitro* assays, the flavones presented low cytotoxicity, inducing moderate hemolysis at the highest concentrations (500 and 1000 μg/mL) and, except for flavone, protected erythrocytes in the presence of hypotonic solution (NaCl 0.24%), indicating that phenols do not compromise the structure or function of the erythrocyte membrane.

The genotixicity test showed that 200 mg/kg flavone did not induce either aneugenic or clastogenic effects in mice erythroblasts. Certain pharmacological effects were influenced by the electron donating group (OH) as well as variation of its position on the flavonoids' benzopyran rings. As flavone significantly reduced the formation of micronuclei in cells, we suggest further research regarding cancer inhibition as promoted by this compound.

CONFLICT OF INTEREST

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

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Artigo de pesquisa cientifica

Determinação da toxicidade aguda de diferentes fases do extrato etanólico de *Sida santaremnensis* frente à *Artemia salina* Leach

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RESUMO

Introdução: as plantas são largamente utilizadas como recurso terapêutico para o tratamento de pessoas com enfermidades. Entretanto, muitas servem como terapia sem os devidos estudos que comprovem sua eficácia e segurança. Com isso, é necessário buscar alternativas que auxiliem na pesquisa da toxicidade das plantas. Nesse contexto, insere-se os estudos com a *Sida santaremnensis*. **Objetivo:** investigar o potencial toxicológico das fases hexânica, diclorometânica, acetato de etila e hidroalcóolica do extrato etanólico bruto de *Sida santaremnensis*, visando a utilização segura dessa espécie em estudos farmacológicos posteriores. **Metodologia:** foi adotado o bioensaio com *Artemia salina* Leach. para determinação da concentração letal 50% (CL_{50}), por ser um teste bastante aceito na literatura científica. A *A. salina* foi utilizada na forma de náuplios e cada concentração das fases foi testada em triplicata e repetida em três experimentos. A CL_{50} foi determinada por regressão não-linear, com intervalo de confiança de 95%. **Resultados:** o extrato etanólico bruto apresentou uma toxicidade elevada representada por uma CL_{50} = 24,4 (20,0-28,9) µg/mL. As fases hexânica, diclorometânica e acetato de etila demonstraram toxicidades moderadas com CL_{50} de 256,2 (188,6-372,9), 123,7 (77,9-196,4) e 100,0 (93,0-107,6) µg/mL, respectivamente. Com a fase hidroalcóolica, obteve-se uma CL_{50} = 566,0 (509,4-629,0) µg/mL, sendo assim considerada de baixa toxicidade. Em todas as avaliações, observou-se uma toxicidade concentração-resposta. **Conclusão:** as fases hexânica, diclorometânica e acetato de etila apresentaram resultados promissores com uma potencial bioatividade para estudos específicos, enquanto a hidroalcóolica é a mais indicada para testes farmacológicos *in vivo* por ter baixa toxicidade.

***Palavras-chaves*:** mortalidade; plantas medicinais; bioatividade.

RESUMEN

Determinación de la toxicidad aguda de diferentes fases del extracto etanólico de *Sida santaremnensis* contra *Artemia salina* Leach

Introducción: las plantas son ampliamente utilizadas como recurso terapéutico para el tratamiento de personas con enfermedades. Muchas sirven como terapia sin los estudios necesarios que demuestren su eficacia y seguridad. Es necesario buscar alternativas que ayuden en la investigación de la toxicidad de las plantas. En este contexto, se insertan estudios sobre el *Sida santaremnensis*. **Objetivo:** investigar el

potencial toxicológico de las fases hexano, diclorometano, acetato de etilo e hidroalcohólica del extracto etanólico crudo de *Sida santaremnensis*, buscando su uso seguro en estudios farmacológicos. **Metodología:** se adoptó el bioensayo con *Artemia salina* Leach. para determinar la concentración letal del 50% (CL_{50}), ya que es una prueba ampliamente aceptada en la literatura científica. Se utilizó *A. salina* en forma de nauplios y la concentración de cada fase se probó por triplicado y se repitió en tres experimentos. La CL_{50} se determinó mediante regresión no lineal, con un intervalo de confianza del 95%. **Resultados:** el extracto etanólico mostró alta toxicidad representada por una CL_{50} = 24,4 (20,0-28,9) μg/mL. Las fases de hexano, diclorometano y acetato de etilo demostraron toxicidades moderadas con CL_{50} de 256,2 (188,6-372,9), 123,7 (77,9-196,4) y 100,0 (93,0-107,6) μg/ml, respectivamente. Con la fase hidroalcohólica se obtuvo una CL_{50} = 566,0 (509,4-629,0) μg/mL, por lo que se considera de baja toxicidad. En todas las evaluaciones se observó toxicidad concentración-respuesta. **Conclusión:** las fases hexano, diclorometano y acetato de etilo mostraron resultados prometedores con potencial bioactividad para estudios específicos, mientras que la fase hidroalcohólica es la más adecuada para pruebas farmacológicas *in vivo* debido a su baja toxicidad.

Palabras clave: mortalidad; plantas medicinales; bioactividad.

SUMMARY

Determination of the acute toxicity of different phases of the ethanolic extract of *Sida santaremnensis* against *Artemia salina* Leach

Introduction: plants are widely used as a therapeutic resource for treating people with illnesses. However, many serve as therapy without the necessary studies to prove their effectiveness and safety. Therefore, it is necessary to look for alternatives that help in researching plant toxicity. In this context, studies on *Sida santaremnensis* are inserted. **Objective:** to investigate the toxicological potential of the hexane, dichloromethane, ethyl acetate and hydroalcoholic phases of the crude ethanolic extract of *Sida santaremnensis*, aiming for the safe use of this species in subsequent pharmacological studies. **Methodology:** the bioassay with *Artemia salina* Leach was adopted to determine the 50% lethal concentration (LC_{50}), as it is a widely accepted test in the scientific literature. *A. salina* was used in the form of nauplii and each phase concentration was tested in triplicate and repeated in three experiments. LC_{50} was determined by non-linear regression, with a 95% confidence interval. **Results:** the crude ethanolic extract showed high toxicity represented by an LC_{50} = 24.4 (20.0-28.9) μg/mL. The hexane, dichloromethane and ethyl acetate phases demonstrated moderate toxicities with LC_{50} of 256.2 (188.6-372.9), 123.7 (77.9-196.4) and 100.0 (93.0-107.6) μg/mL, respectively. With the hydroalcoholic phase, an LC_{50} = 566.0 (509.4-629.0) μg/mL was obtained, thus being considered of low toxicity. In all assessments, concentration-response toxicity was observed. **Conclusion:** the hexane, dichloromethane and ethyl acetate phases showed promising results with potential bioactivity for specific studies, while the hydroalcoholic phase is the most suitable for *in vivo* pharmacological tests due to its low toxicity.

Keywords: mortality; medicinal plants; bioactivity.

1. INTRODUÇÃO

Ao longo dos séculos, os seres humanos têm explorado o uso de diferentes partes dos vegetais como fitomedicamentos. As plantas desempenham um papel fundamental, uma vez que possuem uma diversidade de constituintes bioativos, abrangendo compostos primários e secundários. Notavelmente, os metabólitos secundários exibem uma impressionante riqueza química e taxonômica. Esses metabólitos têm encontrado aplicações multifacetadas em várias áreas, incluindo terapia humana, agricultura, pesquisa científica e medicina veterinária [1].

O Brasil, caracterizado por sua grande diversidade de espécies vegetais, conta com inúmeras plantas medicinais que se aplicam como matérias-primas para a fabricação de fitoterápicos e outros produtos com fins medicamentosos. Esse fato, associado ao crescimento da fitoterapia, correlaciona-se aos avanços ocorridos na área científica, que permitiram a busca por terapias menos industrializadas e mais naturais, destinadas ao atendimento primário à saúde, bem como, ao desenvolvimento de fitoterápicos reconhecidos pela segurança e eficácia [2].

No território brasileiro, apesar do uso de plantas medicinais ser bem difundido, há uma necessidade de estudos maiores para investigar os verdadeiros benefícios das plantas. Existem muitos vegetais que não possuem registros na literatura sobre seus metabólitos, propriedades químicas, farmacológicas e toxicológicas que possam assegurar o uso pela população, sem o risco de apresentar alguma reação indesejada. Diante disso, os estudos em que se avaliam a toxicidade de plantas medicinais são fundamentais, pois colaboram com a elucidação sobre seu uso, eficácia e segurança [3].

Sendo assim, o uso de plantas medicinais muitas vezes é baseado na confiança que a população possui nos conhecimentos tradicionais, acreditando na baixa toxicidade e nos possíveis poucos efeitos indesejáveis das plantas quando comparadas aos medicamentos químicos industrializados [4].

Nesse cenário, pode-se incluir o estudo de *Sida santaremnensis* H. Monteiro, espécie vegetal pertencente à família Malvaceae e conhecida popularmente como "vassourinha" ou "guanxuma". É encontrada no Brasil especialmente nas regiões Nordeste e Sudeste. Caracteriza-se como uma planta herbácea, semiarbustiva, considerada uma erva daninha com pouco valor comercial. Os constituintes químicos descritos para *S. santaremnensis* correspondem a uma mistura de sitosterol 3-O-β-D-glucopiranosídeo e estigmasterol 3-O-β-D-glucopiranosídeo, isolados da fase hexânica do extrato etanólico bruto, bem como kaenperol 3-O-β-D-glicose-6''-α-L-ramnosídeo advindo da fase acetato de etila [5].

Estudos realizados com *S. santaremnensis* revelaram atividade antiulcerogênica, antinociceptiva e antiedematogênica. Além disso, pesquisas relatam como o extrato etanólico, obtido a partir das partes aéreas da *S. santaremnensis*, apresenta atividade inibidora *in vitro* da enzima conversora de angiotensina e promove vasodilatação mediada pelo aumento na produção de óxido nítrico, além de ausência de citotoxicidade, enfatizando a importância em se investigar mais seu potencial farmacológico e o toxicológico. Adicionalmente, pesquisas recentes demonstraram que *S. santaremnensis* possui elevada ação contra macrófagos, atividade anti-inflamatória, e ausência de toxicidade aguda em ratos [6]. Porém, pesquisas de toxicidade com diferentes fases do extrato etanólico bruto de *S. santaremnensis* (EEB*Ss*) ainda não foram publicadas na literatura científica.

Levando-se em consideração o potencial tóxico das plantas, a comunidade científica tem priorizado testes que têm a capacidade de avaliar parâmetros de toxicidade, mas que não utilizam animais maiores. Um dos testes frequentemente utilizados é o da toxicidade aguda com *Artemia salina* Leach. Este bioensaio utiliza um microcrustáceo sensível às variações ambientais, de fácil aquisição e com cistos que permanecem viáveis no estado seco por um considerável período. Ademais, apresenta baixo custo e fornece uma grande quantidade de náuplios, sendo uma alternativa a outros ensaios com animais. Por meio desse bioensaio, é obtida a concentração letal média (CL_{50}), que é a concentração de um produto que causa a letalidade de 50% de uma população em teste, em um tempo pré-estabelecido [7].

As informações anteriormente citadas foram relevantes para a elaboração desse estudo, aliadas ao fato da necessidade constante de investigações que permitam a utilização segura de substâncias vegetais, direcionem os estudos farmacológicos e evitem pesquisas comumente

desencorajadas pelos comitês de ética, por utilizar determinados animais. Sendo assim, o presente trabalho objetivou avaliar a toxicidade de diferentes fases do EEB*Ss* por meio da determinação da CL_{50} no bioensaio com *A. salina*, visando selecionar as fases para estudos farmacológicos posteriores.

2. METODOLOGIA

2.1. Tipo de estudo e obtenção dos extratos/fases

Corresponde a um estudo experimental, quantitativo, realizado em condições laboratoriais específicas e padronizadas. O EEB*Ss* e as fases hexânica, diclorometânica, acetato de etila e hidroalcóolica foram obtidos e cordialmente cedidos pelo Laboratório de Farmacognosia da Unidade Acadêmica de Saúde do Centro de Educação e Saúde / Universidade Federal de Campina Grande.

A espécie *S. santaremnensis* e suas partes aéreas foram coletadas no Parque Piauí na cidade de Teresina/PI. A planta foi devidamente identificada, sendo uma exsicata depositada no Herbário Graziella Barroso, sob o código 21867.

2.2. Incubadora e solução salina

A incubadora para eclosão dos cistos de *A. salina* Leach. consistiu de um recipiente retangular de vidro com uma divisória contendo orifícios de aproximadamente 0,02 cm de espessura e distribuídos uniformemente. O meio para eclosão dos cistos foi uma solução salina preparada com 39 g de sal marinho (Marinex®) e 1 L de água destilada com um pH dentro da faixa limite ideal para eclosão dos cistos, compreendido entre 8,0 e 8,5.

2.3. Obtenção dos náuplios de *A. salina* Leach.

As larvas de *A. salina* foram utilizadas na forma de náuplio. Os cistos foram colocados na incubadora, junto com a solução salina, em um dos lados da incubadora. A parte do sistema contendo os cistos foi recoberta com papel alumínio, para que as larvas, após a eclosão dos cistos, fossem atraídas pela luz do outro lado do sistema, forçando-as a atravessar à divisória. O conjunto permaneceu em incubação sob luz artificial por 24 horas com auxílio de uma lâmpada de 40 W. Com isso, os cistos que eclodiram foram coletados com auxílio de pipetas de Pasteur.

2.4. Preparação da solução inicial

O EEB*Ss* e as suas fases foram solubilizados em água salina artificial a fim de se obter uma solução inicial de 4 mg/mL. Para a solubilização da fase hexânica e acetato de etila foi utilizado um volume de 20 μL do agente solubilizante Cremofor®. O volume da solução inicial foi determinado pela quantidade de tubos e a concentração que se desejou alcançar até um volume final de 5 mL.

Para o EEB*Ss* e cada uma das fases, mediu-se 0,02 g solubilizados em 5 mL da solução salina, obtendo-se a solução inicial. Em seguida, nos tubos de ensaio, foram adicionados 60, 120, 240, 480, 960, 1920, 3840 μL da solução inicial, acrescentando-se 5 mL da solução salina, totalizando 10 mL em cada tudo. Sendo assim, resultou em concentrações de 47, 94, 183, 350, 644, 1110 e 1738 μg/mL para cada extrato/fase testada.

2.5. Bioensaios com *A. salina* Leach.

Para a realização dos bioensaios foi adotada a metodologia descrita por Meyer *et al*. (1982) [8]. Após a obtenção dos náuplios pela eclosão dos cistos, foram adicionados 10 náuplios em cada tubo de ensaio com 10 mL de solução de cada concentração a ser analisada. As concentrações

foram testadas em triplicata e em três testes posteriores, resultando em nove tubos de ensaio para cada concentração de extrato/fase. Em seguida, aguardou-se 24 horas, sendo, então, procedida a contagem de náuplios vivos ou mortos para posterior análise em programa estatístico.

2.6. Grupo controle

Os grupos controles consistiram da mesma solução salina utilizada para a preparação das soluções iniciais. Os grupos controles para as fases hexânica e acetato de etila foram preparados contendo o volume de 20 μL de cremofor®. Dessa forma, os controles consistiram do solvente e agente solubilizador de cada extrato/fase.

2.7. Análise estatística

Os valores de CL_{50} foram calculados por meio da expressão dos resultados como uma percentagem dos controles, e determinados graficamente a partir das curvas concentração-resposta por regressão não-linear, com intervalo de confiança de 95%, utilizando-se o programa *GraphPad Prism*.

3. RESULTADOS

Com a avaliação dos dados obtidos no bioensaio com *A. Salina* e levando-se em consideração a letalidade causada pelo EEB*Ss* e suas fases, observou-se que, todos os extratos causaram um determinado grau de toxicidade, efeito contrário aos grupos controles em que não houve morte dos náuplios de *A. salina*. Ademais, com o aumento das concentrações das fases testadas, houve aumento da toxicidade para *A. Salina*, como demonstrado na tabela 1.

Tabela 1. Quantidade de náuplios mortos pelo tratamento com o EEB*Ss* e suas fases no bioensaio com *A. salina* Leach. (N = 90).

Tratamento	**Concentrações (μg/mL)**							
	0	**47**	**94**	**183**	**350**	**644**	**1110**	**1738**
EEB*Ss*	0	49	70	85	90	90	90	90
Fase hexânica	0	0	6	21	38	53	62	63
Fase diclorometânica	0	0	34	48	50	78	90	90
Fase acetato de etila	0	0	27	57	63	65	67	66
Fase hidroalcoólica	0	0	1	19	37	68	90	90

Os resultados para o EEB*Ss* foram plotados em um gráfico, obtendo-se a figura 1. Ademais, foi possível calcular uma CL_{50} de 24,4 μg/mL com intervalo de confiança de 20,0-28,9 μg/mL.

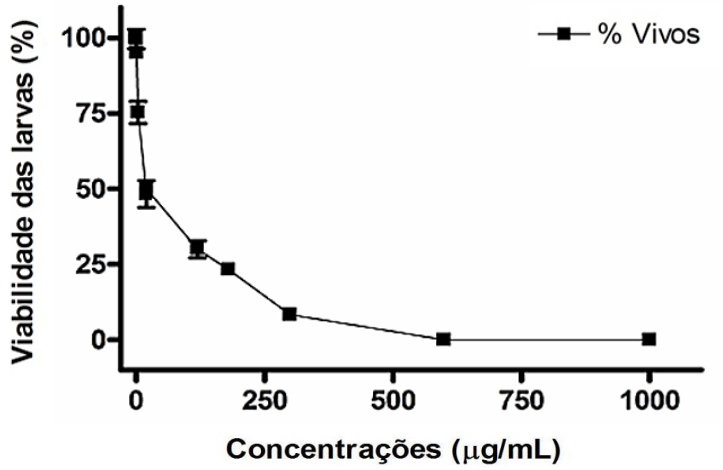

Figura 1. Curva concentração-resposta de diferentes concentrações do EEB*Ss* no bioensaio com *A. salina* Leach.

Na figura 2, constata-se a letalidade para *A. salina* frente à fase hexânica de EEB*Ss*. Por meio da análise estatística, pode-se determinar uma CL_{50} com o valor de 256,2 com intervalo de confiança entre 188,6 e 372,9 μg/mL.

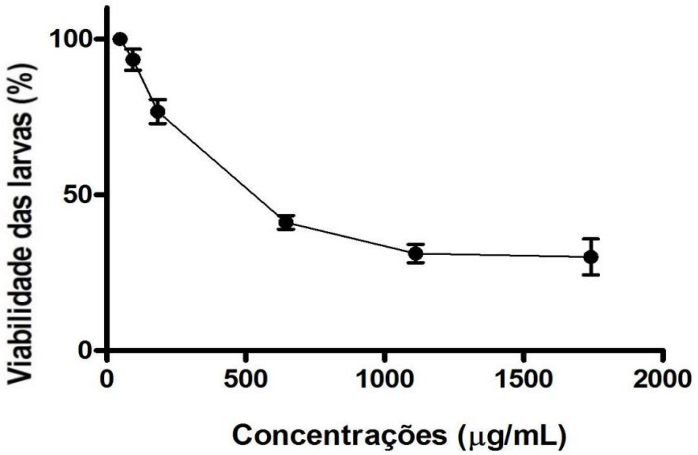

Figura 2. Curva concentração-resposta de diferentes concentrações da fase hexânica do EEB*Ss* no bioensaio com *A. salina* Leach.

Para a fase diclorometânica, foi possível calcular uma CL_{50} de 123,7 (77,9-196,4) μg/mL. Os dados para essa fase estão representados na figura 3.

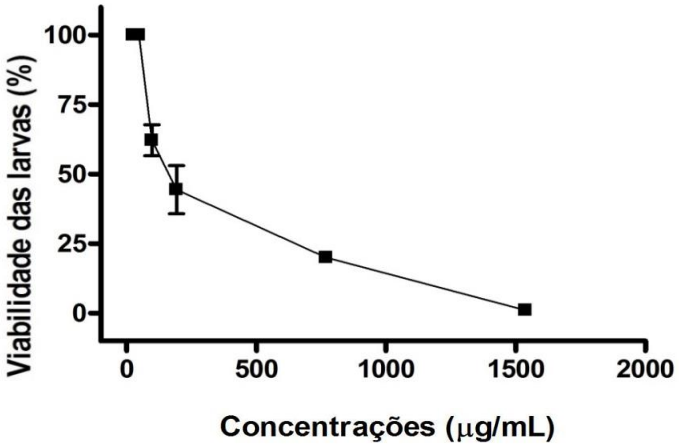

Figura 3. Curva concentração-resposta de diferentes concentrações da fase diclorometânica do EEB*Ss* no bioensaio com *A. salina* Leach.

Na figura 4, pode-se notar o perfil de letalidade da fase acetato de etila para *A. Salina*. Para essa fase, registrou-se uma CL_{50} com o valor de 100,0 (93,0-107,6) μg/mL.

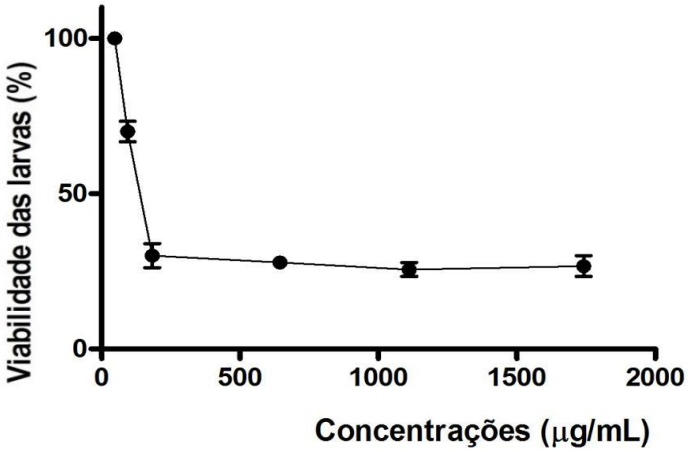

Figura 4. Curva concentração-resposta de diferentes concentrações da fase acetato de etila do EEB*Ss* no bioensaio com *A. salina* Leach.

A CL_{50} para a fase hidroalcóolica de EEB*Ss* foi determinada em 566,0 μg/mL com variação de 509,4 a 629,0 μg/mL. A curva concentração-resposta está representada na figura 5.

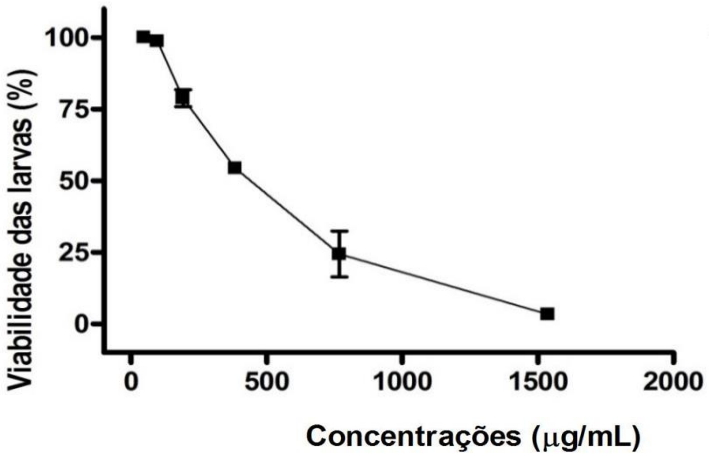

Figura 5. Curva concentração-resposta de diferentes concentrações da fase hidroalcoólica do EEB*Ss* no bioensaio com *A. salina* Leach.

4. DISCUSSÃO

As plantas medicinais são utilizadas de forma isolada ou como matéria prima para síntese de fármacos ou formulação de fitoterápicos, com finalidade de tratamento, cura e prevenção de doenças. O consumo nacional de produtos à base de plantas medicinais atinge cerca de 82% da população brasileira. Com um alto consumo de medicamentos à base de plantas, o uso descontrolado pode representar um risco grave para a saúde da população, pois as plantas medicinais e fitoterápicos representam misturas complexas de substâncias que podem muitas vezes interagir com outras e ter efeito indesejável [9].

Os compostos, incluindo os de origem vegetal, para serem estudados sobre suas potencialidades terapêuticas, necessitam ser avaliados por ensaios de segurança. Para avaliação da toxicidade, tem-se, como exemplo, o teste da *A. salina* que consiste de uma triagem inicial para a avaliação do potencial tóxico de inúmeras substâncias [10]. No presente estudo, utilizou-se esse bioensaio para determinar a toxicidade das fases do EEB*Ss.*

A análise dos resultados obtidos no bioensaio com *A. salina* mostrou a eficiência desse teste, uma vez que, pode-se determinar a toxicidade aguda das fases testadas, semelhante a determinação da toxicidade de outras espécies vegetais e até substâncias sintéticas [11, 12].

A relação entre o grau de toxicidade e a CL_{50} apresentada por extratos de plantas sobre náuplios de *A. salina*, tanto extratos orgânicos, quanto extratos aquosos são bastante aceitos por pesquisadores. Os extratos com valores de CL_{50} acima de 1000 μg/mL, são considerados sem toxicidade significativa, de baixa toxicidade quando a CL_{50} for superior a 500 μg/mL, toxicidade moderada para CL_{50} entre 100 a 500 μg/mL e muito tóxico para valores inferiores a 100 μg/mL [13].

No caso de extratos e substâncias, naturais ou sintéticas, que em concentração de 1000 μg/mL não causam letalidade em nenhum dos 10 náuplios transferidos por tubo, não há necessidade de se calcular a CL_{50}, por serem considerados isentos de toxicidade relevante para *A. salina*. Sendo assim, calculou-se a CL_{50} das fases do EEB*Ss*, já que nas concentrações testadas foi observada morte de náuplios do microcrustáceo.

O EEB*Ss* foi considerado bastante tóxico, com uma CL_{50} classificada como bastante alta, com um valor inferior a 100 μg/mL. A *A. salina* tem a característica de apresentar reduzida tolerância a alterações ambientais e alta especificidade a interferências externas, garantindo a

expressão de resultados nítidos em face de pequenas variações de qualidade do ambiente. Desta forma, foi possível determinar a CL_{50}, observando a sensibilidade do microcrustáceo ao EEB*Ss*, utilizando um teste de letalidade que é considerado essencial como bioensaio preliminar no estudo de compostos com potencial atividade biológica [14].

No tocante as fases de EEB*Ss*, essas foram analisadas seguindo a ordem crescente de polaridade, ou seja, do extrator menos polar para o que apresentava maior polaridade. Assim, seguiu-se a sequência partindo-se da fase hexânica, diclorometânica, acetato de etila e, por fim, a hidroalcóolica. Os solventes mais apolares, como o hexano e diclorometano, apresentam a propriedade de extrair constituintes mais lipofílicos. Por outro lado, solventes polares, a exemplo do acetato de etila, removem constituintes com características mais hidrofílicas, que geralmente são constituídos de um núcleo aglicona acompanhado de um ou mais resíduos de açúcar.

As fases hexânica, diclorometânica e acetato de etila exibiram uma toxicidade considerada moderada, de acordo com os valores de CL_{50}. Por conseguinte, extratos e substâncias com toxicidade moderada, podem ser considerados com uma interessante bioatividade, já que a toxicidade para *A. salina* tem demonstrado uma boa correlação com outras atividades biológicas, como a citotóxica contra tumores humanos, leishmanicida e larvicida [15]. Além disso, permite ainda um direcionamento para outros estudos, de maneira que, substâncias tóxicas de extratos vegetais para o microcrustáceo podem ser testadas para outros fins como: herbicidas e inseticidas [16].

Para a fase hidroalcóolica, obteve-se uma CL_{50} acima de 500 μg/mL. Isto posto, essa fase é considerada de baixa toxicidade para *A. salina*, de acordo com a literatura. Os compostos que demonstram ausência de toxicidade devem prosseguir para estudos farmacológicos e toxicológicos *in vivo* para comprovação da sua eficácia e segurança, bem como, baixa toxicidade demostra o potencial para futuros estudos de identificação de compostos e de determinação de outras atividades biológicas [16]. Essa informação permite inferir que a fase hidroalcóolica é a mais indicada para estudos posteriores com finalidade farmacológica *in vivo*, por ter demonstrado o menor potencial tóxico. Nos grupos controles, não foi observada letalidade, incluindo os grupos que continham Cremofor®, demonstrando que este agente solubilizante não interferiu nos testes.

Os resultados quando convertidos em gráficos demonstraram melhor que o aumento da concentração das fases, provocava crescimento da letalidade em *A. salina* de forma concentração-resposta, indicando toxicidade dependente da concentração [17].

A mortalidade de *A. salina* pelas fases pode estar diretamente ligada a dois fatores: as concentrações testadas, como também a presença de metabólitos prejudiciais à *A. salina* [18].

É provável que o EEB*Ss*, por possuir a totalidade de constituintes extraídos pelo etanol, e que, em seguida, foram divididos nas diferentes fases, tenha provocado uma maior letalidade de *A. salina*, levando a ponderar que esse efeito esteja ocorrendo por sinergismo entre seus metabólitos secundários. O EEB*Ss*, ao ser tratado com soluções extrativas de diferentes polaridades, teve seus constituintes separados nas diversas fases avaliadas nesse estudo. Destarte, cada fase possui constituintes de acordo com a capacidade extrativa de suas características químicas, principalmente, a polaridade. É conhecido que solventes de característica apolar extrai constituintes como esteroides e terpenos de natureza química mais lipofílica. Entretanto, solventes mais polares removem constituintes a exemplo de flavonoides, taninos e substâncias glicosiladas em geral.

Desse modo, na obtenção da fase hidroalcóolica, é possível que os componentes mais tóxicos do EEB*Ss* para *A. salina*, não estejam presentes, visto que essa fase causou menos letalidade, assim como, os constituintes mais polares sejam menos tóxicos para *A. salina*.

Embora a atividade toxicológica de uma planta dependa diretamente dos seus metabólicos contidos em sua composição química, a toxicidade dos compostos sofre influência de outros fatores, como: quantidade a ser ingerida, tolerância, condições de caráter ambiental e a própria intolerância do organismo intoxicado [19]. Por conseguinte, *S. santaremnensis* teve seu perfil fitoquímico estudado e até o momento foram isolados uma mistura de saponinas esteroidais, um flavonoide aglicona e um flavonoide glicosilado [20, 5]. Porém, relacionar a toxicidade de cada fase diretamente a constituintes específicos ainda é temerário, pois requerem estudos mais detalhados.

É importante destacar que esses dados demonstram que, apesar de serem produtos naturais, os extratos e fases vegetais apresentam toxicidade e, por esse motivo, necessitam ser testados, para que sejam utilizados sem riscos [7].

Adicionalmente, o bioensaio também contribui para o monitoramento dos fracionamentos cromatográficos fitoquímicos, ou seja, determinar a fase obtida do EEB*Ss* mais viável para dar continuidade a determinados estudos [21].

Esse trabalho permitiu avaliar a toxicidade e/ou bioatividade de fases do EEB*Ss*, por meio de uma metodologia simples e confiável, sem a prática de utilizar animais vertebrados superiores, proporcionando mais confiança para estudos farmacológicos seguintes.

5. CONCLUSÃO

De acordo com os resultados que permitiram a determinação das CL_{50} das fases do EEB*Ss* por meio do bioensaio com *A. salina*, pode-se concluir que as fases hexânica, diclorometânica e acetato de etila apresentaram toxicidade moderada com potencial bioatividade para estudos específicos. A fase hidroalcoólica apresentou baixa toxicidade, sendo a mais recomendada para estudos farmacológicos que não requeiram atividade tóxica. A toxicidade das fases demonstrou propriedades do tipo concentração-resposta.

Pode-se sugerir uma relação entre a toxicidade e/ou bioatividade das fases com suas propriedades de polaridade. É notória a importância de estudos mais detalhados sobre *S. santaremnensis*, que avaliem o potencial farmacológico e tóxico, não só de seus extratos e fases, mas também de compostos isolados, para uma melhor distinção de suas propriedades químicas e biológicas.

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CONFLITOS DE INTERESSE

Os autores não têm conflitos de interesse a declarar.

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Scientific research article

Peculiarities of the effect of the ethanol extract of quinquelobate motherwort on the behaviour of rats

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SUMMARY

Introduction: Modern medicine is actively researching herbal preparations to correct psychoemotional disorders because they have high bioavailability, mild action, and fewer side effects than synthetic analogues. The quinquelobate motherwort, which contains flavonoids, alkaloids, and iridoids, is a promising sedative, and it is being studied in animal models through behavioral tests. **Objective:** The purpose of this research is to evaluate the peculiarities of the neurotropic activity of quinquelobate motherwort extract on experimental behaviour models for laboratory animals. **Materials and methods:** For the manufacture of the drug, dry herbal starting material (leaves) was used, the extraction of which was carried out in a Soxhlet apparatus with ethyl alcohol 70%. To reduce the effect of alcohol itself on the emotional status of animals, it was evaporated in a water bath; the preparation was replenished with distilled water. **Results:** In the black and white chamber test, after administration of the quinquelobate motherwort extract, there was an increase in the time spent in the light compartment by almost 6 times and a simultaneous decrease in the duration of the animals' stay in the dark compartment; the number of exits to the light compartment significantly increased and the number of urinations decreased. In the elevated cruciform maze test, the latent period significantly decreased, which indicates a decrease in the decision-making time for the implementation of an active or passive behaviour strategy; the number of freezing acts significantly increased. In the open field test system, after administration of the tested extract, a significant increase in horizontal motor activity was observed; at the same time, the number of visits to peripheral squares and the time spent in the centre increased. **Discussion:** In accordance with the existing interpretation of such data, the dynamics in behavioural reactions after administration of the herbal preparation indicated a decrease in fear and anxiety responses in all experimental models used, as well as an increase in orientative-trying activity, which collectively indicates the anti-stress effect of the studied extract. **Conclusion:** In addition to the well-known sedative properties of quinquelobate motherwort, its anxiolytic effects — demonstrated through significant antiphobic and antidepressant impact — have been observed in a series of stress-inducing behavioural tests.

Keywords: quinquelobate motherwort; laboratory rats; emotional behaviour; open field; elevated cruciform maze; black and white chamber.

RESUMEN

Peculiaridades del efecto del extracto etanólico de agripalma quinquelobato en el comportamiento de ratas

Introducción: La medicina moderna investiga activamente las preparaciones herbales para corregir trastornos psicoemocionales debido a su alta biodisponibilidad, acción suave y menos efectos secundarios que los análogos sintéticos. La agripalma quinquelobato, que contiene flavonoides, alcaloides e iridoides, es un sedante prometedor y se está estudiando en modelos animales mediante pruebas de comportamiento. **Objetivo:** El propósito de esta investigación es evaluar las peculiaridades de la actividad neurotrópica del extracto de agripalma quinquelobato en modelos experimentales de comportamiento en animales de laboratorio. **Materiales y métodos:** Para la fabricación del fármaco, se utilizó materia prima herbácea seca (hojas), cuya extracción se realizó en un aparato Soxhlet con alcohol etílico al 70 %. Para reducir el efecto del alcohol en el estado emocional de los animales, se evaporó al baño maría; la preparación se reabasteció con agua destilada. **Resultados:** En la prueba de la cámara blanca y negra, tras la administración del extracto de agripalma quinquelobato, se observó un aumento de casi 6 veces en el tiempo de permanencia en el compartimento iluminado y una disminución simultánea de la duración de la estancia de los animales en el compartimento oscuro; el número de salidas al compartimento iluminado aumentó significativamente y el número de micciones disminuyó. En la prueba del laberinto cruciforme elevado, el período de latencia disminuyó significativamente, lo que indica una disminución del tiempo de decisión para la implementación de una estrategia de comportamiento activo o pasivo; el número de actos de inmovilización aumentó significativamente. En el sistema de prueba de campo abierto, tras la administración del extracto probado, se observó un aumento significativo de la actividad motora horizontal; al mismo tiempo, aumentó el número de visitas a los cuadrados periféricos y el tiempo de permanencia en el centro. **Discusión:** De acuerdo con la interpretación existente de estos datos, la dinámica de las reacciones conductuales tras la administración del preparado herbal indicó una disminución de las respuestas de miedo y ansiedad en todos los modelos experimentales utilizados, así como un aumento de la actividad orientadora, lo que, en conjunto, indica el efecto antiestrés del extracto estudiado. Conclusión: Además de las conocidas propiedades sedantes de la agripalma quinquelobato, sus efectos ansiolíticos, demostrados mediante un significativo efecto antifóbico y antidepresivo, se han observado en una serie de pruebas conductuales de inducción de estrés.

Palabras clave: agripalma quinquelobato; ratas de laboratorio; comportamiento emocional; campo abierto; laberinto cruciforme elevado; cámara en blanco y negro.

RESUMO

Peculiaridades do efeito do extrato etanólico da erva-mãe quinquelobato sobre o comportamento de ratos

Introdução: A medicina moderna pesquisa ativamente preparações fitoterápicas para corrigir distúrbios psicoemocionais, pois apresentam alta biodisponibilidade, ação suave e menos efeitos colaterais do que análogos sintéticos. A erva-mãe quinquelobato, que contém flavonoides, alcaloides e iridoides, é um sedativo promissor e está sendo estudada em modelos animais por meio de testes comportamentais. **Objetivo:** O objetivo desta pesquisa é avaliar as peculiaridades da atividade neurotrópica do extrato da erva-mãe quinquelobato em modelos experimentais de comportamento com animais de laboratório. **Materiais e métodos:** Para a fabricação do fármaco, utilizou-se material vegetal seco (folhas), cuja extração foi realizada em aparelho Soxhlet com álcool etílico 70%. Para reduzir o efeito do álcool no estado emocional dos animais, este foi evaporado em banho-maria; a preparação foi completada com água destilada. **Resultados:** No teste de câmara preta e branca, após a administração do extrato de erva-mãe quinquelobato, houve um aumento no tempo gasto no compartimento de luz em quase 6 vezes e uma diminuição simultânea na duração da permanência dos animais no compartimento escuro; o número

de saídas para o compartimento de luz aumentou significativamente e o número de micções diminuiu. No teste do labirinto cruciforme elevado, o período latente diminuiu significativamente, o que indica uma diminuição no tempo de tomada de decisão para a implementação de uma estratégia de comportamento ativo ou passivo; o número de atos de congelamento aumentou significativamente. No sistema de teste de campo aberto, após a administração do extrato testado, foi observado um aumento significativo na atividade motora horizontal; ao mesmo tempo, o número de visitas a quadrados periféricos e o tempo gasto no centro aumentaram. **Discussão:** De acordo com a interpretação existente de tais dados, a dinâmica nas reações comportamentais após a administração da preparação à base de ervas indicou uma diminuição nas respostas de medo e ansiedade em todos os modelos experimentais usados, bem como um aumento na atividade de tentativa de orientação, o que indica coletivamente o efeito antiestresse do extrato estudado. **Conclusão:** Além das conhecidas propriedades sedativas da erva-mãe quinquelobata, seus efeitos ansiolíticos — demonstrados por meio de impacto antifóbico e antidepressivo significativo — foram observados em uma série de testes comportamentais indutores de estresse.

Palavras-chave: erva-mãe quinquelobata; ratos de laboratório; comportamento emocional; campo aberto; labirinto cruciforme elevado; câmara preta e branca.

1. INTRODUCTION

Numerous disorders of the central nervous system affecting the psychoemotional state of an individual and triggering various somatic manifestations — such as insomnia, headaches, and emotional instability — highlight the need for the development of modern targeted medicines. On the other hand, the emergence of the 'drug disease/syndrome' has enhanced interest in herbal medicines. The use of innovative technologies in the production of herbal remedies, including the selection of plant starting materials, processing methods, isolation and use of secondary plant metabolites, etc. significantly improve the quality of modern medicinal products. Phytotherapy is currently used both in the treatment and in prevention of various diseases, contributing to an increase in the adaptive and protective-compensatory capabilities of the body [1, 2].

Drugs that actively influence the central nervous system hold a special place among phytopreparations. These neurotropic compounds modulate various processes of the brain, affecting both mental and motor activities with diverse action profiles. Among the wide range of drugs with neurotropic effects, plant-based medicines occupy a large niche. This is due to their high bioavailability to the human body, possible complex organ-protective effect, minimal number of adverse reactions, as well as the relative cheapness of herbal starting materials in contrast to synthetic ones [3-5].

Sedatives of plant origin are widely used for correction of psychoemotional conditions of various genesis; they can be sold in pharmacies without prescriptions and do not require medical advice. The quinquelobate motherwort *(Leonurus quinquelobatus)* meets these requirements, possessing, as it is believed, sedative and less pronounced antidepressant properties [6]. It is a perennial herbaceous plant of the *Labiaceae* family with a wide natural area; its herbage and leaves are used for practical purposes. The wide range of the plant's chemical components (flavonoids, alkaloids, iridoids) determines the variety of its optimizing and medicinal effects on the human body. In particular, the neurotropic effects of the motherwort are due to the presence of iridoids that determine the bitterness of drugs made on its basis (marubin, leonuride, garpagide, ajugol, ayugoside, etc.). In its chemical structure, *Leonurus quinquelobatus* has tannins, saponins, sugars, vitamins of group C and A, flavonoids, essential oils [7, 8].

The physiological effects of sedation on the nervous system are well known to be medi-

ated by enhancing either excitation or inhibition processes. Depending on the drug's mechanism of action, this can lead to different functional outcomes, ranging from drowsiness to increased alertness, with various intermediate tonic effects possible. The existing data on the possibility of using quinquelobate motherwort as a component of a complex plant preparation creates the need to quantify manifestations of its pharmacological activity *in vivo*. The high validity of physiological behavioural tests and the authors' skills in using such test models had determined the choice of an experimental approach to assess the immediate effects of motherwort on animal behavioural reactions on three test systems: black and white chamber (BWC), elevated cruciform maze (ECM) and open field (OF).

2. MATERIALS AND METHODS

The purpose of this research is to determine the peculiarities of the neurotropic activity manifestation of the ethanol extract of quinquelobate motherwort on behavioural models for rodents.

The studies were performed on 30 albino Wistar rats (males, weight 180-220 g). Animal care in a vivarium, the conditions of their adaptation to the beginning of the experiment and the experiment procedure itself met the requirements for work with animals in accordance with Order No. 199н of the Ministry of Healthcare of the Russian Federation dated 01.04.16 On Approval of Rules of Good Laboratory Practice [9]. The experiment procedure took place under standard conditions for maintaining the constancy of environmental factors in the laboratory environment, including illumination, sound insulation, conditions, feeding patterns (balanced food appropriate for rodents), etc.

The object of the study is an ethanol extract of quinquelobate motherwort made in a Soxhlet extractor. To do this, the dry herbal starting material was crushed and poured into a cartridge of filter paper. The filled cartridge was placed in the apparatus and extracted with a 70%-solution of ethyl alcohol for 14-15 cycles. To avoid the influence of ethyl alcohol on the emotional status of animals, ethanol was evaporated in a water bath; the volume of the evaporated extractant was then replenished with distilled water. The animals received the tested phytosolution by voluntary consumption. To do this, each rat was individually trained to drink from a syringe, and later, at a fixed time, the animals consumed 1 ml of the extract [10]. All experiments were performed at the same time (in the period from 12:00h to 16:00h).

The emotional status of the animals was evaluated before and after administration of the solution (60 minutes after administration) on three behavioural models: BWC, ECM and OF. Testing and interpretation of the obtained results were carried out according to the methods described in previously published studies [10, 11]. Based on the literature on the stress associated with behavioural models and the results of our own research, the entire cycle of experimental studies was conducted under a scheme that incorporates the following sequence of test systems: BWC – ECM – OF (from least to most stressful). A digital video system No. VS1304 was used to record the behaviour of animals during testing.

Statistical analysis of the data was carried out using the Excel and SPSS software according to the Student's t-criterion [12]. The differences were considered significant at $p \leq 0.05$.

3. RESULTS

At the first stage, the behavioural characteristics of the albino laboratory rats were analysed before and after the administration of the extract in the black and white chamber test (Table 1). The test allows us to evaluate the anxiety level in test animals and identify the possible anxiolytic effect of the drug under study. In the emotional status of intact animals, we observed anxiety-related responses caused by the stress of the experimental model. Those animals preferred to be in the dark compartment of the chamber and rarely peeked out from there; urination and defecation were recorded during the test period. After administration of the prescribed dosage of dealcoholized motherwort extract, there was an increase in the time spent in the light compartment of the chamber by almost 6 times and a simultaneous significant decrease in the duration of the animals' stay in the dark chamber ($p<0.001$). Along with these changes, there was a significant increase in the time and number of exits from the dark compartment of the system ($p<0.05$) and a decrease in the number of urinations and defecations ($p<0.01$).

Table 1. Features of the effect of herbal extract of the quinquelobate motherwort on the behaviour of rats in the black and white chamber test

Behavioural characteristics	**Control (intact) n=15**		**Experiment n=15**
	Statistical indicators		
	M±m	**p**	**M±m**
Latent period of entering the dark compartment, with	11.04±3.55	p>0.05	11.83±3.1
Number of peeks from the dark compartment, pcs	2.10±0.74	p>0.05	3.3±0.55
Duration of peeks from the dark compartment, s	5.81±3.10	**p<0.001**	32.6±4.7
Number of exits from the dark compartment, pcs	0.75±0.11	**p<0.05**	1.7±0.32
Time spent in the light compartment, s	19.8±3.22	**p<0.001**	125.86±15.14
Time spent in the dark compartment, s	272.71±11.4	**p<0.001**	130.57±9.53
Number of defecations, pcs	0.16±0.1	**p<0.05**	0±0
Number of urinations, pcs	1.88±0.32	**p<0.01**	0±0

Source: compiled by the authors

Note: hereafter M is the arithmetic mean, m is the sampling error; p is the result significance level.

These facts collectively suggest a reduction in anxiety levels and a decrease in autonomic nervous system reactions to stress following the administration of *Leonurus quinquelobatus* extract.

Table 2 shows the characteristics of animal behaviour in the elevated cruciform maze test before and after administration of the extract. As can be seen from the data in the table, the latent period significantly decreased, which indicates a decrease in the decision-making time for the implementation of an active or passive behaviour strategy. The number of freezing acts has also significantly increased which indicates a possible sedative effect of the motherwort extract. The change in other parameters was not statistically significant.

Table 2. Features of the effect of herbal extract of the quinquelobate motherwort on the behaviour of rats in the elevated cruciform maze test

Behavioural characteristics	**Control (intact) n=15**		**Experiment n=15**
	Statistical indicators		
	M±m	**p**	**M±m**
Latent period, s	2.79±0.29	**p>0.05**	0.23±6.78
Number of visits to open maze arms, pcs	1±0	p>0.05	1.0±0.4
Number of visits to closed maze arms, pcs	2.75±0.64	p>0.05	2.0±0.4
Duration of visits to open maze arms, s	9.95±5.46	p>0.05	74.67±10.2
Duration of visits to closed maze arms, s	282.34±9.71	p>0.05	158.3±32.4
Duration of the acts of freezing behaviour, s	68.89±19.35	p>0.05	164.5±21.1
Number of the acts of freezing behaviour, pcs	3.75±0.73	**p<0.05**	6.5±0.4
Number of rears, pcs	1.75±0.21	p>0.05	2.5±0.6
Number of defecations, pcs	0±0	p>0.05	0±0
Number of urinations, pcs	1±0.35	**p<0.05**	0±0

Source: compiled by the authors.

Table 3 shows the results obtained on the open field test system. This model allows us to evaluate the motor activity of rats, expressed in the number of intersections of squares and racks; research activity – by the number of examinations of holes in the bottom of the arena. Among the three models used in this study, the open field model induces the most pronounced stress. This is due to both the size of the chamber and its illumination, as a brightly lit open space is an inherently extreme stimulus for rats, triggering psychomotor and autonomic responses characteristic of fear and stress reactions (Table 3, control group of animals).

Table 3. Features of the effect of herbal extract of the quinquelobate motherwort on the behaviour of rats in the open field test

Behavioural characteristics	**Control (intact) n=15**		**Experiment n=15**
	Statistical indicators		
	M±m	**p**	**M±m**
Latent period of exits to the periphery, s	2.99±0.49	p>0.05	2.4±0.16
Horizontal motor activity, pcs	20±2.93	**p<0.05**	33.5±3.8
Vertical motor activity, pcs	1.75±0.41	**p<0.05**	2.50±2.06
Number of grooming acts, pcs	1±0.35	**p<0.05**	3.0±0.8
Duration of grooming acts, s	15.19±5.15	**p<0.05**	27.7±1.06
Number of the acts of freezing behaviour, pcs	6±0.5	p>0.05	4.0±0.5
Duration of the acts of freezing behaviour, s	104.68±25.91	p>0.05	67.95±12.12
Number of defecations, pcs	0.25±0.21	**p<0.05**	0±0
Number of urinations, pcs	0.25±0.21	**p<0.05**	0±0
Number of well inspections, pcs	1±0.35	**p<0.05**	1.5±0.4
Number of crossed central squares, pcs	2.75±0.41	**p<0.05**	4.0±1.6
Number of crossed peripheral squares, pcs	17.25±2.76	**p<0.05**	29.5±2.76
Time spent at the centre, s	0.25±0.21	**p<0.05**	0.5±0.4

Source: compiled by the authors.

After administration of the tested herbal preparation, the animals showed an increase in horizontal and vertical motor activity ($p<0.05$); the number of grooming acts and its duration

($p<0.005$), as well as the time spent in the centre ($p<0.05$), increased. The number of the inspected wells and the number of the crossed central and peripheral squares significantly increased ($p<0.05$). At the same time, the number of defecations and urinations significantly decreased ($p<0.05$).

4. DISCUSSION

Drugs with a strong anxiolytic effect are known to manage psychoemotional tension by reducing anxiety and fear (antiphobic effect), and associated symptoms, while also providing a sedative effect [13, 14]. It is believed that the main effects of anxiolytic drugs are achieved by reducing the excitability of subcortical emotionogenic structures of the brain, including the limbic system and the diencephalic brain (thalamus, hypothalamus). The main neurochemical effect is realized through the inhibitory GABAergic system, less pronounced effect – through the noradrenergic, dopaminergic and serotonergic systems. Some anxiolytics are capable of a sedative (calming) effect due to their impact on the reticular activating system of the brain. Various herbal preparations have neurotropic effects ambiguous in terms of their nature and severity.

Motherwort tincture belongs to the category of the most commonly used drugs with a pronounced sedative effect. It is believed to regulate the functional state of the central nervous system by reducing nerve cell excitability, offering a calming effect and, depending on the dose, a hypnotic effect. We analysed the features of behavioural and vegetative reactions of animals in stressful situations after administration of the tincture of *Leonurus quinquelobatus*, taking into account its sedative effect as well as possible targeted anxiolytic action. It is believed that the open field, elevated cruciform maze and dark/light chamber tests are important for assessing anxiety, which is an endophenotype of depression and is important for antidepressant screening [12]. The series of experimental behavioural models made it possible to identify a set of physiological reactions of rats that are most clearly manifested under the influence of a herbal preparation. The experimental models themselves, due to the features of their construction (large, brightly lit, open spaces, heights), were stress-inducing for animals, causing manifestations of fear, anxiety, discomfort, etc.

In general, the set of animal reactions evaluated according to these test systems can be grouped into three behavioural blocks: locomotor or motor activity (horizontal motor activity, latent period of the first movement), exploratory and searching activity (rears and dippings from the open maze arms, inspection of wells), and emotional reactivity (time, number of visits of open and closed maze arms, time at the centre, the number and duration of freezing behaviour acts, frequency of urination and defecation). Such behavioural and vegetative reactions were observed to varying degrees across all three experimental models used for testing. For instance, in the BWC test, after administration of the drug, the duration of staying in the dark / light compartments of the chamber changed significantly: the duration of peeking and coming out of the dark part increased, the time spent in the light part increased (accordingly, the time in the dark compartment decreased). At the same time, a decrease in urination and defecation was observed. This dynamics of animal behaviour in a stressful situation can be regarded as a manifestation of a significant antiphobic effect mediated by the action of the quinquelobate motherwort extract [15]. Naturally, the decrease in the fear reaction was accompanied by other anti-stress manifestations (reduction of anxiety and uneasiness). This fact is supported by the fact of changes in vegetative reactions (urination, defecation). In the ECM test, after administration of the herbal preparation, the changes were less pronounced, however,

the latent period of being in the centre decrease, which, according to the accepted test interpretation of behaviour, is regarded as an increase in the speed of decision-making in a stressful situation [16]. According to the OF test, significant differences before and after administration of the phytopreparation were recorded in almost all parameters, both in the behaviour of the animals and in their vegetative reactions. An increase in the frequency of horizontal (sum of crossed squares) and vertical motor activity indicates an increase in locomotor activity; an increase in the number of inspected wells, sniffing of the wells and vertical motor activity indicate an increase in the exploratory and searching reactions [17]. Such behaviour caused less frequent urination and defecation and lowered the number of freezing acts in rats. These changes are the result of shifts in the animals' emotional state, marked by reduced anxiety, fear, and depression caused by the situation of the experimental model that is inadequate for animals. This dynamics in the behavioural and vegetative reactions of albino laboratory rats due to the intake of dealcoholized ethanol extract of *Leonurus quinquelobatus* serve as a confirmation of a pronounced anxiolytic effect of the drug. The increase in locomotor, exploratory and searching activity and the nature of the dynamics of vegetative reactions after administration of the drug are indicative of the animals' recovery from stress caused by the abnormal conditions of the experimental setup.

5. CONCLUSION

The quinquelobate motherwort extract induced both sedative and anxiolytic effects in rats, as evidenced by various behavioural and physiological responses across all three stress-inducing behavioural models. The most significant reactions of the anti-stress activity of ethanol extract from *Leonurus quinquelobatus* were found in the models of BWC and OF. After administration of the studied drug, the behaviour of animals in the BWC test system significantly changed: rats started preferring the open test spaces (reduction of anxiety and fear), and peeking out of the dark compartment of the chamber became more frequent (exploratory activity). In the OF test, an increase in locomotion and exploratory and searching activity was observed in rats due to the tested phytopreparation. An increase in the number of freezing acts in the ECM can be considered as a sedative effect of the motherwort extract. In all three test systems, the administration of the herbal preparation led to changes in the animals' vegetative status, indicating a reduction in emotional excitability. Behavioural reactions indicating a decrease in decision-making time, regardless of the active or passive strategy of behaviour (latent period), allow us to speak about the preservation of sufficient performance capabilities of cortical cells when exposed to a herbal medicinal product with a pronounced sedative effect.

CONFLICT OF INTEREST

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

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Artículo de investigación tecnológica

Elaboración de una barra de cacao (*Theobroma cacao*) naturalmente endulzada y su efecto hipoglucemiante

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RESUMEN

Introducción: El cacao (*Theobroma cacao*), contiene abundantes flavonoides que son responsables de la reducción del estrés oxidativo. Las barras de chocolate con una mezcla de cacao superior al 45% son buenas para la salud humana debido a su alto contenido de antioxidantes. El uso de modelos que estudian el efecto del aumento de la concentración de glucosa en sangre ha permitido establecer que los procesos oxidativos están involucrados en la patogénesis, complicaciones y mal pronóstico de la *diabetes mellitus*. Se ha entendido que, la sobreproducción de especies reactivas de oxígeno debido al aumento de la actividad de la cadena transportadora de electrones, la vía del sorbitol, la autooxidación de la glucosa, la glicación de proteínas y la reducción de las defensas antioxidantes, generan un estado prooxidante que causa daño oxidativo a ácidos nucleicos, proteínas, carbohidratos y lípidos, contribuyendo al desarrollo de la diabetes. **Objetivo:** Evaluar el efecto hipoglucemiante de barras de chocolate elaboradas con cacao (*Theobroma cacao*) endulzadas con diferentes edulcorantes naturales en un modelo de ratas diabéticas. **Métodos:** Se elaboraron barras de cacao usando dos concentraciones; 50% y 85% endulzadas con algarrobina y yacón. Estas barras fueron evaluadas organoléptica y microbiológicamente. Se indujo diabetes experimental en ratas inyectando aloxano monohidratado (100 mg/kg) por vía intraperitoneal y se evaluó su efecto hipoglucemiante. **Resultados:** Las barras de cacao elaboradas al 50% y 85% demostraron buenas características organolépticas e inocuidad microbiológica. Se evidenció mayor efecto hipoglucemiante con las barras de cacao de mayor concentración. **Conclusiones:** Se ha podido evidenciar un efecto hipoglucemiante en las ratas tratadas con las barras de cacao al 85% endulzadas con algarrobina.

Palabras clave: diabetes; hipoglucemiante; hiperglicemia; edulcorante natural; cacao.

SUMMARY

Preparation of a cocoa bar (*Theobroma cacao*) naturally sweetened with a hypoglycemic effect

Introduction: Cocoa (*Theobroma cacao*) contains abundant flavonoids responsible for reducing oxidative stress. Chocolate bars with a cocoa mix of more than 45% are good for human health due to their high antioxidant content. Using models studying the effect of increased blood glucose concentration has established that oxidative processes are involved in the pathogenesis, complications, and poor prognosis of *diabetes mellitus*. It has been understood that the overproduction of reactive oxygen species due to increased activity of the electron transport chain, the sorbitol pathway, autoxidation of glucose, glycation of proteins, and reduction of antioxidant defenses generate a pro-oxidant state that causes oxidative damage to nucleic acids, proteins, carbohydrates, and lipids, contributing to the development of diabetes. **Objective:** To evaluate the hypoglycemic effect of chocolate bars made with cocoa (*Theobroma cacao*) sweetened with different natural sweeteners in a diabetic rat model. **Methods:** Cocoa bars were elaborated using 50% and 85% concentrations, sweetened with algarrobin and yacon. These bars were evaluated organoleptically and microbiologically. Experimental diabetes was induced in rats by injecting alloxan monohydrate (100 mg/kg) intraperitoneally, and the hypoglycemic effect was assessed. **Results:** Cocoa bars prepared at 50% and 85% showed good organoleptic characteristics and microbiological safety. A more significant hypoglycemic effect was evidenced with the cocoa bars of higher concentration. **Conclusions:** A hypoglycemic effect was evidenced in rats treated with the 85% cocoa bars sweetened with algarrobin.

Keywords: diabetes; hypoglycemic; hyperglycemia; natural sweetener; cocoa.

RESUMO

Produção de uma barra de cacau naturalmente adoçada (*Theobroma cacao*) e seu efeito hipoglicemiante

Introdução: O cacau (*Theobroma cacao*) contém abundantes flavonoides que são responsáveis por reduzir o estresse oxidativo. Barras de chocolate com teor de cacau superior a 45% são boas para a saúde humana devido ao seu alto teor de antioxidantes. A utilização de modelos que estudam o efeito do aumento da concentração de glicose no sangue estabeleceu que processos oxidativos estão envolvidos na patogênese, complicações e mau prognóstico do diabetes mellitus. Entende-se que a superprodução de espécies reativas de oxigênio devido ao aumento da atividade da cadeia de transporte de elétrons, da via do sorbitol, da autoxidação da glicose, da glicação de proteínas e da redução das defesas antioxidantes, gera um estado pró-oxidante que causa danos oxidativos aos ácidos nucleicos, proteínas, carboidratos e lipídios, contribuindo para o desenvolvimento do diabetes. **Objetivo:** Avaliar o efeito hipoglicemiante de barras de chocolate feitas com cacau (*Theobroma cacao*) adoçadas com diferentes adoçantes naturais em um modelo de rato diabético. **Métodos:** Barras de cacau foram produzidas utilizando duas concentrações; 50% e 85% adoçados com alfarroba e yacon. Essas barras foram avaliadas organolepticamente e microbiologicamente. O diabetes experimental foi induzido em ratos pela injeção intraperitoneal de monoidrato de aloxana (100 mg/kg) e seu efeito hipoglicêmico foi avaliado. **Resultados:** Barras de cacau elaboradas com 50% e 85% apresentaram boas características organolépticas e segurança microbiológica. Um maior efeito hipoglicêmico foi evidente com barras de cacau com maior concentração. **Conclusões:** Foi observado efeito hipoglicemiante em ratos tratados com barras de cacau 85% adoçadas com alfarroba.

Palavras-chave: diabetes; hipoglicêmico; hiperglicemia; adoçante natural; cacau.

1. INTRODUCCIÓN

Durante las últimas décadas, el cacao y los productos a base de cacao, especialmente el chocolate, se han convertido en uno de los alimentos más atractivos [1]. Los granos de cacao contienen componentes químicos importantes como la manteca de cacao, polifenoles (flavonoides), teobromina (en pequeñas cantidades), cafeína, pectina, y ácidos grasos [2]. Estos componentes influyen en el sabor particular del cacao y le otorgan beneficios como hipoglucemiante, con gran potencial en las dietas para los diabéticos [3, 4].

Existen numerosos estudios sobre los efectos saludables de los polifenoles del cacao, y han demostrado que la suplementación con extracto de cacao a ratas diabéticas obesas produce a corto plazo el control de la glucosa postprandial reduciendo la oxidación plasmática debido a sus polifenoles y metilxantinas [5]. Asimismo, los flavonoides como la epicatequina reducen los niveles de glucosa y triglicéridos posprandiales en sangre, disminuyen el estrés oxidativo, mejoran el equilibrio de la glucemia disminuyendo la absorción intestinal y la digestión de los carbohidratos [6].

La *diabetes mellitus*, es un grave trastorno metabólico crónico que se caracteriza por una hiperglucemia resultante de una deficiencia en la secreción de insulina y/o disminución de la reacción de los órganos a la insulina. Además de la dieta y el estilo de vida, el control y la prevención de la hiperglucemia son enfoques primarios en la gestión de la *diabetes mellitus* [7]. Según Sheehan (2003) [8], los agentes que pueden contribuir a mejorar el control glucémico funcionan como secretagogos de insulina (aumentan la secreción de insulina), como sensibilizadores de insulina (aumentan la acción de la insulina) o como inhibidores de la absorción de glucosa (disminuyen la necesidad de insulina). Últimamente se extraen cada vez más compuestos naturales de origen animal y vegetal con propiedades antidiabéticas [9-13]. Se sabe que los granos de cacao son una fuente rica de polifenoles, especialmente (-)-epicatequina [14, 15], en un estudio anterior se demostró que una dieta con extractos ricos en polifenoles de cacao reducía los niveles de glucosa y los perfiles lipídicos en ratas diabéticas inducidas por estreptozotocina (STZ) [16]. Con esta investigación se pretende proporcionar una nueva alternativa alimentaria apetecible y sana para la población en general, y especialmente para los diabéticos, a base del fruto del cacao (*Theobroma cacao*), adicionado de edulcorantes naturales como el yacón (*Smallanthus sonchifolius*) y la algarrobina, endulzantes con múltiples propiedades beneficiosas para la salud entre ellos efectos hipoglucemiantes.

2. MATERIALES Y MÉTODOS

2.1. Obtención de la muestra

Aproximadamente 20 kilogramos de granos de cacao chuncho (*Theobroma cacao*) fueron obtenidos del distrito de Echarate ubicado a 1162 m.s.n.m., en la provincia de la Convención, región del Cusco (Figura 1a y 1b).

Figura 1. (a) Árbol de cacao con mazorcas maduras; **(b)** Mazorca de cacao con semillas; **(c)** Secado de granos de cacao**.**

2.2. Obtención de la pasta de cacao

Luego de inspeccionar los frutos para establecer la madurez de la pulpa y su aspecto, se procedió a despulpar y extraer los granos de cacao. Se colocaron en bandejas para el secado (Figura 1c). Al cabo de aproximadamente 5 días, los granos de cacao se tostaron a fuego lento durante 15 a 20 minutos (Figura 2a). Se quitaron las cáscaras y se molieron obteniéndose la pasta de cacao (Figura 2b).

2.3. Formulación de las barras de cacao

Las barras de cacao fueron formuladas usando dos concentraciones de pasta de cacao 50% y 85% y se endulzaron con yacón y algarrobina. Se obtuvieron barras de 30 gramos (Figura 2c). En la Tabla 1 se detallan los componentes de las barras de cacao:

Tabla 1. Composición de las barras de cacao

Componentes	Porcentaje	Función
Pasta de cacao	50% y 85%	Ingrediente activo
Algarrobina y yacón	43% y 10%	Edulcorantes
Lecitina de soya	3.3%	Emulsionante
Stevia	0.2%	Edulcorante secundario
Leche c.s.p	30 g	Saborizante

2.3.1. Procedimiento

Se pesó la pasta de cacao, lecitina de soya, algarrobina o yacón, estevia y leche en polvo, de acuerdo a la formulación mostrada en la Tabla 1.

Se agregó la stevia a la pasta de cacao y se llevó a baño maría, removiendo hasta lograr una consistencia uniforme. En otro recipiente se mezcló la lecitina de soya y los edulcorantes (algarrobina o yacón) según corresponda. Una vez mezclado el edulcorante con la lecitina se agregó removiendo constantemente a la mezcla de cacao y stevia. Se bate y se agrega la leche en polvo previamente disuelta. Se removió hasta lograr una consistencia homogénea. Se colocó en un molde de 30 g y se dejó enfriar hasta su completa solidifcación.

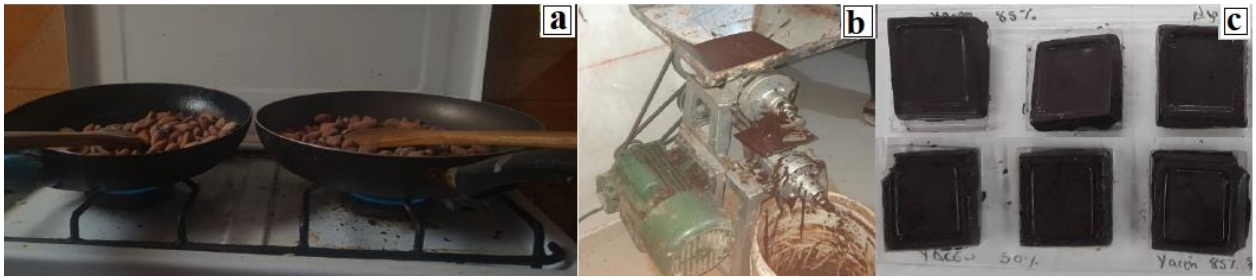

Figura 2. (a) Tostado de los granos de cacao; **(b)** Molienda de los granos tostados de cacao; **(c)** Barras de cacao de 30 g en moldes.

2.4. Control de calidad de las barras de cacao

Se realizó el control de calidad organoléptico y microbiológico siguiendo los protocolos establecidos.

2.4.1. Control de calidad organoléptico

Las propiedades organolépticas son atributos de calidad de un producto que pueden valorarse mediante un examen organoléptico o análisis sensorial que utiliza los sentidos humanos como herramienta principal para medir la aceptación de un producto [17]. Estas técnicas sensoriales son aplicadas por evaluadores o consumidores formados, con materiales de referencia relacionados con las características del producto objeto de estudio [18]. En ese sentido, el control de calidad organoléptico del chocolate de este estudio se llevó a cabo de acuerdo a los parámetros recomendados por Antonio, catador de chocolates elaborados con el cacao recolectado en la zona de estudio [19].

2.4.2. Control de calidad microbiológico

Siguiendo las normas sanitarias sobre los criterios microbiológicos de calidad sanitaria, por la DIGESA-Perú [20]; donde se establece que para productos de confitería, se debe realizar un análisis microbiológico en el que se determine la presencia de *Escherichia coli* (< 10 UFC/g), *Salmonella sp*. (ausencia/25 g) y mohos (< 10^3 UFC/g).

2.5. Determinación del efecto hipoglucemiante de las barras de cacao

2.5.1. Animales

Se obtuvieron ratas macho Holtzman de 170 - 200 g de peso del bioterio del Centro Nacional de Productos Biológicos del Instituto Nacional de Salud. Todas las ratas se mantuvieron en jaulas metálicas en condiciones ambientales estándar de temperatura, humedad relativa y un ciclo de 12 h de oscuridad/12 h de luz. Se les dio libre acceso a una dieta estándar y agua ad libitum. Las ratas se utilizaron para los experimentos tras una semana de aclimatación. Todos los animales fueron cuidados de acuerdo con principios éticos, respetando las pautas para el uso y cuidado de los animales con fines científicos publicados por el Comité Consultivo Nacional para la Investigación con Animales de Laboratorio [21].

2.5.2. Inducción de diabetes experimental en las ratas

Tras una noche de ayuno, se inyectó por vía intravenosa Aloxano monohidratado (100 mg/kg de masa corporal) disuelto en suero fisiológico. El mecanismo de inducción de diabetes del aloxano es provocar la formación de radicales libres especialmente peróxido de hidrógeno

(H_2O_2), oxígeno singlete (O_2) e hidroxilo (OH-) que destruyen selectivamente las células β-pancreáticas, causando su necrosis y generando muerte celular [22].

Tres días después de la inyección de Aloxano, se midió el nivel de glucemia en ayunas de las ratas a partir de la punta de la cola y se comprobó el nivel de glucosa plasmática usando un glucómetro digital y los valores obtenidos se expresaron en mg/dL.

2.5.3. Diseño experimental

Los experimentos con los animales se llevaron a cabo tras una semana de inyección de Aloxano. El experimento se realizó con ratas diabéticas y normales que se dividieron en 8 grupos de cinco ratas cada uno (para determinar el número de ratas, se ha tomado en cuenta las recomendaciones del Informe del Consejo de Bioética de Nuffield y las recomendaciones de Rusell y Burch conocidas como las 3R) [23], teniendo en cuenta el peso y la hiperglucemia que presentaron [24], como se muestra en la Tabla 2.

Tabla 2. Grupos experimentales para evaluar el efecto hipoglucemiante

Grupo	Tratamiento	Dosificación
1	Suero fisiológico	2 mL por vía oral*
2	Aloxano	Sin tratamiento*
3	Aloxano + insulina	4 UI/kg en ayunas por vía subcutánea*
4	Aloxano + glibenclamida	5 mg/kg por vía oral en ayunas*
5	Aloxano + cacao 50% + yacón	30 g/70 kg por vía oral en ayunas
6	Aloxano + cacao 85% + yacón	30 g/70 kg por vía oral en ayunas
7	Aloxano + cacao 50% + algarrobina	30 g/70 kg por vía oral en ayunas
8	Aloxano + cacao 85% + algarrobina	30 g/70 kg por vía oral en ayunas

*Suero fisiológico (control positivo); Aloxano (control negativo); Aloxano + insulina (Patrón 1); Aloxano + glibenclamida (Patrón 2).

Se midieron los niveles de glucosa en sangre a las 2 h, 48 h y hasta los 8 días posteriores a la administración de los tratamientos.

2.6. Determinación de la eficacia hipoglucemiante

La eficacia hipoglucemiante se obtuvo utilizando la siguiente fórmula:

$$\% \text{ de Eficacia hipoglucemiante} = \frac{\text{Control} - \text{tratamiento}}{\text{Control}} \times 100 \qquad \text{Fórmula (1)}$$

2.7. Análisis estadístico

Los resultados finales obtenidos en las fichas de recopilación de información se analizaron con el programa SPSS versión 21. Los datos fueron procesados usando ANOVA y la prueba de Tukey para comprobar si existe similitud o diferencia entre los grupos de experimentación utilizando un nivel de confiabilidad del 95% y un nivel de significancia $p<0,05$.

3. RESULTADOS Y DISCUSIÓN

3.1. Formulación de las barras de cacao

En la Tabla 3, se muestran las fórmulas de las barras de cacao al 50% y 85% edulcoradas con yacón y algarrobina.

Tabla 3. Fórmulas cuali-cuantitativas de las barras de cacao

Porcentaje de pasta de cacao	Insumo	Cantidad
50%	Pasta de cacao	15,0 g
	Algarrobina o yacón	13,0 g
	Lecitina de soya	1,0 g
	Stevia	0,05 g
	Leche en polvo	0,95 g
85%	Pasta de cacao	25,5 g
	Algarrobina o yacón	3,0 g
	Lecitina de soya	1,0 g
	Stevia	0,05 g
	Leche en polvo	0,45 g

Se formularon dos concentraciones diferentes de chocolate, a 50% y 85% de cacao más un edulcorante natural; teniendo en cuenta que los chocolates con una mezcla de pasta de cacao superior al 45% son buenos para la salud por su altísimo contenido en antioxidantes [25].

3.2. Control de calidad organoléptico de las barras de cacao

El resultado del control de calidad organoléptico de las barras de cacao elaboradas con pasta de cacao a las concentraciones de 85% y 50%, endulzadas con algarrobina y yacón se presentan en la tabla 4.

Tabla 4. Resultados de la evaluación organoléptica de las barras de cacao

Parámetros	Especificaciones*	Barra de cacao al 85%		Barra de cacao al 50%	
		Algarrobina	Yacón	Algarrobina	Yacón
Olor	Su aroma es intenso y entre sus ingredientes no debe incluir saborizantes.	Conforme	Conforme	Conforme	Conforme
Color	Marrón muy oscuro y brillante, uniforme, sin ningún tipo de mácula, burbujas o hendiduras	Conforme	Conforme	Conforme	Conforme
Sabor	La acidez debe predominar sobre el amargor y el dulzor	Conforme	Conforme	Conforme	Conforme
Textura o consistencia	Firme, al partirse el sonido debe ser seco y quebradizo.	Conforme	Conforme	Conforme	Conforme

* Blog de Pumatiy- empresa de chocolates [19].

El control de calidad organoléptico de los chocolates a través de parámetros sensoriales es importante no sólo para su caracterización, sino también para garantizar su calidad en el mercado. Esta caracterización debe realizarse teniendo en cuenta la gran diversidad y variación botánica de cada región [26]. Permata e Hidayah (2024) establecieron las propiedades organolépticas de los granos de cacao procedentes de tres localidades, 5 panelistas evaluaron el aroma, sabor, textura y color. Los chocolates elaborados con los granos de cacao recolectados

en las diferentes zonas de producción se distinguieron por su escasa acidez y astringencia, así como por su aroma medio. Esta evaluación demostró que las condiciones de las distintas zonas de producción pueden garantizar una buena calidad organoléptica de los chocolates resultantes [27]. En otro estudio se determinó que, los chocolates producidos con granos de las regiones Transamazónica y Nordeste del Brasil presentaron una fuerte acidez, amargor, astringencia y sabor, características dependientes de la zona de producción de los granos de cacao [28].

3.3. Control de calidad microbiológico de las barras de cacao

El resultado del control de calidad microbiológico de las barras de cacao elaboradas a base de cacao endulzado con algarrobina y yacón se muestra en la Tabla 5.

Tabla 5. Resultados de la evaluación microbiológica de las barras de cacao

Microorganismo	Límites aceptados	Barra de cacao al 85%		Barra de cacao al 50%		Resultado
		Algarrobina	Yacón	Algarrobina	Yacón	
E. coli	<10 UFC/g	<10 UFC/g	<10 UFC/g	<10 UFC/g	<10 UFC/g	Conforme
Salmonella sp.	Ausencia /25g	Ausente	Ausente	Ausente	Ausente	Conforme
Mohos	$< 10^3$ UFC/g	$< 10^3$ UFC/g	$< 10^3$ UFC/g	$< 10^3$ UFC/g	$< 10^3$ UFC/g	Conforme

El resultado de la evaluación microbiológica mostró la inocuidad de las barras de cacao elaboradas, debido a que se observó que los microorganismos evaluados se encontraban dentro de los límites aceptados por la entidad peruana autorizada.

3.4. Efecto hipoglucemiante de las barras de cacao

En la Tabla 6 se aprecian los valores de glicemia de los diferentes grupos experimentales. El tratamiento tuvo una duración de 8 días.

Tabla 6. Glicemia basal, glicemia post aloxano y glicemia post tratamiento

Grupo	Tratamiento	Nivel de glucosa basal (mg/dL)	Nivel de glucosa post aloxano (mg/dL)	Nivel de glucosa post tratamiento (mg/dL)
1	Suero fisiológico	118,8	114,2 ± 6,18	98 ± 8,72 [a]
2	Aloxano	113,6	347 ± 38,01	205,8 ± 15,22 [b]
3	Aloxano + insulina	114,2	275,6 ± 71,81	132,8 ± 16,27 [c]
4	Aloxano + glibenclamida	118,4	277,6 ± 89,01	157 ± 48,94 [d]
5	Aloxano + cacao 85% + yacón	119,8	259,4 ± 13,79	197,4±12,14 [e]
6	Aloxano + cacao 50% + yacón	123,8	289,8 ± 47,14	202,6 ± 21,62 [b]
7	Aloxano + cacao 85% + algarrobina	108,2	260,2 ± 40,06	137,4 ± 46,04 [c]
8	Aloxano + cacao 50% + algarrobina	117	259,4 ± 29,62	141,2 ± 8,64 [c]

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

Los niveles de glucosa basal y post administración de aloxano son diferentes según los grupos experimentales: Grupo 1: no hay diferencia entre el nivel basal y nivel post aloxano, puesto que este grupo solo recibió suero fisiológico y no se indujo diabetes experimental con aloxano, la glicemia final medida a los 8 días mostró un nivel de glicemia de 98,0 ± 8,72 mg/dL. El Grupo 2 presentó una glucosa basal de 113,6 mg/dL y luego de la administración de aloxano mostró una hiperglicemia de 347,0 ± 38,0 mg/dL, este grupo al no recibir ningún tratamiento, mostró un nivel de glicemia de 205,8 ± 15,2 mg/dL, lo que demuestra que no hubo una disminución del nivel de glicemia significativa al cabo de los 8 días de tratamiento. El Grupo 3 presentó una glucosa basal de 114,2 mg/dL y luego de la administración de aloxano mostró un valor de glicemia de 275,0 ± 71,8 mg/dL, luego del tratamiento con insulina se midió un nivel de glicemia de 132,8 ± 16,3 mg/dL mostrando una disminución significativa. El Grupo 4 presentó una glucosa basal de 118,4 mg/dL, y una glucosa post aloxano de 277,6 ± 89,0 mg/dL y luego de recibir el tratamiento con glibenclamida mostró un nivel de glucosa de 157,0 ± 48,9 mg/dL. El Grupo 5 mostró un nivel de glucosa basal de 119,8 mg/dL, y luego de la inyección de aloxano mostró un nivel de glucosa de 259,4 ± 13,8, y al cabo de los 8 días de tratamiento con cacao 85% + yacón se obtuvo un nivel de glucosa de 197,4 ± 12,1 mg/dL. El Grupo 6 mostró un nivel de glucosa basal de 123,8 mg/dL, un nivel de glucosa post aloxano de 289,8 ± 47,1 mg/dL y luego del tratamiento con cacao 50% + yacón durante 8 días mostró un nivel de glucosa de 202,6 ± 21,6 mg/dL. El Grupo 7 tuvo un nivel de glucosa basal de 108,2 mg/dL, un nivel de glucosa post aloxano de 260,2 ± 40,1 mg/dL y luego de tratamiento con cacao 85% + algarrobina durante 8 días mostró un nivel de glucosa de 137,4 ± 46,0 mg/dL. Finalmente, el Grupo 8 mostró un nivel de glucosa basal de 117 mg/dL, un nivel de glucosa post aloxano de 259,4 ± 29,6 mg/dL y luego del tratamiento con cacao 50% + algarrobina durante 8 días mostró un nivel de glucosa de 141,2 ± 8,6 mg/dL.

Por los resultados antes detallados se puede evidenciar que los grupos 7 y 8 ambos endulzados con algarrobina y con porcentajes de pasta de cacao de 85% y 50% respectivamente presentaron mejor efecto hipoglucemiante. Lo que fue corroborado también por el análisis estadístico, el cual estableció que no hubo diferencias significativas para estos dos grupos y para el grupo tratado con insulina, con el que tuvieron valores de glucosa muy cercanos.

En el estudio desarrollado por Chikezie (2015) en el cual se usó el modelo de diabetes inducida por aloxano en ratas para establecer la actividad hipoglucemiante del extracto etanólico de *Theobroma cacao*, se encontró que las dosis de 100, 200 y 400 mg/kg provocaron un descenso de la glucosa de 258,10 ± 3,27 mg/dL a 138,00 ± 3,27 mg/dL; 254,58 ± 2,01 mg/dL a 118,58 ± 2,69 mg/dL y 253,94 ± 2,13 mg/dL a 113,50 ± 3,55 mg/dL respectivamente, demostrando una significativa reducción de los niveles de glucosa en sangre [29]. El extracto de cacao rico en polifenoles totales obtenido mediante extracción con etanol al 80% a las dosis de 1% y 3% redujo significativamente ($p < 0,05$) los niveles plasmáticos de glucosa en ratas diabéticas [4]. El consumo de cacao se ha asociado con la disminución de los niveles de glucosa en sangre y con la disminución de lípidos como el colesterol total y los triglicéridos (TG), promoviendo la translocación del transportador de glucosa 4 (GLUT4) en tejidos sensibles a la insulina a través de la activación de la adenosinmonofosfato quinasa activada por adenosinmonofosfato (AMPK) que forma parte del mecanismo por el que disminuye la hiperglucemia [30].

Asimismo, el consumo de catequinas se ha asociado con una disminución de la hiperglucemia y la resistencia a la insulina a través de la modulación de citoquinas proinflamatorias como IL-1β, IL-6 y TNF-α, y la activación de vías de señalización vías que permiten mantener

una función adecuada de la cadena respiratoria mitocondrial, y con ello, proteger las células islotes y mejorar la resistencia a la insulina [31].

3.5. Porcentaje de eficacia hipoglucemiante

En la Tabla 7, se muestra el porcentaje de eficacia calculado para los diferentes grupos de tratamiento.

Tabla 7. Porcentaje de eficacia hipoglucemiante de los diferentes tratamientos

Grupo	Tratamiento	Porcentaje de eficacia
1	Suero fisiológico	--
2	Aloxano	--
3	Aloxano + insulina	35,47 % [a]
4	Aloxano + glibenclamida	23,71 % [b]
5	Aloxano + cacao 85% + yacón	4,08 % [c]
6	Aloxano + cacao 50% + yacón	1,55 % [d]
7	Aloxano + cacao 85% + algarrobina	33,24 % [a]
8	Aloxano + cacao 50% + algarrobina	31,39 % [a]

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

Como se aprecia en la Tabla 7, los grupos 7 y 8, tratados con cacao al 85% y 50% endulzados con algarrobina, presentaron porcentajes del 33,24% y 31,39% respectivamente, ambos muy cercanos al porcentaje de eficacia de la insulina.

Trabajos previos demostraron que la algarrobina presentaba efecto hipoglucemiante [32], lo que explica nuestros resultados, porque al incluirla en la formulación de las barras de cacao aumentaron el efecto hipoglucemiante del cacao.

Las pruebas demuestran que el cacao contribuye a mejorar el perfil cardiometabólico, al reducir la glucemia HbA1c, perfil lipídico, marcadores de inflamación y estrés oxidativo.

Investigaciones previas sugieren que, debido a las características de los principales compuestos bioactivos del cacao, éstos podrían ejercer efectos beneficiosos sobre la *Diabetes mellitus*. Sin embargo, aún no se han realizado ensayos clínicos que respalden el uso del cacao o de alguno de sus compuestos bioactivos para prevenir, tratar o mejorar esta patología [33].

En la Figura 3(a) se observa que el tratamiento con la barra de cacao al 85% endulzada con algarrobina hace que el nivel de glucosa descienda hasta llegar casi al mismo nivel logrado por la insulina a los 8 días. Sin embargo, el tratamiento con la barra de cacao al 85% endulzada con yacón muestra que los niveles de glucosa no descienden significativamente, y a los 8 días de tratamiento están muy cercanos a los niveles del grupo que no recibió tratamiento alguno luego de la inducción con aloxano. En la Figura 3(b) igualmente se observa que el tratamiento con la barra de cacao al 50% endulzada con algarrobina alcanza niveles de glucosa por debajo de 150 mg/dL a los 8 días, en tanto que el tratamiento con la barra de cacao al 50% endulzada con yacón alcanza niveles de glucosa por encima de 200 mg/dL.

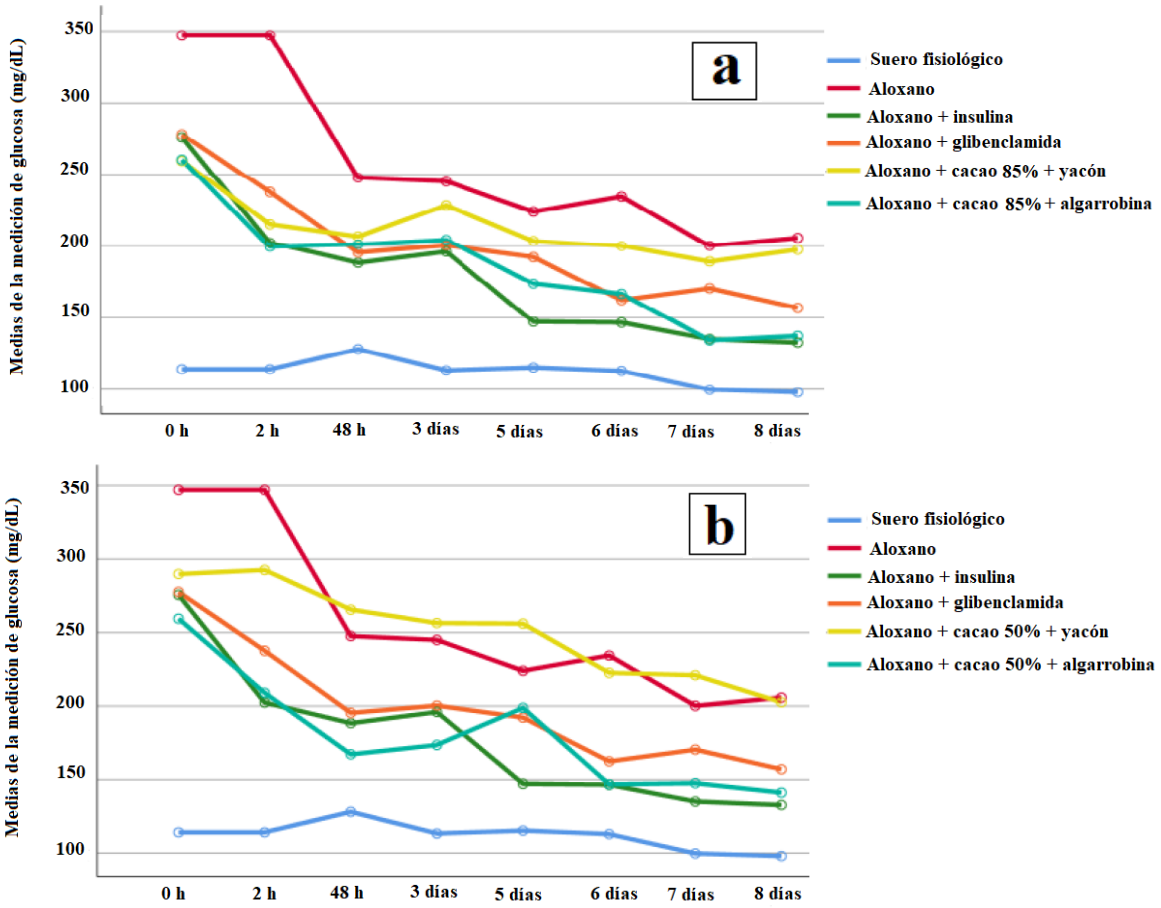

Figura 3. Comparación del efecto hipoglucemiante de las barras de cacao *(Theobroma cacao)* al 85% (a) y al 50% (b) respecto a los edulcorantes yacón y algarrobina y al tiempo de tratamiento.

En la investigación desarrollada por Olooto (2014) se determinó que el extracto acuoso de cacao en polvo mostró una reducción de glucosa plasmática tras su administración oral, resaltando que a la dosis de 300 mg/kg mostró un efecto hipoglucemiante signiticativo reduciendo los niveles de glucosa a 170 mg/dL a los 120 minutos de administrado el tratamiento [34]. La hipoglucemia observada en las ratas diabéticas inducidas por aloxano podría deberse a la actividad antioxidante de los compuestos polifenólicos presentes en las barras de cacao. Se observaron resultados similares en ratas diabéticas inducidas por estreptozotocina [4, 35]. El efecto hipoglucemiante del cacao en polvo, también puede deberse al tipo o clase de flavonol presente [34]. Asimismo, se ha descrito que el cacao es rico en flavonoles de la clase de las catequinas, que reducen la biodisponibilidad de los carbohidratos de la dieta, ya sea por inhibición de los transportadores intestinales de glucosa o por inhibición de la α-amilasa pancreática reduciendo así la absorción de glucosa, con la consiguiente reducción del nivel de glucosa en sangre [4].

4. CONCLUSIONES

La pasta de cacao (*Theobroma cacao*) formulada demostró tener efecto hipoglucemiante en un modelo experimental de diabetes inducida en ratas por inyección intraperitoneal de una solución de Aloxano monohidratado. Este efecto es dependiente del procentaje de pasta de cacao y del tipo de edulcorante usado. De esta forma se ha establecido que la concentración de pasta de cacao del 85% en la barra de cacao endulzada con algarrobina logra un mejor efecto

hipoglucemiante comparado con la insulina usada por vía subcutánea. Este efecto podría deberse a los metabolitos secundarios y componentes presentes en los granos de cacao, especialmente los polifenoles, teobromina y otros alcaloides purínicos. Asimismo, la algarrobina podría ser responsable del aumento del efecto hipoglucemiante del cacao en este modelo, existiendo estudios previos que ya le han atribuido propiedades hipoglucemiantes. Esta es una nueva alternativa alimentaria apetecible y sana para la población en general, y especialmente para los pacientes diabéticos.

Teniendo en cuenta los resultados obtenidos es factible recomendar el consumo de cacao en individuos con *Diabetes mellitus* ya que se han demostrado efectos beneficiosos sobre las citoquinas proinflamatorias y la glucemia, utilizando dosis que están disponibles en el mercado y que son fácilmente consumibles.

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CONFLICTOS DE INTERÉS

Los autores declaramos no tener ningún conflicto de interés.

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Review article

Promising potential of the antifungal action of essential oils and its challenges: a systematic review

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SUMMARY

Introduction: Fungal infections have increased, especially in immunocompromised patients, while therapeutic options have decreased due to resistance to available antifungals. The World Health Organization (WHO) warns of the urgent need for new therapies. In this context, essential oils (EOs) have been the subject of study to find new treatments. **Objective:** A systematic review was carried out to investigate the antifungal activity of EOs according to the Cochrane Handbook guidelines (2023). The search, selection and extraction of data of interest were carried out in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) in articles selected in biomedical databases (Pubmed/Medline, Scopus, Virtual Health Library, ScienceDirect and Online Electronic Scientific Library - SciELO). The descriptors used were defined by Medical Subject Heading (MeSH) and Descriptors in Health Sciences (DeCS). **Results:** Of the 419 articles initially found, 35 were included in the review. Most studies (54.2%) were conducted in Brazil, investigating mainly aromatic plants such as lemongrass, oregano, and citronella. Although EOs have shown antifungal activity in some studies, research has revealed a lack of standardization in testing methods and a paucity of studies for certain pathogens. However, some EOs have shown significant potential against *Candida* spp., *Aspergillus* spp., and dermatophytes. **Conclusion:** There is a growing interest in investigating EOs as therapeutic alternatives against resistant fungal infections. However, more research is needed, especially to explore less studied plants to identify promising new antifungal agents.

Keywords: Volatile oil; phytochemical compounds; antifungal agent

RESUMO

Potencial promissor da ação antifúngica dos óleos essenciais e seus desafios: uma revisão sistemática

Introdução: As infecções fúngicas aumentaram, especialmente em pacientes imunocomprometidos, enquanto as opções terapêuticas diminuíram devido à resistência aos antifúngicos disponíveis. A Organização Mundial da Saúde (OMS) alerta para a necessidade urgente de novas terapias. Nesse contexto, os

óleos essenciais (OEs) têm sido objeto de estudo para encontrar novos tratamentos. **Objetivo:** Foi realizada uma revisão sistemática para investigar a atividade antifúngica de OEs de acordo com as diretrizes do *Cochrane Handbook* (2023). A busca, seleção e extração dos dados de interesse foram realizadas de acordo com o *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) em artigos selecionados em bases de dados biomédicas (Pubmed/Medline, Scopus, Biblioteca Virtual em Saúde, *Science Direct* e *Online Electronic Scientific Library* - SciELO). Os descritores utilizados foram definidos pelo *Medical Subject Heading* (MeSH) e Descritores em Ciências da Saúde (DeCS). **Resultados:** Dos 419 artigos encontrados inicialmente, 35 foram incluídos na revisão. A maioria dos estudos (54,2%) foi conduzida no Brasil, investigando principalmente plantas aromáticas, como capim-limão, orégano e citronela. Embora os OEs tenham demonstrado atividade antifúngica em alguns estudos, a pesquisa revelou uma falta de padronização nos métodos de teste e uma escassez de estudos para certos patógenos. No entanto, alguns OEs mostraram potencial significativo contra *Candida* spp., *Aspergillus* spp. e dermatófitos. **Conclusão:** Há um interesse crescente em investigar EOs como alternativas terapêuticas contra infecções fúngicas resistentes. No entanto, mais pesquisas são necessárias, especialmente para explorar plantas menos estudadas para identificar novos agentes antifúngicos promissores.

Palavras-chave: Óleo volátil; compostos fitoquímicos; agente antifúngico

RESUMEN

Potencial prometedor de la acción antifúngica de los aceites esenciales y sus desafíos: una revisión sistemática

Introducción: Las infecciones por hongos han aumentado, especialmente en pacientes inmunocomprometidos, mientras que las opciones terapéuticas han disminuido debido a la resistencia a los antifúngicos disponibles. La Organización Mundial de la Salud (OMS) advierte de la urgente necesidad de nuevas terapias. En este contexto, los aceites esenciales (AEs) han sido objeto de estudio para encontrar nuevos tratamientos. **Objetivo:** Se llevó a cabo una revisión sistemática para investigar la actividad antifúngica de los AEs según las directrices del Manual Cochrane (2023). La búsqueda, selección y extracción de datos de interés se realizaron de acuerdo con el *Preferred Reporting Items for Systematic Reviews and Meta-Analyses* (PRISMA) en artículos seleccionados en bases de datos biomédicas (Pubmed/Medline, Scopus, Biblioteca Virtual em Saúde, *Science Direct* y Biblioteca Científica Electrónica en Línea - SciELO). Los descriptores utilizados fueron definidos por *Medical Subject Heading* (MeSH) y *Health Sciences Descriptors* (DeCS). **Resultados:** De los 419 artículos encontrados inicialmente, 35 fueron incluidos en la revisión. La mayoría de los estudios (54,2%) se realizaron en Brasil, investigando principalmente plantas aromáticas, como limoncillo, orégano y citronela. Aunque los AEs han demostrado actividad antifúngica en algunos estudios, las investigaciones han revelado una falta de estandarización en los métodos de prueba y una escasez de estudios para ciertos patógenos. Sin embargo, algunos AEs han mostrado un potencial significativo contra *Candida* spp., *Aspergillus* spp. y dermatofitos. **Conclusión:** Existe un interés creciente en investigar los AE como alternativas terapéuticas contra las infecciones fúngicas resistentes. Sin embargo, se necesita más investigación, especialmente para explorar plantas menos estudiadas e identificar nuevos agentes antifúngicos prometedores.

Palabras clave: Aceite volátil; compuestos fitoquímicos; agente antifúngico

1. INTRODUCTION

Antimicrobial resistance (AMR) is one of the biggest challenges of the 21st century, with an imminent risk of a lack of treatment for even minor infections [1]. In this context, the need for research aimed at finding potentially active substances, that have characteristics such as non-toxic and coming from renewable sources [2], stands out.

In this sense, the plant kingdom is considered extremely relevant and plants are an alternative, as they produce many bioactive compounds whose activity has not yet been completely clarified. Furthermore, the use of compounds derived from plants brings the advantage of resource availability, especially in Brazil [3].

It has been reported that many plants have active natural substances, exerting, for example, antimicrobial action. Among plant-derived products, essential oils (EOs) play a prominent role, with use in various industrial segments, including food and pharmaceuticals. Furthermore, they are environmentally friendly, that is, they degrade quickly in soil and water, and are low toxic to humans [4].

EOs are considered plant secondary metabolites and can be found in the leaves, flowers, stems, buds, seeds, fruits, roots, or bark of many plants [5]. The most commonly used extraction methods for EOs can be hot (steam distillation, hydro-diffusion and hydrodistillation) or cold (solvents), and their respective products can vary in their activity due to the type and stereochemistry of their molecules [4] and the extraction method used can account for the variation in its activity due to the types of molecules and their stereochemical structures [6].

According to Bilia *et al.* [7], various compounds are found in EOs, including hydrocarbons (monoterpenes, sesquiterpenes) and oxygenated ones (alcohols, ethers, esters, ketones, aldehydes, phenols, lactones, and others), which may be phenolic or terpenic. Phenols and terpenes are related to the antifungal action of EOs. The literature shows that phenolic compounds and sesquiterpenes have potential activity against fungi, with fungicidal or fungistatic activity depending on the concentration used [8,9].

The number of fungal infections has increased, particularly in immunocompromised patients, largely due to the indiscriminate use of antibiotics and greater access to therapies that alter the immune system [8-10]. Therefore, the availability of therapeutic options for the treatment of these fungal infections has been decreasing, since both yeast-like fungi, such as *Candida auris* [10], and filamentous fungi with a high level of resistance or even complete resistance to all classes of available antifungal drugs [11]. This fact led the World Health Organization [12] to publish a list that warns of resistant fungi that must be monitored and for which new therapies must be urgently sought. For example, in this list *C. auris* and *Candida albicans* are mentioned as critical priorities, the latter considered one of the main and most frequent etiological agents of fungal infections.

Therefore, it is necessary to increase knowledge about the antifungal potential of highly relevant bioactive compounds. Therefore, this work aims to report the findings of a systematic review on the antifungal potential of EOs found in the literature, which may help and encourage new research and the use of these compounds in the fight against fungal infections.

2. MATERIAL AND METHODS

2.1. Search strategy

A systematic review was conducted according to the Cochrane Handbook guidelines [13]. The research, selection, and extraction of data of interest were carried out according to the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) [14]. Articles that aimed to investigate the antifungal activity of essential oils were searched from biomedical databases (Pubmed/Medline, Scopus, Virtual Health Library, ScienceDirect, and Online Electronic Scientific Library - SciELO). The descriptors used were defined by Medical Subject Heading (MeSH) (descriptors in English) and Descriptors in Health Sciences (DeCS) (descriptors in Portuguese and Spanish), being: ("Volatile Oils" OR "Oil, Essential" OR "Essential Oil" OR "Oils, Essential" OR "Essential Oils" OR "Volatile Oil" OR "Oil, Volatile") AND ("Agents, Antifungal" OR "Therapeutic Fungicides" OR "Fungicides, Therapeutic" OR "Antifungal Agent" OR " Agent, Antifungal" OR "Antibiotics, Antifungal" OR "Antifungal Antibiotics'). The descriptors were combined using the AND and OR symbols between them, being inserted in the search tabs of each base according to their characteristics and limitations. Limits were established for articles published in Portuguese, Spanish, and English, published until December 2023.

2.2. Eligibility criteria

The general selection criteria for studies obtained after searching previously defined databases was determined by the "PVE" strategy, as follows: "Population" – plants; "Variable": antifungal activity; "Study": studies using essential oils.

2.3. Exclusion criteria

Review articles, notes, emails, editorials, letters, works presented at scientific events, and articles that did not present original material were excluded. Furthermore, other articles were excluded based on the following criteria: (i) studies that do not identify fungi at the genus or species level; (ii) studies that did not present the minimum inhibitory concentration (MIC) for EOs. If the study complies with the inclusion criteria, but the original text is not available, the corresponding author was contacted by email up to 3 times (with an interval of 14 days between them), and the study was excluded if it was not sent after the last contact.

The selected articles were subjected to a full analytical reading to identify and extract the variables of interest: reference, study location, studied plant, origin, identification of the fungus, EO's concentration, and MIC obtained. The data were summarized in a table for subsequent critical analysis.

3. RESULTS AND DISCUSSION

In this study, a systematic review of the literature was conducted to investigate the activity of essential oils against fungi. As shown in Figure 1, 419 articles were retrieved in the initial search in the selected databases. After excluding repeated studies, 407 were screened according to the exclusion criteria. After reading the title, keywords, and summary, 332 articles were excluded and the remaining 75 continued to read the full text. At this stage, 40 articles were

excluded after evaluating their agreement with the defined eligibility criteria, and the remaining 35 articles were included in this review.

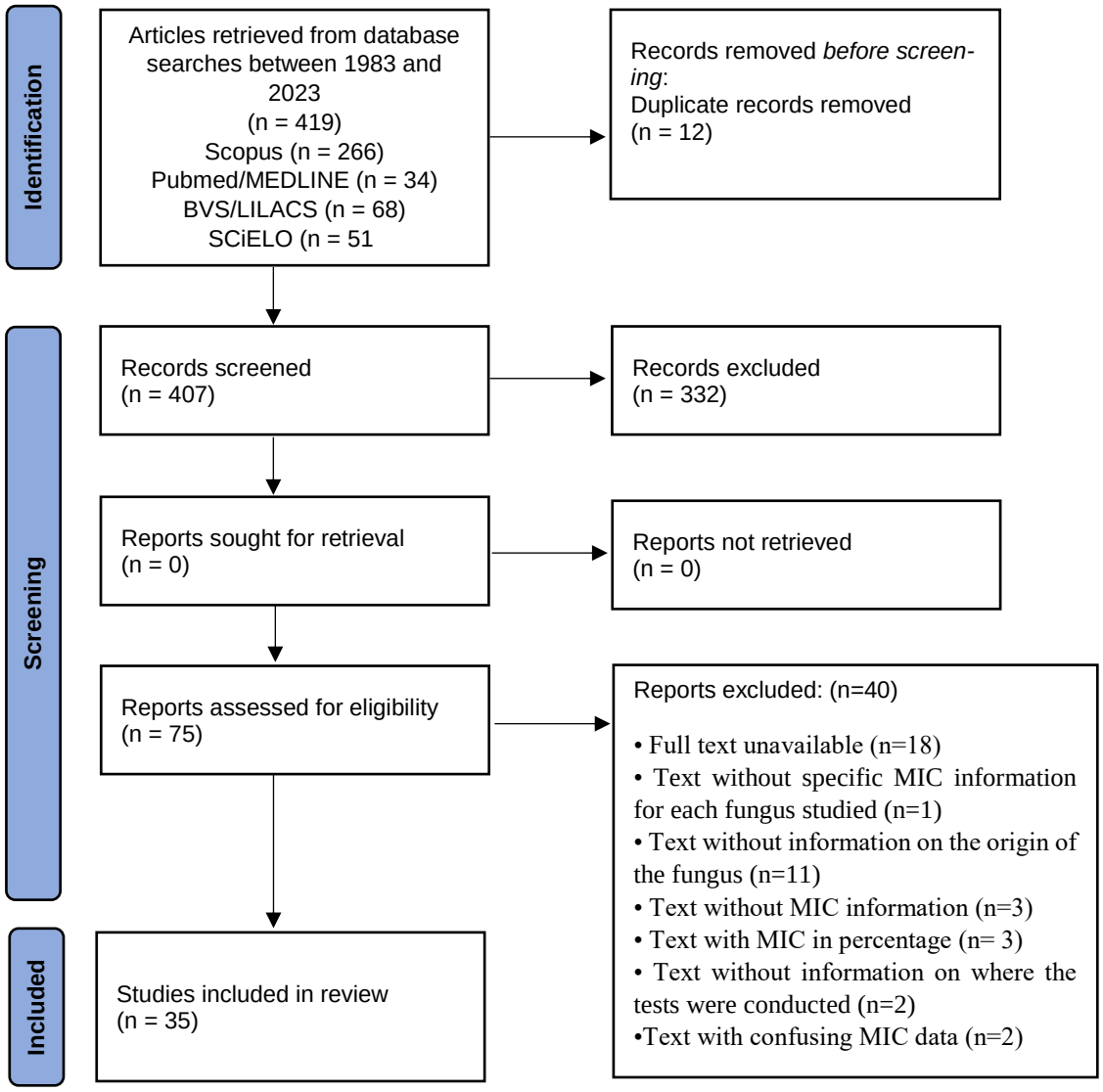

Figure 1. Flowchart of articles selected for the systematic review according to the PRISMA criteria.

Although the selection period was not limited, it was observed that only from 2005 (Table 1) were studies found that covered the topic of this review, with a greater number of publications from 2015 (21/34) onwards. As reviewed by Suleyman and Alangaden [15], fungal infections have increased, especially healthcare-associated infections (HAIs), due to the greater number of immunocompromised patients due to chronic diseases with the consequent need for organ transplants, prolonged use of therapy antibacterial and invasive medical devices. In parallel, antifungal resistance is also growing, which is an additional challenge, combined with the toxicity of the already limited therapeutic options [16]. Therefore, new strategies for the treatment of these infections have to be sought and, in this context, EOs tend to be more explored.

Table 1. Antifungal activity of essential oils extracted from different plants.

Study reference	**Study location**	**Essential oil study plant/ popular name/ basic composition according to the authors**	**Genus or species of fungus/origin**	**Essential oil activity concentration**
Lemos *et al*. [17]	Brazil	*Ocimum gratissimum*/ manjericão/ eugenol	*Cryptococcus neoformans* (clinical isolate)	CIM_{50} and CIM_{90} 250 μg/mL
Fontenelle *et al*. [18]	Brazil	*Croton nepetifolius*/ marmeleiro vermelho/ methyl eugenol and bicyclogermacrene *Croton argyrophylloides*/ marmeleiro prateado/ spatulenol and bicyclogermacrene *Croton zehntneri*/ canela-brava/ methyl-chavicol and anethole	*Candida albicans* *Candida tropicalis* *Microsporum canis* (veterinary isolates)	*C. nepetifolius*: *C. albicans* and *C. tropicalis* undetected activity *M. canis* > 5000 μg/mL *C. argyrophylloides*: *C. albicans and C. tropicalis* undetected activity *M. canis* 9 - > 19 μg/mL *C. zehntneri:* *C. albicans* > 5000 μg/mL *C. tropicalis* 2500 μg/mL *M. canis* 620 – 1250 μg/mL
Cleff et *al*. [19]	Brazil	*Origanum vulgare*/ oregano/ carvacrol	*Sporothrix schenckii* (clinical isolate human and veterinary)	250 μl/mL
Souza *et al*. [20]	Brazil	*Origanum vulgare*/ orégano/carvacrol and thymol *Origanum majorana*/ marjoram and terpinen-4-ol	*Candida albicans* ATCC-7645 *Candida tropicalis* LM-14 *Cryptococcus neoformans* FGF-5 *Aspergillus flavus* LM-02 *Aspergillus fumigatus* IPP-21 *Trichophyton rubrum* ATCC-28184 *Trichophyton mentagrophytes* LM-64 *Microsporum gypseum* ATCC 184 *Microscoporum canis* LM-36 *Cladosporium herbarium* ATCC 26362 (reference strains)	*O. vulgare*: 80 μg/mL (except for *T. mentagrophytes* and *M. gypseum*) *O. majorana:* 160 μL/mL (except for *T. rubrum, M. canis, C. herbarium* and *A. fumigatus*)
Custódio *et al*. [21]	Brazil	*Pimenta pseudocaryophyllus*/ louro-cravo/ eugenol *Tynanthus micranthus*/ cipó-cravo/ eugenol	*Candida albicans* *Candida krusei* (clinical isolate)	*P. pseudocaryophylus:* 4 μL/mL *T. micranthus*: 17 μL/mL
Anaruma *et al*. [22]	Brazil	*Cymbopogon citratus*/ capim-limão/ geranial and neral	*Colletotrichum gloeosporioides* (vegetable isolate)	0.25 μg/mL

Deus *et al*. [23]	Brazil	*Copaifera multijuga*/ copaiba/ α-copaene, caryophyllene oxide and β-caryophyllene	*Aspergillus flavus* IOC-3974 *Aspergillus niger* IOC-200 *Aspergillus tamarii IOC*-186, IOC-187 *Aspergillus terreus* IOC-217 *Candida guilliermondii* IOC-2889 *Candida tropicalis* IOC-3610 *Candida parapsilosis* IOC-2882 (reference strains)	*A. flavus* 0.08 mg/mL *A. niger* 0.1 mg/mL *A. tamarii* 0.3 – 0.5 mg/mL *A. terreus* 0.3 mg/mL *C. guilliermondii* 0.1 mg/mL *C. tropicallis* 0.5 mg//mL *C. parapsilosis* 0.1 mg/mL
Castro, Lima [24]	Brazil	*Ocotea odorifera*/ canela-sassafrás/ NA *Rosmarinus officinalis*/ alecrim/ NA	*Candida albicans* ATCCs 90028, 76615, 76645, 76485,13803, LM-42V, 18F, MD-37, LM-968, ICB-12 *Candida tropicalis* ATCC-13803 and LM-708, 14, 028, 37, 13, 759. (reference strains)	*O. odorífera:* *C. albicans* 2.5 – 5.0 mg/mL *C. tropicalis* 2.5 - > 5.0 mg/mL *R. officinalis:* *C. albicans* 2.5 - > 5.0 mg/mL *C. tropicalis* ≥ 5 mg/mL
Khosravi *et al*. [25]	Iran	*Cuminum cyminum*/ cominho/ pinene, cineole and linalool *Nigella sativa*/ cominho preto/ trans-anethole and p-cymene *Ziziphora clinopodioides*/ tomilho de folha estreita/ pulegone, 1,8-cineole and limonene	*Aspergillus fumigatus* ATCC 16913 *Aspergillus flavus* ATCC 16013 (reference strains)	*C. cyminum:* *A. fumigatus* and *A. flavus* CIM_{90} 0.25 – 1.5 mg/mL *N. sativa:* *A. fumigatus* CIM_{90} 1.5 mg/mL *A. flavus* CIM_{90} 1.5 – 2.0 mg/mL *Z. clinopodioides:* *A. fumigatus* CIM_{90} 0.5 – 1.5 mg/mL *A. flavus* CIM_{90} 1.5 mg/mL
Pereira *et al*. [26]	Brazil	*Cymbopogon winterianus*/ citronella de java/ citronellal and geraniol	*Trichophyton rubrum* (clinical isolate)	CIM_{50} 312 μg/mL
Oliveira *et al*. [27]	Brazil	*Cymbopogon winterianus*/ citronella de java/ citronellal and geraniol	*Candida albicans* ATCCs: 76485, ATCC 76615, ATCC 13803, LMV 42, ICB 12, M101, LM 968, LM 68, LM 16, LM 018, LM 023, LM 601, LM 290, LM 052, LM 087 (reference strains)	78 – 625 μg/mL
Tadić *et al*. [28]	Serbia	*Sideritis scardica*/ chá grego da montanha/ hexadecanoic acid, myristicin, menthol, caryophyllene oxide and τ-muurolol	*C. albicans* (clinical isolate)	2.560 μg/mL
Vizcaya *et al*. [29]	Venezuela	*Vismia baccifera* subsp. dealbata/ caryophyllene oxide and β-caryophyllene	*C. albicans* ATCC 90028 *C. glabrata* ATCC 90030 *C. tropicalis* ATCC 50658 *C. krusei* ATCC 6258 *Candida parapsilosis* ATCC 22019 (reference strains) *Cryptococcus neoformans* (clinical isolate)	*C. albicans, C. tropicalis, C. parapsilosis, C. neoformans* 1000 μg/mL *C. glabrata* 200 μg/mL *C. krusei* 160 μg/mL

Aguiar *et al*. [30]	Brazil	*Cymbopogon nardus*/ citronela/ citronellal *Corymbia citriodora*/ eucalipto-limão/ citronellal	*Aspergillus* spp. *Pyricularia grisea* *Colletotrichum musae* (isolated from plants and fruits)	*C. nardus*: *Aspergillus* spp. – 100 ppm *P. grisea* – 100 ppm *C. musae* – 50 ppm *C. citriodora:* *Aspergillus* spp. – 200 ppm *P. grisea* – 200 ppm *C. musae* – 50 ppm
Bogavac *et al*. [31]	Serbia	*Coriandrum sativum*/ coentro/ linalyl acetate and linalol *Thymus vulgaris*/tomilho/ thymol	*Candida albicans* (clinical isolate) *Candida albicans* ATCC 10231 (reference strains)	*C. sativum:* *C. albicans* 0.11 - > 0.45 µl/mL *C. albicans* 10231 > 0.45 µl/mL *T. vulgaris:* *C. albicans* and *C. albicans* 10231 0.11 µl/mL
Costa *et al*. [32]	Brazil	*Ocimum selloi*/ alfavacaanis/ methyl chavicol	*Alternaria alternata* CML 184 *Colletotrichum gloeosporioides* CML 459 *Moniliophthora perniciosa* CEPLAC 1188 (reference strains)	*A. alternata, C. gloeosporioides* > 1000 ppm *M. perniciosa* 250-500 ppm
Nascimento *et al*. [33]	Brazil	*Piper arboreum*/ pariparoba/ curcumene, cis-cadin-4-en-7-ol and germacrene	*Microsporum gypseum* URM6199 *Epidermophyton floccosum* URM 3182 (reference strains)	*M. gypseum* 156.25 µg/mL *E. floccosum* 62.5 µg/mL
Brito *et al*. [34]	Brazil	*Lippia sidoides*/ alecrim pimenta/ thymol	*Candida albicans* LM 62 *Candida tropicalis* LM 20 *Candida krusei* LMBM 01 and LMBM 02. (reference strains)	*C. krusei* 01 – 128 µg/mL *C. albicans* 62 – 256 µg/mL *C. tropicalis* 20 – 128 µg/mL
Baptista *et al*. [35]	Brazil	*Eucalyptus smithii*/ eucalipto/ α-pinene, ϱ-cymene, 1,8-cineole, linalool, terpinen-4-ol and α-terpineol	*Microsporum canis* ATCC 32903 *Microsporum gypseum* ATCC 14683 *Trichophyton mentagrophytes* ATCC 9533 *T. mentagrophytes* ATCC 11481 *Trichophyton rubrum* CCT 5507 (reference strains)	*M. canis* ATCC32903 500 µg/mL *M. gypseum* ATCC 14683 1000 µg/mL *T. mentagrophytes* ATCC 9533 250 µg/mL *T. mentagrophytes* ATCC 11481 125 µg/mL *T. rubrum* CCT 5507 62.5 µg/mL

Houël *et al*. [36]	French Guiana	*Achetaria guianensis*/ NA *Cymbopogon citratus*/ capim-limão/ geranial and neral *Mikania micrantha*/ cipó-amargo/NA *Otacanthus azureus*/ erva copaíba/ β-copaen-4-α-ol, α-humulene, α-copaene, myrtenal, viridiflorol and trans-pinocarveol *Piper hispidum*/ falso jaborandi/ curzerene, furanosesquiterpenes and β-caryophyllene *Protium heptaphyllum*/ amescla/ limonene, α-pinene, β-pinene, p-cymene, trans-carveol, β-myrcene and carvone *Vouacapoua americana*/ NA *Andira aubletii*/ acapu/ NA	*Trichophyton mentagrophytes* LMGO 1931 *Microsporum gypseum* LMGO 10 *Microsporum canis* LMGO 22 (reference strains)	*Microsporum gypseum:* > 500 μg/mL *Achetaria guianensis* 16 μg/mL *Cymbopogon citratus* 250 μg/mL *Mikania micrantha* 16 μg/mL *Otacanthus azureus* 125 μg/mL *Piper hispidum* 62 μg/mL *Protium heptaphyllum*, *Vouacapoua Americana*, and *Microsporum canis* > 500 μg/mL *Achetaria guianensis* 62 μg/mL *Cymbopogon citratus* > 500 μg/mL *Mikania micrantha* 125 μg/mL *Otacanthus azureus* 500 μg/mL *Piper hispidum* 62 μg/mL *Protium heptaphyllum* 500 μg/mL *Vouacapoua americana* *Trichophyton mentagrophytes:* > 500 μg/mL *Achetaria guianensis* 8 μg/mL *Cymbopogon citratus* 125 μg/mL *Mikania micrantha* > 8 μg/mL *Otacanthus azureus* 62 μg/mL *Piper hispidum* 31 μg/mL *Protium heptaphyllum* 62 μg/mL *Vouacapoua Americana*
Pinheiro *et al*., [37]	Brazil	*Laurus nobilis*/ louro/ isoeugenol, myrcene, chavicol and methyl eugenol	*Cryptococcus neoformans* FCF10, LM, 0109, LM 310, LM 2601, LM 2301, LM1909 and LM 0310 (reference strains)	256 μg/mL
Cavalcanti *et al*. [38]	Brazil	*Mentha arvensis*/ hortelã japonesa/ NA *Pimpinella anisum*/ anis verde/ NA *Eucalyptus citriodora*/eucalipto limão/ NA *Cymbopogon winterianus*/java citronela/ NA	*Candida albicans* ATCC 289065 *Candida krusei* ATCC 40042 *Candida tropicalis* ATCC40147 (reference strains)	*C. albicans:* 1.25 μg/mL *M. arvensis* and *E. citriodora* 5 μg/mL *P. anisum* 625 μg/mL *C. winterianus* *C. krusei:* 2.5 μg/mL *M. arvensis* and *C. winterianus* 5 μg/mL *P. anisum* and *E. citriodora* *C. tropicalis:* 2.5 μg/mL *M. arvensis* and *E. citriodora* 10 μg/mL *P. anisum* 1.25 μg/mL *C. winterianus*
Bukvicki *et al*. [39]	Serbia	*Thymus algeriensis*/ maralina do campo/ carvacrol	*Aspergillus fumigatus* (clinical isolate) *Penicillium aurantiogriseum.* (isolated from food) *Aspergillus versicolor* ATCC 11730, *Aspergillus ochraceus* ATCC12066, *Aspergilius niger* ATCC 6275, *Trichoderma viride* IAM 5061, *Penicillium funiculosum* ATCC 36839, *Penicillium ochrochloron* ATCC 9112. (reference strains)	*A. fumigatus, A. ochraceus, A. niger, T. viride, P. funiculosum, P. ochrochloron* 0.01 mg/mL *A. versicolor* 0.04 mg/mL *P. aurantiogriseum* 0.02 mg/mL

Gadban *et al*. [40]	Argentina	*Tagetes filifolia*/ cravinho da serra/ anethole	*Colletotrichum truncatum* *Trichoderma harzianum* (isolated from grains and soil)	*C. truncatum* 4000 µl/L *T. harzianum* 7400 µl/L
Hosseini *et al*. [41]	Iran	*Allium sativum*/alho/allyl trisulfide, diallyl disulfide and diallyl sulfide *Rosmarinus officinalis*/ alecrim/ α-pinene, bornyl acetate, camphor and 1,8-cineole	*Colletotrichum nymphaeae* CCTUCCh32 (reference strains)	*A. sativum* CIM_{50}: 3.74 -10.10 µL/L *R. officinalis* CIM_{50}: 43.77 -75.49 µL/L
Elgat *et al*. [42]	Egypt	*Citrus aurantium*/ laranja da terra/ linalyl acetate, α-terpineol, linalool, neryl acetate, geranyl acetate and caryophyllene *Eucalyptus camaldulensis*/ eucalipto vermelho/ inalool and 1,8-cineole *Citrus sinensis* /laranjeira/limonene	*Aspergillus flavus* AFI375 *Aspergillus niger* FC24771 *Aspergillus terreus* V0103 *Fusarium culmorum* CBS128,537. (reference strains)	*C. aurantium:* *A. flavus* 25 µL/mL *A. niger* 8 µL/mL *A. terreus, F. culmorum* 12 µL/mL *E. camaldulensis:* *A. flavus* 12 µL/mL *A. niger* 7 µL/mL *A. terreus* 10 µL/mL *F. culmorum* 8 µL/mL *C. sinensis:* *A. flavus* 12 µL/mL *A. niger, A. terreus, F. culmorum* 6 µL/mL
Abdullahi *et al*. [43]	Malasia	*Zingiber officinale*/ gengibre/isoeugenol	*Fusarium oxysporum* *Pyricularia oryzae* *Colletotrichum falcatum* *Ganoderma boninense* *Rigidoporus microporus* (isolated from plants)	1 µL/mL
Sobrinho *et al*. [44]	Brazil	*Vernonia chalybaea*/ cambarazinho-roxo/ β-caryophyllene	*Trichophyton rubrum* LABMIC 0201; LABMIC 0202; LABMIC 0203 and LABMIC 0204. (reference strains)	1,25 mg/mL
Oliveira *et al*. [45]	Brazil	*Lippia gracilis*/ alecrim-selvagem/ carvacrol and thymol	*Fusarium oxysporum* URM6704 *Fusarium solani* URM6749 *Colletotrichum gloeosporioides* URM6176 Colletotrichum lindemuthianum URM5771. (reference strains)	*F. oxysporum, F. solani* 0.625 mg/mL *C. gloeosporioides*, *C. lindemuthianum* 0.31 mg/mL
Pusceddu *et al*. [46]	Italy	*Thymus capitatus*/ tomilho de creta/ carvacrol *Thymus herba-barona*/ tomilho-alcarávia/carvacrol *Cinnamomun zeylanicum*/ canela da índia/ e-cinnamaldehyde	*Ascosphaera apis* 3136 (reference strains)	*T. capitatus*, *T. herba-borona* 100 µg/mL *C. zeylanicum* 200 µg/mL

Marinas *et al*. [47]	Romania	*Amorpha fruticosa*/ falso indigo/ linalool, citronellol, β -caryophyllene, caryophyllene oxide, α- and γ -muurolene, germacrene D, δ - cadinene and τ -cadinol	*Candida famata* *Candida albicans*, *Candida utilis* (clinical isolate) *Candida famata* CMGBy.14 *Candida albicans* ATCC 101103 (reference strains)	*C. famata* 945 – 14.75 mg/mL *C. albicans* 393 – 7.38 mg/mL *C. utilis* 15 – >29.50 mg/mL C. famata CMGBy.14 > 29.50 mg/mL *C. albicans* ATCC 101103 7.38 mg/mL
Tripathi, Yami, Shukla [5]	India	*Ageratum conyzoides*/ erva de são joão/alkaloids, coumarins, flavonoids, chromenes, benzofurans, sterols and terpenoids *Lantana camara* /cambará/alkaloids, phenolics, terpenoids, phytosterols, tannins and saponins *Litsea cubeba* /pimenta chinesa/ citral *Piper mullesua*/ NA	*Alternaria alternata* MTCC 149 *Mucor haemilis* MTCC 157 *Helminthosporium solani* MTCC 1899 *Humicola grisea* MTCC 352 *Botrytis cinerea* MTCC 359. (reference strains)	*A. conyzoides:* *A. alternata* 500 ppm *H. solani* 300 ppm *M. haemilis, H. grisea, B. cinerea* 400 ppm *L. camara:* *A. alternata* 200 ppm *M. haemilis, H. solani* 300 ppm *H. grisea, B. cinerea* 400 ppm *L. cubeba:* *A. alternata, H. grisea* 300 ppm *M. haemilis* 400 ppm *H. solani, B. cinerea* 200 ppm *P. mullesua:* *A. alternata, H. grisea* 400 ppm *M. haemilis* 200 ppm *H. solani, B. cinerea* 300 ppm
Vuko *et al*. [3]	Croatia	*Dittrichia viscosa*/ tádega/ 1,8-cineole and caryophyllene oxide	*Candida albicans* ATCC 90029 *Aspergillius niger* food isolate	*C. albicans* CIM_{50}: 2.8 mg/mL *A. niger* CIM_{50}: 0.09 mg/mL
Cibanal *et al*. [48]	Argentina	*Origanum vulgare*/ oregano/ carvacrol and thymol	*Penicillium allii*/ vegetable isolate	1,5 μL/mL
Valková *et al*. [4]	Slovakia	*Cymbopogon citratus*/ capim-limão/ citral, geraniol and 1,8-cineole	*Candida albicans* CCM 8261, *Candida krusei* CCM 8271, *Candida glabrata* CCM 8270 *Candida tropicalis* CCM 8223 (reference strains)	*C. albicans* CIM_{50}: 212.35 μL/mL *C. krusei* CIM_{50}: 196.18 μL/mL *C. glabrata* CIM_{50}: 205.26 μL/mL *C. tropicalis* CIM_{50}: 208.34 μL/Ml

Of the 35 articles included (Table 1), 19 (54.2%) are from Brazilian studies, three from Argentina, and one from French Guiana, which suggests a superiority of interest in the antifungal activity of EOs in the Americas, but also marked in several parts of the world [49].

Of the EOs analyzed for their antifungal activity, it was noted that most studies focused on aromatic plants, with emphasis on herbs and spices. The genera *Eucaliptus, Origanum, Piper* and *Cymbopogon* stand out, the latter being the most discussed and referenced in studies from Brazil (five), Slovakia and French Guiana. In fact, according to Butzge *et al*. [50] this genus stands out, with the species *Cymbopogon citratus* (lemongrass) and *Cymbopogon winterianus* (citronella) related to various biological activities, including antifungal.

Regarding the fungi included in the research, the majority were reference strains, but human, veterinary, and plant isolates were also included. However, it was observed that five articles included both the reference strain and the isolates above. The use of a reference strain has the advantage of providing a complete phenotypic description of the species, favoring the

selection of the microorganism based on the hypothesis of biological activity. However, the inclusion of microorganisms of human, veterinary, and environmental origin microorganisms can promote differences in results considering the adaptation attributes that they are using [51].

Of the yeast-like fungi, *Candida spp* and *Cryptococcus neoformans* were included in 16/35 articles, and among the filamentous fungi, dermatophytes (*Trichophyton* spp., *Epidermophyton floccosum*, and *Microscoporum* spp.) and *Aspergillus* spp. Most of the work carried out in Brazil was also explored (Table 1). These fungi, in addition to *Fusarium* spp. and other agents of opportunistic mycoses (*Penicillium* spp., *Alternaria alternata, Mucor haemilis*, among others) are extremely important in the human clinic, especially in cases of invasive infections facilitated by numerous risk factors related to the patient's clinical history [15].

Interestingly, considering the wide distribution of the disease caused by it among humans and animals, especially the increase in cases in southern Brazil [52], only one study [19] investigated the activity of OE on *Sporothrix schenckii*. Furthermore, the report of resistance of this fungus to itraconazole, one of the first treatment options for the infection [53], indicates a gap in the investigation of the antifungal activity of EOs against this pathogen. In this review, it was noted that only the EO of *Origanum vulgare* (oregano) was studied for this purpose.

The EOs for which antifungal action was investigated originated from different plants and it was observed that there is no standardization regarding the concentration unit of the EO to be tested, which makes analysis and categorization as good or bad antifungal activity difficult also references in literature are not found for all units.

However, in most studies (21/35), concentration units in μg/mL (12/35) or mg/mL (9/35) were adopted, allowing a more precise analysis of the results. This is because Kuete [54] classified biocompounds based on their antimicrobial activity as significant (MIC < 100 μg/mL), moderate (100 < MIC ≤ 625 μg/mL), or weak (MIC > 625 μg/mL), while Mbaveng *et al.* [55] considered a MIC below 10 μg/mL to be significant, moderate between 10 and 100 μg/mL, and low when greater than 100 μg/mL.

Thus, considering the articles subject to analysis based on these references, it was observed that only 12 (34.2%) studies were able to demonstrate significant antifungal activity according to the categorization of Kuete [54]. Considering a study carried out by Mbaveng *et al*. [55], which we will use for analysis in this review, the scenario of potential antifungal candidates is more drastic with only five (14.2%) articles included, three of which are articles from Brazil [18, 34, 36, 38, 39]. In these studies, antifungal activity was identified in EOs from plants commonly found in Brazil (*C. citratus, L. medoides* Cham, *C. winterianus, E. citriodora* and *M. arvensis*), against fungi of great clinical relevance, including *C. albicans, C. krusei, C. tropicalis*, *A. fumigatus, A. niger*, in addition to the dermatophytes *M. canis* and *T. mentagrophytes* (Table 1). These results highlight Brazil's potential in researching new bioactive compounds with antifungal properties.

Moderate activity (MIC 10 - 100 μg/mL) [55] was attributed to EOs in nine (25.7%) articles [18, 20, 23, 27, 34-36, 39, 46]. In these studies, emphasis is placed on the activity of OE from oregano, lemongrass, copaiba, and citronella against *Candida* spp and *Aspergillus* spp. (Table 1), which despite the "moderate" classification could be explored for use in association with other compounds after investigating synergism between them, a strategy supported by Cordisco *et al*. [56].

Interestingly, some authors have shown an absolute lack of EO activity against filamentous and yeast-like fungi [18, 24, 25, 28, 30, 47] reporting that concentrations of EOs ≥ 2,000

μg/mL from plants with some known antimicrobial activity, including rosemary, lemon eucalyptus, and cumin, under the conditions used in the studies cited, do not have antifungal action.

Five studies (14.3%) determined the MIC_{50} or MIC_{90}, which are not considered ideal parameters for antimicrobial evaluation by Kuete [54] considering the possible heterogeneity of the population included. Even so, all studies found high MIC values, that is, with moderate or low antifungal activity of EOs, extracted from plants with reports of antimicrobial activity, such as *Cymbopogon* spp., *O. gratissimum*, and *C. cyminum*. Also, these studies included both clinical isolates and reference strains, which suggests that regardless of the origin of the fungus, the antifungal activity of these EOs is not even significant.

Furthermore, two studies also investigated the antifungal action of compounds in ppm concentration, but when converting the results into μg/mL it was observed that the EOs of the plants studied (Table 1) showed good antifungal activity, varying, for example, from 0.2 – 0.5 μg/mL [5]. Completing the relevance of these findings, it should be mentioned that the action was demonstrated against *B. cinerea* and *A. alternata*, important phytopathogens [57], the latter being related to the worsening of respiratory diseases in humans [58].

It should be noted that, although most authors consider that there is a basic component in the EO that could be related to the antifungal action (Table 1), even when the same EO is studied by more than one author, it was not possible to make inferences about the antifungal action and the composition of the EO. This is because the fungal agents included in the studies were very diverse in terms of type (yeast-like and filamentous) and origin (reference species and human, animal or plant isolates), making it impossible to compare the antifungal activity obtained without bias.

This review has some limitations due to the non-uniformity of the studies, especially related to the terroir of the plants originating from the EOs, the extraction methods, and the experimental conditions, especially the concentrations studied. It is known that as essential oil is a secondary metabolite of plants, this variation in antimicrobial potential is expected. However different studies show antifungal activity at low concentrations, while others show no activity at high concentrations of the same plant. Therefore, different factors may be associated with this variation, such as the MIC assessment methods used, the use of standard and correct protocols, and the microorganisms used, among others. However, the data from this work indicate that different OEs are promising antifungals and that, due to the high content of different phytochemical compounds in the composition of the same oil, fungal resistance is less likely. Therefore, given the context of fungi resistance to available drugs, more research must be conducted to establish the best concentrations, essential oils, and the resistance mechanism of these fungi.

4. CONCLUSION

Based on the analysis of the reviewed articles, we can conclude that there is a growing interest in investigating the antifungal activity of EOs against fungal pathogens, especially those resistant to conventional treatments. However, there is a significant gap in research related to the evaluation of EOs against certain fungi, as demonstrated by the scarcity of studies specific to certain pathogens. This gap highlights the need for future research to explore the potential of EOs as effective therapeutic alternatives against resistant fungal infections. Furthermore, these investigations must consider a wide range of essential oils, including those derived from less explored plants, to identify promising new antifungal agents.

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COMPLIANCE WITH ETHICAL STANDARDS

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Scientific research article

In vitro evaluation of 2-bromo-*N*-phenylacetamide for antifungal activity against *Candida glabrata* oral cavity isolates

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SUMMARY

Objective: To evaluate the antifungal potential of 2-bromo-*N*-phenylacetamide (A1Br) against *Candida glabrata* strains isolated from the oral cavity. **Methods**: Minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of A1Br were determined by the broth microdilution technique and via the checkerboard method for pharmacological interaction assessment. Micromorphological changes induced by the compound and the effect of its combination with standard antifungals (nystatin and miconazole) were also evaluated using the checkerboard method, with determination of the fractional inhibitory concentration index (FICI). **Results**: A1Br showed an MIC of 16 μg/mL and an MFC of 32–64 μg/mL against all tested *C. glabrata* strains, indicating predominantly fungicidal activity. The A1Br–nystatin combination exhibited antagonism (FICI = 4.5), whereas the A1Br–miconazole combination showed indifference (FICI = 1.25). Micromorphological analysis revealed reduction of all fungal structures, achieving 100% inhibition at 4× MIC. **Conclusion**: 2-Bromo-*N*-phenylacetamide demonstrated excellent antifungal activity against *C. glabrata* and emerges as a potential drug candidate.

Keywords: Oral candidiasis; *Candida glabrata*; acetanilide; mycobioma.

RESUMO

Avaliação *in vitro* da atividade antifúngica da 2-bromo-*N*-fenilacetamida contra isolados de *Candida glabrata* da cavidade oral

Objetivo: Avaliar o potencial antifúngico da 2-bromo-*N*-fenilacetamida (A1Br) contra cepas de *Candida glabrata* isoladas da cavidade oral. **Métodos:** A concentração inibitória mínima (CIM) e a concentração fungicida mínima (CFM) da A1Br foram determinadas pela técnica de microdiluição em caldo e pelo método checkerboard para avaliação da interação farmacológica. As alterações micromorfológicas induzidas pelo composto e o efeito de sua combinação com antifúngicos padrão (nistatina e miconazol) também foram avaliados pelo método checkerboard, com determinação do índice de concentração inibitória fracionada (ICF). **Resultados:** A1Br apresentou uma CIM de 16 μg/mL e uma CFM de 32–64 μg/mL contra todas as cepas de *C. glabrata* testadas, indicando atividade predominantemente fungicida. A combinação A1Br-nistatina apresentou antagonismo (FICI = 4,5), enquanto a combinação A1Br-miconazol demonstrou indiferença (FICI = 1,25). A análise micromorfológica revelou redução de todas as

estruturas fúngicas, atingindo 100% de inibição a 4 × CIM. **Conclusão:** A 2-Bromo-*N*-fenilacetamida demonstrou excelente atividade antifúngica contra *C. glabrata* e surge como um potencial candidato a fármaco.

Palavras-chave: Candidíase oral; *Candida glabrata*; acetanilida; micobioma.

RESUMEN

Evaluación *in vitro* de 2-bromo-*N*-fenilacetamida para actividad antifúngica contra aislados de *Candida glabrata* de la cavidad oral

Objetivo: Evaluar el potencial antifúngico de la 2-bromo-*N*-fenilacetamida (A1Br) frente a cepas de *Candida glabrata* aisladas de la cavidad oral. **Métodos**: Se determinaron la concentración inhibitoria mínima (CIM) y la concentración fungicida mínima (CFM) de A1Br mediante la técnica de microdilución en caldo y mediante el método de checkerboard para la evaluación de interacciones farmacológicas. También se evaluaron los cambios micromorfológicos inducidos por el compuesto y el efecto de su asociación con antifúngicos estándar (nistatina y miconazol) mediante el método de checkerboard, con la determinación del índice de concentración inhibitoria fraccionada (FICI). **Resultados**: A1Br presentó una CIM de 16 μg/mL y una CFM de 32–64 μg/mL en todas las cepas de *C. glabrata* evaluadas, evidenciando una acción predominantemente fungicida. La combinación A1Br–nistatina mostró antagonismo (FICI = 4,5), mientras que la combinación A1Br–miconazol demostró indiferencia (FICI = 1,25). El análisis micromorfológico reveló reducción de todas las estructuras fúngicas, alcanzando un 100 % de inhibición a 4× CIM. **Conclusión**: La 2-bromo-N-fenilacetamida demostró excelente actividad antifúngica contra *C. glabrata* y se perfila como un posible candidato a fármaco.

Palabras clave: Candidiasis oral; *Candida glabrata*; acetanilida; micobioma.

1. INTRODUCTION

The infectious process called candidiasis is caused by fungi of the Candida genus; opportunistically manifesting in the host in different anatomical regions such as the vaginal or oral mucosa, the respiratory tract, orin various organs [1]. Species such as *Candida albicans* are most often associated with candidiasis lesions, but other species such as *C. tropicalis*, *C. glabrata* and *C. krusei* are also frequently identified [2].

Prosthetic stomatitis is a pathological condition characterized as an inflammatory process that affects the oral mucosa. Colonization of the fungi in the oral cavity is favored by the presence of acrylic resin that due to its porosity favors biofilm accumulation and consequent fungal infection. In the presence of a total prosthesis, tissue changes are observed, including palate injuries and soft tissue damage with the presence of petechiae or reddish areas in the region covered by the prosthesis, often associated with a lack of hygiene. Some patients are asymptomatic for the infection; however, they usually report a variety of symptoms including pain, swelling, xerostomia, halitosis, and bleeding, which often make the prosthesis impossible to use [3].

Epidemiological data reveal that in Brazil, the absence of teeth (edentulism) is indeed present in the young, resulting in the need to use total dentures in 13.7% in the 15-19 year old age group. Other age groups reveal a significant increase evolving to 68.8% in 35-44 year olds, and reaching 92.7% in 65-74 year olds. In total, 17.9% use at least one full denture in one arch and 15.4% in both arches. In a large number of dental prosthesis users, the need for early care is strongly associated with pathological lesions and candidiasis [4].

Candida albicans and *C. glabrata* are responsible for most systemic candidiasis infections, followed by *C. parapsilosis* and *C. tropicalis*. Distinctions between *C. albicans*, *C. parapsilosis,* and *C. tropicalis* relate to CUG (a single leucine-serine exchange).*C.glabrata* maintains several distinctions: in genome duplication, pathogenicity, division, and cell structure [5].

Interactions between the pathogenic fungi *C. albicans* and *C.glabrata* increase with inflammation, suggesting synergistic interactions between the two species and expression of virulence-related genes which may depend on the co-culture composition [6]. In associations of *C. glabrata* with *C. albicans,* the cells do not adhere to each other, but *C. glabrata* presents adhesion along the length of the *C. albicans* hyphae, demonstrating an interdependent relationship. *Candida glabrata* can utilize the destruction of epithelial tissue caused by *C. albicans* to harness nutrients, and even to access the bloodstream [7]. This can bring serious clinical consequences since as *C. glabrata* reaches the internal organs, its high resistance to commonly used antifungals makes treatment more difficult [5-7].

Patients with oral candidiasis are often treated with nystatin or miconazole, antifungals used topically to treat superficial infections. Other agents such as fluconazole, itraconazole, or voriconazole are mainly indicated for deep infections yet are also used in cases of recalcitrant oral candidiasis when topical treatment has failed [3, 8]. Reduced susceptibility of *C. glabrata* to azoles can become a problem in combating infections, especially when infections are associated with patients with HIV [9].

Synthesis of new acetamide substances, is of great interest to researchers since they present several biological activities, among them antibacterial and antifungal [10]. Within the amide class we find the 2-halo-N-arylacetamides, which are an important class of amides used as intermediates in organic synthesis of different products, following principles established by Peixoto [11] and Kristensen [12]. The high incidence of fungal resistance motivates current research to synthesize and discover new substances. The antimicrobial character of bis-pyramidine acetamide has been observed as comparable to the common drugs, cefadroxil (antibacterial) and fluconazole (antifungal) [13].

The high prevalence of candidiasis in users of complete dentures, coupled with the rise of resistant strains, highlights the urgent need for new therapeutic alternatives. Especially *Candida glabrata*, one of the main agents responsible for resistant oral infections, requires the development of effective substances for its control. In this context, acetamides emerge as a promising class, with antifungal properties already observed in previous studies and used in drug formulations [14-16]. This study is justified by the search for new molecules that can expand the therapeutic arsenal available for the treatment of oral candidiasis, particularly in patients with dental prostheses.

The aim of this study was to evaluate the antifungal potential of 2-bromo-*N*-phenylacetamide (A1Br) and its association with other antifungals against *Candida glabrata* strains isolated from the oral cavity.

2. METHODOLOGY

2.1. Chemistry

All reagents and solvents were purchased from commercial sources (Sigma-Aldrich, Brazil) and used without further purification. The progress of the reaction was monitored by thin layer chromatography (TLC) on silica gel plates. ^{1}H-NMR and ^{13}C-NMR spectra were obtained

using a Bruker Avance Ultrashield™ instrument (400 MHz for ^{1}H and 101 MHz for ^{13}C). Deuterated chloroform ($CDCl_3$) was used as solvent and tetramethylsilane (TMS) was used for the internal standard. Chemical shifts (δ) were measured in parts per million (ppm), and the coupling constants (J), in hertz (Hz). The compound was purified by recrystallization and confirmed by determining the melting point range on an MQAPF-3 brand hotplate.

2.2. Preparation of 2-bromo-*N*-phenylacetamide (A1Br)

The preparation of 2-bromo-*N*-phenylacetamide (A1Br), as show in Fig. 1, was obtained according to the procedure of Kaushik *et al.* [17]. In a 50 mL flask containing aniline (1.86 g, 0.020 moles) and K_2CO_3 (3.31 g, 0.024 moles) solubilized in 20 mLofCH_2Cl_2 (dichloromethane) at a temperature of 0 °C, 2-bromoacetyl bromide (4.84 g, 0,024 moles) is slowly added. The ice bath was then removed, and there action stayed under agitation for 20 h at room temperature. The reaction mixture was monitored by TLC (hexane/methyl acetate 1:1). At the end of the reaction, there action mixture was filtered and the solvent was evaporated under reduced pressure, yielding a precipitate. The solid was recrystallized from an ethanol/water mixture (8:2). Yield: 80% M.P. 130-132 °C (Lit. 129-131°). ^{1}H NMR (400 MHz, $CDCl_3$) δ 9.93 (s, 1H), 7.61 (d, J = 8.7 Hz, 2H), 7.30 (t, J = 8.0 Hz, 2H), 7.09 (t, J = 7.4 Hz, 1H), 3.97 (s, 2H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 164.53, 137.76, 128.25, 123.73, 119.36, 29.29 [17].

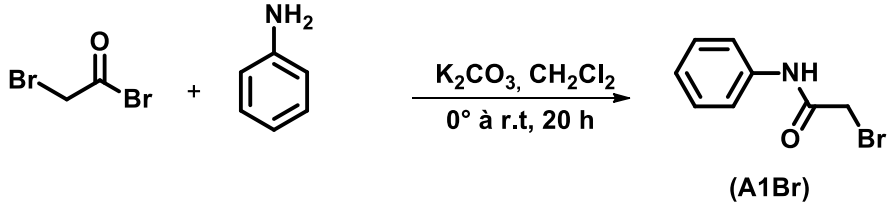

Figure 1. Synthetic route to obtain the target molecule.

The products were weighed and properly solubilized in 5% dimethyl sulfoxide (DMSO) and 2% Tween 80. The final volume was completed with sterile distilled water to yield an emulsion of the products for initial concentrations of from 0.5 μg/mL to 1024 μg/mL [18-20]. Nystatin and miconazole used in the assays as controls were purchased from Sigma-Aldrich® with batches SLCC2040 (nystatin) and BCBD5966D (miconazole), Cotia/SP, CNPJ: 68.337.658/0001-27.

2.3. Culture mediums

For maintenance of strains for assays of antifungal activity, Sabouraud Dextrose Agar – ASD, and RPMI 1640 (INLAB, São Paulo, Brazil) were used, which were prepared according to the manufacturers' descriptions.

2.4. Microorganisms

For the antifungal activity assays, 13 strains of *C. glabrata*, isolated from the oral mucosa were used: LM-16, LM-17, LM-28, LM-35, LM-46, LM-106, LM-108, LM -116, LM-186, LM-188, LM-302, LM-418, LM-533, and a standard ATCC-90030 strain. All were kept on Sabouraud Dextrose-ASD Agar (DIFCO Laboratories LTD/USA) at 4 °C (refrigerator). All belong to the collection of the Antibacterial and Antifungal Activity Research Laboratory, of the Bioactive Natural and Synthetic Products/Health Sciences Center (CCS), of the Department of Pharmaceutical Sciences (DCF), at Paraíba Federal University (UFPB).

2.5. Ethical aspects

As an observational, qualitative-quantitative, analytical/descriptive study, an informed consent waiver was performed since it used *Candida glabrata* strains from the oral cavity; from biological materials collected and stored in the Antibacterial and Antifungal Activity Research Laboratory, of the Bioactive Natural and Synthetic Products/Health Sciences Center (CCS), of the Department of Pharmaceutical Sciences (DCF), at the Federal University of Paraíba (UFPB). The study phases were performed without risk to the research participants. The project linked to the study was approved by the research ethics committee of the Health Sciences Center of the Federal University of Paraíba (CAEE: 12364419.3.0000.5188).

2.6. Inoculum

The inoculum was prepared from colonies obtained from fresh cultures (24-48 hour 35-37°C) of *C. glabrata* in ASD medium. The colonies were suspended in sterile 0.9% physiological solution (Fresenius Kabi Brazil LTDA Barueri-SP), and adjusted according to the 0.5 McFarland scale standard to obtain an inoculum of 10^6 CFU/mL [18, 20-23].

2.7. Determination of Minimum Inhibitory Concentration - MIC

Initially, 100 μL of double concentrated RPMI broth was distributed to the microdilution plate wells. Then 100 μL of each substance was dispensed to the wells of the first line of the plate. By serial dilution at a ratio of two, concentrations of from 0.5 μg/mL to 1024 μg/mL were obtained. Finally, 10 μL of the yeast suspensions were added to the wells, where each column of the plate specifically referred to one yeast. At the same time, the microorganism (RPMI + yeast), and culture medium (RPMI) controls were performed to verify the respective viability of the strains and the sterility of the medium. Controls were also performed with standard antifungals: nystatin and miconazole. The prepared plates were aseptically sealed and incubated at 35-37°C for 24 - 48 hours. MIC determination was performed by the liquid microdilution technique (RPMI broth) with 96 bottom U-shaped wells, (ALAMAR ®). After the assay incubation time, reading was performed and the MIC for each product was defined as the lowest concentration capable of visually inhibiting microbial growth. The antifungal activity of the products was interpreted and considered as being either active or inactive according to the following criteria: 50-500 μg/mL = strong activity; 600-1500 μg/mL = moderate activity; > 1500 μg/mL = weak activity or inactive [24-27]. The assays were performed in duplicate and the results expressed as the arithmetic means of the MFCs obtained in the two assays [27].

2.8. Determination of Minimum Fungicidal Concentration –MFC

After MIC reading, 10 μL aliquots were removed from the well supernatants with no visible fungal growth with the two subsequent dilutions (MIC, MIC × 2 and MIC × 4), and transferred to new microdilution plates containing 100μL of RPMI-1640 in each well. The plates were incubated at 35-37 °C for 24-48 hours. The MFC was considered as the lowest concentration at which there was no growth of yeast in the culture medium. The assays were performed in duplicate and the results expressed as the arithmetic MFC mean [27]. According to Saddiq and Khayyat [28], a substance is fungistatic when the MFC/MIC ratio ≥ 4 and fungicidal when the MFC/MIC ratio<4. For Hafidh and colleagues [29], the respective ratios of 1:1 and 2:1 are considered; where a product is considered both fungicidal and fungistatic at a ratio greater than 2:1.

2.9. Effect of 2-bromo-*N*-phenylacetamide, nystatin, and miconazole on the viability and morphology of *C. glabrata*

This study was performed to analyze *C. glabrata* ATCC-90030 and LM-302 cell viability and morphological characteristics against the products as used in the antifungal activity assays; based on MIC, MIC × 2, and MIC × 4. The fungi were sub-cultured against differing concentrations of 2-bromo-*N*-phenylacetamide, nystatin, and miconazole. First, 100 μL of RPMI 1640 broth was distributed to the wells of the microdilution plates. Then, 100 μL of product was dispensed into the wells of the first line of the plate, this at a concentration corresponding to MIC × 4. By two-fold serial dilutions, the corresponding concentrations of MIC × 2 to MIC were obtained. Then 10 μL of microorganism inoculum was added to the wells (each column of the plate corresponded to a specific fungal lineage). The plates were then incubated at 35 ± 2 °C for 24-48 hours. After the incubation period, a 10 μL aliquot was taken and placed between a slide and coverslip for optical microscopy analysis at 400X magnification. Cell viability assessment of yeast characteristics was thus performed [30].

The experiment was performed in duplicate and the arithmetic mean was calculated from the structures present in five fields (100 μL of RPMI 1640 + 10 μL of inoculum). Evaluation of the results was performed in SPSS® version 13.0statistical software, available as a trial version, and the graphs were elaborated in Excel 2016.

2.10. 2-Bromo-*N*-phenylacetamide (A1Br) molecule association assay (checkerboard method)

The effect of association with standard antifungals was evaluated using microdilution - checkerboard technique for derivation of the FICI (fractional inhibitory concentration index). The tests used solutions of the products in concentrations determined from their respective MICs. Initially, 100 μL RPMI was added to the 96-well plate wells. Then 50 μL of miconazole and nystatin (standard antifungals) at: (MIC × 8, MIC × 4, MIC × 2, MIC, MIC ÷ 2, MIC ÷ 4 and MIC ÷ 8) were added vertically, and then 50 μL of the test product (A1Br) was added horizontally. Finally, 20 μL of fungal suspension was added. The assay was performed in triplicate and the assay incubated at 35-37 °C for 24-48 hours [31].

The FICI (Fractional Inhibitory Concentration Index) was calculated by summing the FIC^A + FIC^B, where A represents the test product and B the standard antifungal. The FIC^A, is calculated as FIC^A= (MIC^A combined)/(MIC^A alone), whereas FIC^B is calculated as FIC^B = (MIC^B combined)/(MIC^B alone). The index is interpreted as follows: synergism (FICI ≤ 0.5), antagonism (FICI> 4.0) and indifference (0.5 <FICI ≤ 4) [32-36].

2.11. Statistical analysis

Analysis of the results was performed according to statistical guidelines for each methodology used; selecting the most appropriate tests in relation to each variable. The results for the micromorphology study were evaluated using SPSS® software version 13.0, available in a trial version. Graphs were elaborated in Excel 2016 for Windows. The non-parametric samples were submitted to statistical analysis using the Mann-Whitney U test, considering significance at $p < 0.05$.

3. RESULTS

Test results for the antifungal activity of the A1Br molecule and the standard antifungals nystatin and miconazole are shown in Table 1. For all studies the media sterility control in RPMI presented no contamination, and all wells in the fungal growth control (no product or antifungal addition) were positive.

The A1Br molecule at a concentration of 16 μg/m inhibited the growth of 13 of the 14 *C. glabrata* strains (92.8%) used in the microbiological assays. The MIC was set at 16 μg/mL and the MFC between 32-64 μg/ml. The antifungal evaluation of results for nystatin were also recorded. The antifungal nystatin inhibited the growth of 11 of the 14 *C. glabrata* strains (78.54%). Its MIC was established at 8 μg/mL. The MFC of nystatin was determined to be between 8-32 μg/mL. For the antifungal activity results of miconazole, the antifungal inhibited yeast growth of11 of the 14 *C. glabrata* strains (78.5%), with an MIC determined at a concentration of 64 μg/mL and the respective MFC at between 512-1204 μg/mL.

Table 1. MIC and MFC results for A1Br, nystatin and miconazole with respective fungicidal or fungistatic activity against *C.glabrata* isolates - microdilution technique.

Strains	Acetamide A1Br (μg/mL)				Nystatin (μg/mL)				Miconazole (μg/mL)			
	MIC[A]	MFC[A]	MFC[a]/MIC[a]	Antifungal Activity	MIC[B]	MFC[b]	MFC[b]/MIC[b]	Antifungal Activity	MIC[c]	MFC[c]	MFC[c]/MIC[c]	Antifungal Activity
ATCC 90030	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 16	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 17	16	32	2	Fungicide	8	8	1	Fungicide	64	512	8	Fungistatic
LM 28	16	32	2	Fungicide	16	32	2	Fungicide	64	1024	16	Fungistatic
LM 35	32	32	2	Fungicide	16	32	2	Fungicide	64	512	8	Fungistatic

LM 533	LM 418	LM 302	LM 188	LM 186	LM 108	LM 106	LM 78	LM 46
16	16	16	16	16	16	16	16	16
32	32	32	64	64	64	64	32	32
2	2	2	4	4	4	4	2	2
Fungicide	Fungicide	Fungicide	Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungicide	Fungicide
8	8	8	8	8	8	8	8	8
8	8	8	8	8	16	16	8	8
1	1	1	1	1	1	1	1	1
Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide	Fungicide
64	64	64	128	64	512	128	64	64
512	512	512	1024	1024	1024	1024	512	512
8	8	8	8	16	2	8	8	8
Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungistatic	Fungicide	Fungistatic	Fungistatic	Fungistatic

Micromorphological analysis was performed to evaluate the presence or absence of hyphae (virulence structures) as well as the fungalshape. For this experiment, a clinical sample - LM-302 was randomly selected from those with the most common MICs of the strains tested (16 μg/mL) and the ATCC 90030 standard. The data are presented in Figure 1, 2 and 3 (A-B), the effects at MIC, MIC × 2 and MIC × 4 revealed no significant differences.

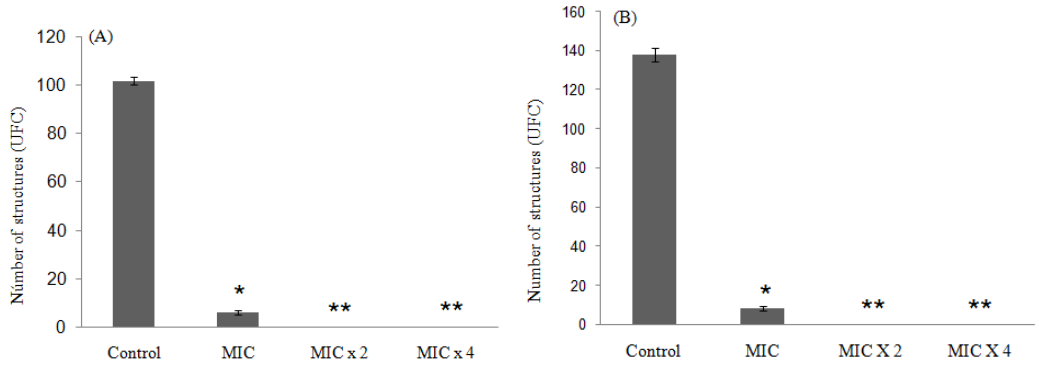

Figure 1. (A-B) - Effect of A1Br at MIC (MIC, MIC × 2, MIC × 4) on standard and clinical lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures.

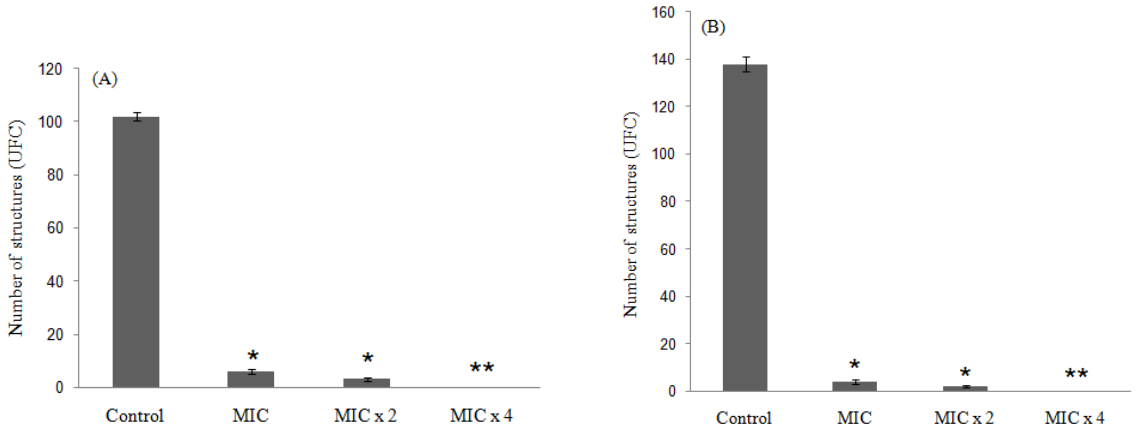

Figure 2. (A-B) - Effect of nystatin at MIC (MIC, MIC × 2, MIC × 4) on standard and clinical lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures.

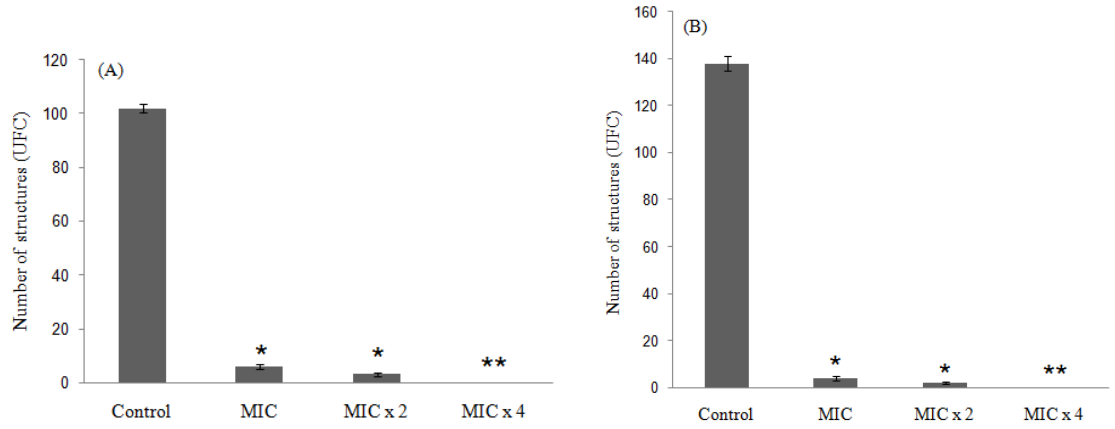

Figure 3. (A-B) - Effect of miconazole at MIC (MIC, MIC × 2, MIC × 4) on the standard and clinical /lineage micromorphology of *Candida glabrata*. (A) *C. glabrata* ATCC® 90030 ™. (B) *C. glabrata* LM-302. * Compared to control (P <0.05), Mann-Whitney Test; ** Absence of structures. Source: Elaborated by the author.

For both the *C. glabrata* strain LM 302 and the ATCC 90030 standard, micromorphology was observed at 400x magnification. Agglomeration of rounded-shaped fungal cells without structural hyphae, consistent with the species was noted.

The association study was performed using the checkerboard technique, which allows construction of a two-dimensional matrix by combining the differing concentrations of the evaluated substances; interactions with nystatin (antagonism)and miconazole (indifference)were respectively obtained (Table 2 and 3).

Table 2. Association study (checkboard) of A1Br molecule with nystatin against *C. glabrata*

Isolated fungi	**MIC (μg/mL)**			**FICI Index**	**Type of Interaction**
	A1Br	Nystatin	A1Br/Nystatin		
C. glabrata ATCC 90030	4	4	2/16	4.5	Antagonism
C. glabrata LM-302	16	2	16/2	4.5	Antagonism

Table 3. Association study (checkboard) of A1Br molecule with miconazole against *C. glabrata*

Isolated fungi	**MIC (μg/mL)**			**FICI Index**	**Type of Interaction**
	A1Br	Miconazole	A1Br/Miconazole		
C. glabrata ATCC 90030	4	128	1/128	1.25	Indifference
C. glabrata LM- 302	16	32	4/32	1.25	Indifference

4. DISCUSSION

The development of antimicrobial research involves analysis of substances with proven effects as well as substances with potential for further development into new compounds. This makes it possible to treat infectious diseases using safer and more effective drugs.

In this context, acetamides and their derivatives appear in several studies revealing antimicrobial and antifungal power [8, 10, 36-39]. Studies such as those by Oliveira and collaborators [37], attaching a methoxide to the amide aromatic ring in the "para" position contributed to antifungal activity, and reinforce the importance of research involving this type of compound; where changes in the molecule's structure may alter its antifungal activity.

The present study confirmed antifungal activity of the A1Br molecule as well as the standard antifungals nystatin and miconazole against clinical samples of *C. glabrata.* When performing the MIC test, the A1Br product was observed inhibiting the growth of 92.8% of the tested strains; this after several dilutions from 1024 μg/mL to 16 μg/mL. The tested substance thus presented excellent antifungal activity values according to the Sartoratto *et al.* classification [25].

When performing the MIC test for antifungals it was observed that nystatin presents an MIC of 8 μg/mL with reductions for 78.54% of the *C. glabrata* strains tested, and presenting low concentration activity against most of these fungi. For miconazole, the most commonly used antifungal for oral cavity fungal infections, an MIC of 64 μg/mL against the tested *C. glabrata* strains was observed. This in relation to the A1Br product, demonstrates inhibition against

fewer fungi. It also corroborates most studies which report reduced azole activity (miconazole fluconazole, itraconazole, voriconazole, and metronidazole). Resistance to these antifungals can lead to systemic infection and death [7, 8].

The results for antifungal activity as obtained by the A1Br molecule in this study corroborate those already obtained by Oliveira and colleagues [37] who tested ten amides from a vanillic group against *Candida albicans* and all showed antifungal effect on the yeast when MIC testing was performed.

Pejchal *et al.* [38] evaluated the MICs of both triazolic fluorobenzene amide and 13 structurally modified derivatives for antimicrobial and antifungal effect, this, in 5 distinct dilutions ranging from 200 μg/mL to 6.25 μg/mL. One of the fungi studied was *C. glabrata* for which 8 of the amides presented no antifungal action at the highest concentration (200 μg/mL); only 5 substances presented a combination effect at 100 μg/mL, 50 μg/mL, and 25 μg/mL. The authors demonstrated the efficacy of amides, but modification of their structure bonds, either by position (ortho and para) or by binding to other elements, can lead to both greater and/or lesser activity against fungi, whether fungicidal or fungistatic.

In yet another case, Çavuşoğlu *et al.* [39] showed that 10 molecular derivatives of an acetamide (obtained from triazole oxadiazoles) against *C. glabrata* presented MICs of from 65.5 μg/mL to 500 μg/mL for 90% of the tested population, with antifungal and apoptotic effect, and presenting good antifungal potential; yet less than A1Br which at 16 μg/mL itself inhibited 90% of the yeast tested.

The MFC was determined. This assay allows understanding at which concentration a given substance is fungicidal, which may result in values higher than the MIC [40].

With respect to the MFC, Bardiot and colleagues [41] found that fungicidal activity is generally preferred over fungistatic activity because it identifies the inhibition of targets essential for fungal growth and/or induces activation of pathways leading to cell death. It also contributes to reducing the emergence of potential resistance phenomena [40]. The MFC obtained for A1Br was 32–64 μg/mL, characterizing the compound as predominantly fungicidal, promoting cell death at low concentrations against *C. glabrata*.

The micromorphological study, in the presence and absence of A1Br, was visualized at 400 X; indicating the presence of rounded yeast, consistent with the structure of *C. glabrata*. To ensure the validity of the study, the standard ATCC 90030 and LM 302 were randomly selected from those with the most prevalent MIC (16μg/mL) for the tested strains. Yeast growth was analyzed by counting their structures when facing A1Br, nystatin, and miconazole at their MIC, MIC × 2, and MIC × 4 concentrations. Fungicidal or fungistatic action was evaluated in function of concentration in relationship to microbial activity [9, 10]

There was a significant decrease in the number of microorganism structures; reaching 100% for the substance A1Br at MIC × 2 for the two strains studied, an excellent result in relation to the antifungals that at a MIC × 2 concentration obtained only partial reductions of the structures. For all of the products tested, and against both strains, A1Br at MIC × 4 presented a 100% reduction in the number of microorganism structures [30]. In this context, it is important to highlight that the morphological changes induced by acetamide are significant findings, as by preventing the formation of invasion and reproduction structures, the infection is halted and controlled.

After confirming the antifungal activity of A1Br, a combination assay was performed using acetamide and drugs commonly used in clinical practice, such as miconazole and nystatin. According to Andrade Júnior *et al*. [42], the combination of antifungal agents can reduce the

selection of resistant strains, in addition to allowing the use of lower concentrations of each individual drug, thereby minimizing adverse effects and enhancing treatment effectiveness.

In accordance with the results obtained in the combination assay, the FICI was 4.5 (four and a half) for the standard strain ATCC 90030 and LM 302; an antagonism ratio of FICI> 4.0for nystatin with A1Br, and demonstrating activity for only one of the substances against the analyzed strains; imply in inhibition. The value was obtained using the Inhibitory Concentration Index (FICI), which considers the isolated and combined MICs of the products and yields the index value. The association of miconazole with A1Br obtained a value of 1.25 for both ATCC 90030 and LM 302, and an indifferent relationship between them (0.5 <FICI<4). Thus, the union to these two compounds neither potentiates nor antagonizes the activity against the studied yeasts [32-36].

Pharmacological indifference occurs when one drug does not interfere with the mechanism of action of the other, neither reducing nor increasing the response, while antagonism happens when there is interference between the mechanisms of action of the drugs. In the latter case, the combined administration of the substances is unfeasible, although their clinical application individually is perfectly possible [42]. In pharmacological indifference, the drugs can be administered together, even without enhancing each other's therapeutic effect.

Furthermore, it is important to highlight that the variability in the responses of substances against fungi of the same species is related to the genetic and phenotypic characteristics of each strain, emphasizing the need for more in-depth research on this topic [43].

Thus, although no synergy was observed in the combinations tested between A1Br and standard antifungals, combination therapy remains a promising strategy, especially against resistant pathogens [44].

There are no studies in the literature reporting on association effects against *Candida glabrata* for A1Br with either nystatin or miconazole, leaving room for further research evaluating both mechanisms of action and pharmacological receptors, and to further evaluate potential effects of this new substance whether by modification of the ligand site, or through its activity in combination with other drugs.

The A1Br molecule demonstrated excellent antifungal activity against *C. glabrata*, with a predominantly fungicidal character. A significant reduction in fungal structures was observed, being more effective in eliminating fungal cells compared to the other antifungals tested. Regarding the combination, antagonism of A1Br with nystatin and indifference with miconazole was observed. Therefore, despite its promising potential against fungi, further studies are needed to better understand the pharmacological and toxicological characteristics of 2-bromo-*N*-phenylacetamide.

CONFLICT OF INTEREST

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

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Review article

Chronic pancreatitis: Pharmacotherapy, prescription of drugs, meta-analysis, latest digital medical technologies of quantum medicine

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SUMMARY

Introduction: Chronic pancreatitis is a progressive inflammatory disease of the pancreas characterized by irreversible morphological changes, chronic pain, exocrine and endocrine insufficiency, and significantly reduced quality of life. Its incidence is increasing worldwide due to environmental, behavioral, and socioeconomic factors. There is a pressing need for evidence-based, accessible, and standardized pharmacotherapy protocols. The study aimed to manage and optimize pharmacotherapy for chronic pancreatitis through meta-analysis and explore the potential of digital quantum medical technologies. **Methodology:** A systematic review of international and national scientific literature was performed using databases such as PubMed, Embase, and the Cochrane Library. Clinical protocols from the EU, France, Kazakhstan, and Ukraine were included. A multivariate meta-analysis was conducted in STATA 13, guided by SMART objectives. Surveys of healthcare providers were conducted in multiple Ukrainian regions, and 77 anonymized patient medical records were analyzed. Medicines were categorized by international non-proprietary names (INNs) and assessed by two key criteria: "Recognition" (presence in clinical protocols) and "Availability" (presence in regulatory and price documents). Additional methodological approaches included normative, documentary, bibliographic, mathematical, and legal analyses. **Results and Discussion:** A total of 46 INNs were identified for use in chronic pancreatitis pharmacotherapy. Only two drugs—Drotaverine and Ibuprofen—achieved the highest combined scores for recognition and availability across all evaluated systems. Enzyme preparations, analgesics, NSAIDs, proton pump inhibitors, and vitamins were also widely used. The study confirms the need for personalized, evidence-based prescribing to improve patient access and optimize outcomes. Additionally, the paper discusses the prospective role of quantum medicine—particularly microwave resonance therapy—as a supplementary digital technology to enhance treatment quality by targeting the body's electromagnetic field. **Conclusion:** The meta-analysis enabled the structured evaluation of pharmacotherapy for chronic pancreatitis based on recognition and availability criteria. The findings support better regulation, economic efficiency, and improved patient outcomes through optimized prescribing

practices. The potential of quantum medicine technologies offers a novel frontier for enhancing care quality, diagnostics, and long-term management strategies in chronic pancreatitis.

Keywords: Chronic pancreatitis; pharmacotherapy; drugs; meta-analysis; quantum medicine; quantum pharmacy; microwave resonance therapy

RESUMEN

Pancreatitis crónica: Farmacoterapia, prescripción de fármacos, metaanálisis, últimas tecnologías médicas digitales de la medicina cuántica

Introducción: La pancreatitis crónica es una enfermedad inflamatoria progresiva del páncreas que se caracteriza por cambios morfológicos irreversibles, dolor crónico, insuficiencia exocrina y endocrina, y una reducción significativa de la calidad de vida. Su incidencia está aumentando a nivel mundial debido a factores ambientales, conductuales y socioeconómicos. Existe una necesidad apremiante de protocolos farmacoterapéuticos basados en la evidencia, accesibles y estandarizados. El estudio tuvo como objetivo gestionar y optimizar la farmacoterapia para la pancreatitis crónica mediante un metaanálisis y explorar el potencial de las tecnologías médicas cuánticas digitales. **Metodología:** Se realizó una revisión sistemática de la literatura científica internacional y nacional utilizando bases de datos como PubMed, Embase y la Biblioteca Cochrane. Se incluyeron protocolos clínicos de la UE, Francia, Kazajistán y Ucrania. Se realizó un metaanálisis multivariante en STATA 13, guiado por objetivos SMART. Se realizaron encuestas a profesionales sanitarios en varias regiones de Ucrania y se analizaron 77 historiales médicos anónimos de pacientes. Los medicamentos se clasificaron según sus denominaciones comunes internacionales (DCI) y se evaluaron según dos criterios clave: reconocimiento (presencia en protocolos clínicos) y disponibilidad (presencia en documentos regulatorios y de precios). Otros enfoques metodológicos incluyeron análisis normativos, documentales, bibliográficos, matemáticos y legales. **Resultados y discusión:** Se identificaron 46 DCI para su uso en la farmacoterapia de la pancreatitis crónica. Solo dos fármacos (drotaverina e ibuprofeno) obtuvieron las puntuaciones combinadas más altas en reconocimiento y disponibilidad en todos los sistemas evaluados. Las preparaciones enzimáticas, los analgésicos, los AINE, los inhibidores de la bomba de protones y las vitaminas también se utilizaron ampliamente. El estudio confirma la necesidad de una prescripción personalizada y basada en la evidencia para mejorar el acceso de los pacientes y optimizar los resultados. Además, el artículo analiza el papel potencial de la medicina cuántica, en particular la terapia de resonancia de microondas, como tecnología digital complementaria para mejorar la calidad del tratamiento mediante la focalización del campo electromagnético corporal. **Conclusión:** El metaanálisis permitió la evaluación estructurada de la farmacoterapia para la pancreatitis crónica con base en criterios de reconocimiento y disponibilidad. Los hallazgos respaldan una mejor regulación, la eficiencia económica y mejores resultados para los pacientes mediante prácticas de prescripción optimizadas. El potencial de las tecnologías de la medicina cuántica ofrece una nueva frontera para mejorar la calidad de la atención, el diagnóstico y las estrategias de manejo a largo plazo en la pancreatitis crónica.

Palabras clave: Pancreatitis crónica; farmacoterapia; fármacos; metaanálisis; medicina cuántica; farmacia cuántica; terapia de resonancia de microondas

RESUMO

Pancreatite crônica: Farmacoterapia, prescrição de medicamentos, meta-análise, as mais recentes tecnologias médicas digitais da medicina quântica

Introdução: A pancreatite crônica é uma doença inflamatória progressiva do pâncreas, caracterizada por alterações morfológicas irreversíveis, dor crônica, insuficiência exócrina e endócrina e redução significativa da qualidade de vida. Sua incidência está aumentando em todo o mundo devido a fatores

ambientais, comportamentais e socioeconômicos. Há uma necessidade urgente de protocolos farmacoterapêuticos baseados em evidências, acessíveis e padronizados. O estudo teve como objetivo gerenciar e otimizar a farmacoterapia para pancreatite crônica por meio de meta-análise e explorar o potencial das tecnologias médicas quânticas digitais. **Metodologia:** Foi realizada uma revisão sistemática da literatura científica internacional e nacional utilizando bases de dados como PubMed, Embase e Biblioteca Cochrane. Protocolos clínicos da UE, França, Cazaquistão e Ucrânia foram incluídos. Uma meta-análise multivariada foi conduzida no STATA 13, guiada pelos objetivos SMART. Pesquisas com profissionais de saúde foram conduzidas em diversas regiões ucranianas, e 77 prontuários médicos anonimizados de pacientes foram analisados. Os medicamentos foram categorizados por nomes não proprietários internacionais (DCIs) e avaliados por dois critérios principais: "Reconhecimento" (presença em protocolos clínicos) e "Disponibilidade" (presença em documentos regulatórios e de preços). Abordagens metodológicas adicionais incluíram análises normativas, documentais, bibliográficas, matemáticas e jurídicas. **Resultados e Discussão:** Um total de 46 DCIs foram identificados para uso na farmacoterapia da pancreatite crônica. Apenas dois medicamentos — Drotaverina e Ibuprofeno — alcançaram as maiores pontuações combinadas de reconhecimento e disponibilidade em todos os sistemas avaliados. Preparações enzimáticas, analgésicos, AINEs, inibidores da bomba de prótons e vitaminas também foram amplamente utilizados. O estudo confirma a necessidade de prescrição personalizada e baseada em evidências para melhorar o acesso do paciente e otimizar os resultados. Além disso, o artigo discute o papel prospectivo da medicina quântica — particularmente a terapia por ressonância de micro-ondas — como uma tecnologia digital suplementar para aprimorar a qualidade do tratamento, direcionando-se ao campo eletromagnético do corpo. **Conclusão:** A meta-análise permitiu a avaliação estruturada da farmacoterapia para pancreatite crônica com base em critérios de reconhecimento e disponibilidade. Os resultados corroboram uma melhor regulamentação, eficiência econômica e melhores resultados para os pacientes por meio de práticas de prescrição otimizadas. O potencial das tecnologias da medicina quântica oferece uma nova fronteira para aprimorar a qualidade do atendimento, o diagnóstico e as estratégias de manejo a longo prazo na pancreatite crônica.

Palavras-chave: Pancreatite crônica; farmacoterapia; medicamentos; meta-análise; medicina quântica; farmácia quântica; terapia por ressonância de micro-ondas

1. INTRODUCTION

Today, the incidence of chronic pancreatitis is increasing all over the world. In the general structure of diseases of the digestive organs, chronic pancreatitis ranks 3rd among all pathologies of the gastrointestinal tract [1]. Increasing influence of adverse factors of the external environment, armed conflicts, increasing medical and pharmaceutical burden, use of psychoactive substances, alcohol, decrease in quality of nutrition and general standard of living are etiological risk factors of chronic pancreatitis. People over 45 years of age usually get sick, although in the last few years there is a trend of "rejuvenation" of the disease. In Ukraine, over the last decade, pancreatitis occurs 3 times more often in young people and adolescents than before. Chronic pancreatitis is dangerous due to its complications. In the first 10 years, about 20% of patients die from complications, and within 20 years – 50% [2].

Publications on the diagnosis and management of acute pancreatitis were found among pancreatitis [3].The incidence of acute pancreatitis is also increasing. Over the past 10 years, hospitalization due to pancreatitis has increased by at least 15% [4]. A severe form of acute pancreatitis covers approximately 20–30% of patients, is a life-threatening disease with a hospital mortality rate of about 15 [5]. Understanding the pathophysiology of pancreatitis pro-

vides an opportunity to expand the methods of pharmacotherapy [6]. Further recommendations for the diagnosis and treatment of acute pancreatitis were made in China [7]. Optimizing the safety of patients with acute pancreatitis has been studied with the use of opioids [8].

Recurrent acute pancreatitis can lead to the development of chronic pancreatitis. Current world data show an increase in the number of patients with chronic pancreatitis. The results of the research concerning the pharmacotherapy of patients with chronic pancreatitis with comorbidity from the pharmacological point of view were described earlier [9]. Chronic pancreatitis is a chronic inflammatory disease of the pancreas, which is accompanied by irreversible structural changes in the development of excretory and incretory insufficiency, manifested by abdominal pain and characterized by a significant reduction in the quality of life of patients [10].

Chronic pancreatitis is a persistent inflammatory disease leading to irreversible morphological changes in the pancreas, progressive functional decline, and significant impact on patients' quality of life. Traditional diagnostic and therapeutic approaches often fall short in fully elucidating the pathogenesis and managing long-term outcomes. In this context, quantum medicine—based on quantum field theory principles—offers novel perspectives by considering the bioresonance properties of biological tissues, energetic imbalances, and the possibility of early preclinical detection and modulation of disease processes. The integration of quantum diagnostics and therapeutic modalities into the management of chronic pancreatitis provides a potentially transformative framework for individualized, non-invasive interventions aimed at harmonizing systemic function and cellular communication.

The use of meta-analysis was carried out to evaluate the appointment of antibiotics and protease inhibitors in acute pancreatitis [11, 12]. In addition, a meta-analysis was conducted in the treatment of alcoholic hepatitis. The results of the meta-analysis were recommended for doctors providing primary, secondary (specialized) medical care [13]. To continue research, the purpose of this study is the management of pharmacotherapy of chronic pancreatitis with the use of meta-analysis, prospects for using the latest digital medical technologies of quantum medicine.

2. METHODOLOGY

The work uses sources of scientific literature using the keywords "pancreatitis", "chronic pancreatitis", "acute pancreatitis", "alcoholic pancreatitis", "treatment", "pharmacotherapy", "drugs", "treatment protocols", "the latest digital medical technologies of quantum medicine" in PubMed, Embase, Cochrane Library, open electronic databases of specialized journals, publications of conferences, professional associations. The search strategy was based on the combination of terms for chronic pancreatitis with a variant of the arbitrary text. In addition, lists of literary sources of all suitable articles and modern systematic reviews were considered. A meta-analysis of the prescription of drugs in the treatment of chronic pancreatitis was conducted according to the principles of evidence-based medicine and evidence-based pharmacy (quality, safety, economy, accessibility). Conducting a meta-analysis to study direct and indirect comparisons of International non-proprietary names of medicines in the management of pharmacotherapy of chronic pancreatitis was conducted using a multivariate meta-analysis model with statistical software STATA 13 (StataCorp. College Station, Texas, USA). A recent update to the multivariate meta-analysis procedure in STATA enables network meta-analysis to be performed in commonly used meta-analysis software. The author's personalized approaches for SMART objectives of meta-analysis: specific (concrete), measurable, verifiable,

acceptable, realistic, timely were used [13]. The selection of countries of the world and evidentiary information of clinical protocols was made objectively with the help of the computer program "dilovodstvo" of the automated document management system, which was developed by the Department of Informatization of Courts and Judicial Statistics of the Council of Judges of Ukraine. EU countries, France, Kazakhstan, and Ukraine were selected for this program. For these countries, the authors administered regulatory documents, evidence-based clinical guidelines, unified clinical protocols, and treatment standards [14-18]. The information base of the study consisted of scientific works of foreign and domestic scientists on the topic of the article. The review of scientific sources of literature was carried out considering the recommendations of the Cochrane Society for PICO: P (population) – the population suffering from chronic pancreatitis; I (intervention) – pharmacotherapy, administration of effective, safe, affordable medicines; C (comparator) – research technology; O (outcomes) – research results. The analysis based on production was performed with the determination of the share of domestic and foreign drugs, by individual countries, manufacturing companies. Drugs clinical and pharmacological groups for pharmacotherapy of chronic pancreatitis with diagnostic codes ATC – Classification (ATC) were selected [19]. International and national medical and technological documents on standardization of medical care of chronic pancreatitis, as well as scientific sources were used for regulatory, documentary and pharmacoeconomic analysis. Clinical protocols and international treatment standards were considered. Diagnostic codes and criteria were analyzed: K86.0; K86.1 – International Classification of Diseases of 10th edition; DS31; DC32 – International Classification of Diseases of 11th edition; D99 – International Classification of Primary Care-2 (ICPC-2). The names of medicines were systematized by International non-proprietary name (INN), trade names, the number of medicines, dosage forms. All drugs were registered in the State Register of drugs of Ukraine as of June 2024. The current research was carried out using the system approach during 2020–2024. The survey of doctors was conducted in hospitals and polyclinics of the Rivne, Kharkiv, Kyiv, and Kirovohrad regions of Ukraine. At the same time, the anonymous analysis of medical cards of patients with chronic pancreatitis was carried out (77 medical cards). Medicines for the management of the pharmacotherapy of chronic pancreatitis are systematized. The experimental study was conducted on the clinical bases of Kharkiv Medical Academy of Postgraduate Education, Lviv Medical University, Luhansk State Medical University (hospitals, pharmacies), Department of Medical and Pharmaceutical Law, General and Clinical Pharmacy of Kharkiv Medical Academy of Postgraduate Education, and Department of Pharmacy of Luhansk State Medical University, European Academy of Digital Medical Technologies “Re-Generation” Center (Kyiv, Ukraine).

In parallel with the meta-analysis, modern research methods were also used clinical and pharmacological, normative and legal, documentary, bibliographic, systemic, comparative, marketing, graphic, mathematical analysis. Mathematical processing and statistical evaluation of data was performed using Microsoft Excel. Inclusion criteria encompassed peer-reviewed articles published between 2010 and 2024, in English or Ukrainian, focusing on chronic pancreatitis, quantum medicine, bioresonance therapy, and related diagnostic or therapeutic modalities. Exclusion criteria included conference abstracts, editorial materials, animal studies, and papers lacking quantitative or clinical data. The databases consulted included PubMed, Scopus, Web of Science, and Google Scholar. Keywords used were: “chronic pancreatitis”, “quantum medicine”, “bioresonance therapy”, “electromagnetic therapy”, and “quantum diagnostics”.

Two reviewers performed data extraction independently. Extracted data included study design, population characteristics, type of intervention, outcomes measured, and quality indicators. Discrepancies were resolved by consensus. The quality of included studies was assessed using the PRISMA guidelines and the GRADE framework.

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The design of the study was carried out according to the methodology developed by the authors of the article and is given in the Table 1.

Table 1. Design of the author's research methodology

No.	Stage	Type of work
1.	Regulatory and legal analysis of the evidence base of management in the pharmacotherapy of chronic pancreatitis in the countries of the world	Collection, systematization, and analysis of prescriptions of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis using the database of the Ministry of Health of Ukraine, MedElement, Duodecim Medical Publications Ltd
2.	Ranking of prescriptions of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis according to the "Recognition" criteria	Conducting a questionnaire of doctors to determine the specific share of prescriptions of International non-proprietary names of medicines according to the "Recognition" criteria
3.	Ranking of prescriptions of International non-proprietary names of medicines for pharmacotherapy of chronic pancreatitis according to the "Availability" criteria	Conducting a questionnaire of doctors to determine the percentage of prescriptions of International non-proprietary names of medicines according to the "Availability" criteria
4.	Determination of the average arithmetic value of the criteria "Recognition" and "Availability"	Systematization of the table 3 data considering the criteria "Recognition" and "Availability" in percentages and further calculation of the average arithmetic value
5.	Building a network of comparisons of International non-proprietary names of medicines in the pharmacotherapy of chronic	Conducting a meta-analysis for the analysis of direct and indirect comparisons of the International non-proprietary names of medicines in the pharma-

	pancreatitis considering the criteria "Recognition" and "Availability"	cotherapy of chronic pancreatitis using a multidimensional meta-analysis model with statistical software STATA 13
6.	Conclusions	Determination of conclusions based on comparison network analysis

3. RESULTS AND DISCUSSION

The diagnosis "chronic pancreatitis" is recommended to be used to define chronic inflammation of the pancreas with chronic, irreversible, inflammatory, and fibrotic changes in the pancreas. It often characterized by severe pain, which reduces the quality of life of patients. The diagnosis of chronic inflammation of the pancreas is confirmed based on the symptoms present in the patient. The results of imaging to determine the structure of the pancreas (ultrasound examination, computed tomography), exocrine and endocrine function tests were considered. Use of psychoactive substances, alcohol, and tobacco are significant factors in chronic pancreatitis [20].

Management during the differential diagnosis of chronic pancreatitis is carried out to prove the presence of this disease, to exclude other diseases that may be accompanied by similar complaints. Differential diagnosis of chronic pancreatitis is associated with the exclusion of disorders of the gastrointestinal tract, cardiovascular, reproductive system, and other disorders. Among disorders of the gastrointestinal tract, it is necessary to exclude acute pancreatitis, acute cholecystitis, gallstones, acute appendicitis, peptic ulcer, tumors of the pancreas, Crohn's disease, paresis of the stomach, nonspecific ulcerative colitis, irritable bowel syndrome. Among disorders of the cardiovascular system, it is necessary to exclude myocardial infarction, ischemic heart disease, and vascular thrombosis. Endometriosis, ectopic pregnancy, ovarian cyst, inflammation of the fallopian tubes, ovarian cancer should be excluded from the disorders of the reproductive system. At the same time, other disorders are excluded – urolithiasis, thoracic radiculopathy [15, 21, 22]. Clinical-pharmacological groups of drugs prescribed for pharmacotherapy of chronic pancreatitis are shown in the Table 2.

Table 2. Drugs based on evidence in the pharmacotherapy of chronic pancreatitis

No.	Clinical and pharmacological group	INNs
1.	Analgesics and antipyretics	Metamizole sodium, Paracetamol
2.	Analogues of somatostatin	Octreotide
3.	Antibacterial agents	lmipenem + Cilastatin, Meropenem, Metronidazole, Cefotaxime, Cefuroxime, Cefoperazone, Ceftriaxone, Ciprofloxacin
4.	Vitamins	Ergocalciferol, Menadione, Retinol, Tocopherol, Phytomenadione, combined vitamin preparations
5.	Proton pump inhibitors	Esomeprazole, Lansoprazole, Omeprazole, Pantoprazole, Rabeprazole
6.	Blood substitutes and perfusion solutions	Sorbitol + Sodium lactate + Sodium chloride + Calcium chloride + Potassium chloride + Magnesium chloride; Albumin; Glucose
7.	Nonsteroidal anti-inflammatory and antirheumatic drugs	Ibuprofen
8.	Opioid analgesics	Tramadol
9.	Enzyme preparations	Pancreatine
10.	Antispasmodics	Drotaverine, Mebeverine, Papaverine

At the next stage of the research, a normative and legal analysis of the evidence base of management in the pharmacotherapy of chronic pancreatitis among selected countries of the world was carried out. The list of evidence-prescribed drugs includes 46 International non-proprietary names of medicines (Table 3).

Table 3. Prescribing drugs in the pharmacotherapy of chronic pancreatitis

No.	INNs	Ukraine	Instruction No. 00209 EU	Kazakhstan	France
1.	Albumin	+			
2.	Alendronic acidum			+	
3.	Cefoperazone	+	+		
4.	Cefotaxime	+	+		
5.	Ceftriaxone	+	+		
6.	Cefuroxime	+	+		
7.	Ciprofloxacin	+	+		
8.	Diazepam			+	
9.	**Drotaverine**	**+**	**+**	**+**	**+**
10.	Ergocalciferol	+			
11.	Esomeprazole	+		+	
12.	Famotidine			+	
13.	Glucose	+	+		+
14.	Ibandronic acid			+	
15.	**Ibuprofen**	**+**	**+**	**+**	**+**
16.	Insulin		+		
17.	Ketoprofen			+	
18.	Ketorolac			+	
19.	Kolekaltsiferolum			+	
20.	Lansoprazole	+		+	
21.	Imipenem + Cilastatin	+	+		
22.	**Mebeverine**	**+**	**+**	**+**	**+**
23.	Menadione	+			
24.	Menadione sodium bisulfite			+	
25.	Meropenem	+	+		
26.	Metamizole sodium	+			
27.	Metronidazole	+	+		
28.	Morphine			+	
29.	Octreotide	+		+	
30.	Omeprazole	+		+	
31.	**Pancreatine**	**+**	**+**	**+**	**+**
32.	Pantoprazole	+		+	
33.	Paracetamol	+		+	
34.	Phytomenadione	+			
35.	Prednisolone			+	
36.	Pregabalin			+	
37.	Rabeprazole	+		+	
38.	Ranitidine			+	
39.	Retinol	+		+	

40.	Sorbitol + Sodium lactate + Sodium chloride + Calcium chloride + Potassium chloride + Magnesium chloride	+			
41.	Sulpiride			+	
42.	Teriparatide			+	
43.	Tocopherol	+		+	
44.	Tramadol	+		+	
45.	Zoledronic acid			+	
46.	Papaverine	+	+		+

Table 3 describes that the four drugs Drotaverine, Ibuprofen, Mebeverine, Pancreatine coincide when prescribed to patients in the management of pharmacotherapy of chronic pancreatitis in the EU countries, Ukraine, Kazakhstan, and France. Analgesics and antipyretics, opioid analgesics, nonsteroidal anti-inflammatory and antirheumatic drugs are used in effective doses with appropriate intervals considering contraindications. Invasive pain treatment is prescribed for patients with chronic pancreatitis when medical treatment is ineffective. Enzyme preparations, proton pump inhibitors are prescribed to patients with steatorrhea, lipid malabsorption, diarrhea, weight loss, or other clinical and laboratory signs of nutrient deficiency. Maintaining an adequate diet, correcting micronutrient deficiencies, using pancreatic enzymes, and treating pain showed a positive effect on the nutritional status of patients with chronic pancreatitis.

To carry out the next stage of the research regarding the ranking of International nonproprietary names of medicines in the pharmacotherapy of chronic pancreatitis according to the "Recognition" criteria, a questionnaire was conducted among doctors who provide medical assistance and prescribe pharmacotherapy to patients with chronic pancreatitis. Questionnaire questions are given in the Table 4.

Table 4. Questionnaire for doctors regarding the ranking of International non-proprietary names of medicines for pharmacotherapy of chronic pancreatitis according to the "Recognition" criteria

No.	Question	Answer, %
1.	In your opinion, how important is the information regarding International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in the evidence-based guidelines of the Ministry of Health of Ukraine	81 – very important 19 – mediocre 0 – not important
2.	In your opinion, how important is the presence of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in proven clinical protocols of MedElement	77 – very important 15 – mediocre 8 – not importan
3.	In your opinion, how important is the presence of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in the evidence protocols of PNDS	69 – very important 10 – mediocre 21 – not important
4.	In your opinion, how important is the presence of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in the evidence-based guidelines of Duodecim Medical Publications Ltd EU	94 – very important 6 – mediocre 0 – not important

Thus, the respondents answered that the presence of International non-proprietary names of medicines in the evidence base for the pharmacotherapy of chronic pancreatitis is mostly very important for the management of prescriptions (range from 69% to 94%).

The next stage of the study regarding the ranking of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis according to the criteria "Availability" from the questions of the questionnaire is given in the Table 5.

Table 5. Questionnaire for doctors regarding the ranking of prescriptions of International non-proprietary names of medicines for pharmacotherapy of chronic pancreatitis according to the "Availability" criteria

No.	Question	Answer, %
1.	In your opinion, how important is the appointment of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in regulatory documents regarding the prices of drugs in the pharmaceutical supply of hospitals	72 – very important 22 – mediocre 6 – not important
2.	In your opinion, how important is the appointment of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in regulatory documents regarding the availability of prescription drugs for patients	90 – very important 10 – mediocre 0 – not important
3.	In your opinion, how important is the appointment of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in regulatory documents regarding the availability of over-the-counter drugs for patients	68 – very important 7 – mediocre 25 – not important

Respondents answered that the presence of prescription and over-the-counter International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis is mostly very important (range from 68% to 90%).

For the next stage of the research, the data in the Table 3 were systematized, considering the criteria "Recognition" and "Availability". An analysis of the use of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis in evidence-based protocols was carried out. The presence of the drug in one of the four treatment protocols was equal to 25.0% according to the "Recognition" criteria. To determine the percentage of the "Availability" criteria, the presence of International non-proprietary names of medicines for the pharmacotherapy of chronic pancreatitis in regulatory documents regarding the prices of drugs in the pharmaceutical supply of hospitals was checked; in regulatory documents regarding the availability of prescription drugs for patients; in regulatory documents regarding the availability of over-the-counter drugs for patients. Further, the average arithmetic value of the "Definiteness" and "Availability" criteria was calculated in percentages (Table 6).

Table 6. Determination of the average arithmetic value of the "Recognition" and "Availability" criteria

No.	INNs	Recognition, %	Availability, %	Arithmetic average value of the "Recognition" and "Availability" criteria, %
1.	Albumin	25.0	33.3	29.2
2.	Alendronic acidum	25.0	33.3	29.2
3.	Cefoperazone	50.0	33.3	41.7

4.	Cefotaxime	50.0	66.7	58.4
5.	Ceftriaxone	50.0	33.3	41.7
6.	Cefuroxime	50.0	33.3	41.7
7.	Ciprofloxacin	50.0	66.7	58.4
8.	Diazepam	25.0	66.7	45.9
9.	**Drotaverine**	**100.0**	**100.0**	**100.0**
10.	Ergocalciferol	25.0	66.7	45.9
11.	Esomeprazole	50.0	33.3	41.7
12.	Famotidine	25.0	33.3	29.2
13.	Glucose	75.0	33.3	54.2
14.	Ibandronic acid	25.0	66.7	45.9
15.	**Ibuprofen**	**100.0**	**100.0**	**100.0**
16.	Insulin	25.0	66.7	45.9
17.	Ketoprofen	25.0	33.3	29.2
18.	Ketorolac	25.0	33.3	29.2
19.	Kolekaltsiferolum	25.0	0.0	12.5
20.	Lansoprazole	50.0	33.3	41.7
21.	lmipenem + Cilastatin	50.0	33.3	41.7
22.	Mebeverine	100.0	33.3	66.7
23.	Menadione	25.0	33.3	29.2
24.	Menadione sodium bisulfite	25.0	33.3	29.2
25.	Meropenem	50.0	66.7	58.4
26.	Metamizole sodium	25.0	100.0	62.5
27.	Metronidazole	50.0	100.0	75.0
28.	Morphine	25.0	66.7	45.9
29.	Octreotide	50.0	33.3	41.7
30.	Omeprazole	50.0	100.0	75.0
31.	Pancreatine	100.0	33.3	66.7
32.	Pantoprazole	50.0	66.7	58.4
33.	Paracetamol	50.0	100.0	75.0
34.	Phytomenadione	25.0	66.7	45.9
35.	Prednisolone	25.0	66.7	45.9
36.	Pregabalin	25.0	33.3	29.2
37.	Rabeprazole	50.0	0.0	25.0
38.	Ranitidine	25.0	66.7	45.9
39.	Retinol	50.0	33.3	41.7
40.	Sorbitol + Sodium lactate + Sodium chloride + Calcium chloride + Potassium chloride + Magnesium chloride	25.0	33.3	29.2
41.	Sulpiride	25.0	33.3	29.2
42.	Teriparatide	25.0	0.0	12.5
43.	Tocopherol	50.0	66.7	58.4
44.	Tramadol	50.0	33.3	41.7
45.	Zoledronic acid	25.0	66.7	45.9
46.	Papaverine	75.0	33.3	54.2

The next stage of the research was the construction of a comparison network of International non-proprietary names of medicines for pharmacotherapy of chronic pancreatitis, considering the criteria "Recognition" and "Availability". To do this, a meta-analysis was conducted to analyze direct and indirect comparisons of International non-proprietary names of medicines for

the management of pharmacotherapy of chronic pancreatitis using a multivariate meta-analysis model.

From the list of 43 drugs, two drugs – namely Drotaverine and Ibuprofen were substantiated with the help of meta-analysis. Only two drugs are associated with a significantly higher number of parameters of the prescription than other International non-proprietary names of medicines. At the same time, studies have shown that lifestyle modification, complete abstinence from the use of psychoactive substances, alcohol, tobacco, and increased physical activity contribute to improving the quality of life of patients with chronic pancreatitis.

Among the ways to find a solution to the problem, the authors explore the introduction of digital medical technologies of quantum medicine. Quantum medicine relies on the foundational principles of biophysical phenomena within living organisms. A primary technology within this field is microwave resonance therapy, which employs low-intensity electromagnetic radiation in the millimeter wavelength range. This radiation targets specific active points and zones of the human body, correcting disturbances in the body's electromagnetic framework. Consequently, it mitigates metabolic alterations triggered by these electromagnetic disruptions. The continuous advancement and proliferation of innovative scientific methods in quantum medicine offer significant opportunities for enhancing healthcare quality, clinical diagnostics, and therapeutic practices [23]. Research conducted within the European Union indicates that weak electromagnetic fields facilitate "communication" with human tissues and organs [24]. A conceptual model describes the human body as being enveloped by an electromagnetic "framework," holding essential data regarding its state and developmental status [25].

Several medical techniques rooted in quantum medicine principles include electrotherapy, electron therapy, laser therapy, proton therapy, plasma therapy, quantum therapy, frequency therapy, frequency wave therapy, electric field therapy, magnetic field therapy, bioresonance therapy, Pulsed Electromagnetic Field (PEMF) therapy, Electro-Capacitive Cancer Therapy (ECCT), oncological electromagnetic therapy, Tumor Treating Fields (TTF), radiofrequency therapy, and ultrahigh frequency therapy. All these therapeutic modalities leverage quantum mechanical concepts and quantum coherence as foundational elements in the expanding field of quantum medicine. Each application of quantum theory and related technology directly involves biological entities, focusing explicitly on their biological responses [26].

4. CONCLUSION

A meta-analysis of the prescription of drugs in the pharmacotherapy of chronic pancreatitis was conducted according to the author's design of the SMART study. Better organized the evidence base of International non-proprietary names of medicines according to the criteria "Recognition" and "Availability". Better systematized the ranking of International non-proprietary names of medicines in the pharmacotherapy of chronic pancreatitis according to the evidentiary data of regulatory documents. Economic impact study. Decrease of financial budget on health system according to drug prices in the pharmaceutical management of hospitals. Social impact research. Increase longevity and quality of life of patients with chronic pancreatitis. Pharmaceutical impact research. The digital tool of availability for patients of prescription and non-prescription drugs is used in fifteen hospitals, fourteen medical stakeholders. Better training of the end-users in long-term pharmacotherapy using Drotaverine, Ibuprofen. Medical impact research. Pathways of training programs on prevention of diseases of chronic pancreatitis and usage of digital tools increasing the access to up-to-date information of doctors

and patients. A few appropriate communications for dissemination and exploitation are planned in further studies with associations of producers, distributors, doctors, pharmacists, lawyers. Among the ways to find a solution to the problem, the authors explore the introduction of digital medical technologies of quantum medicine,

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All authors contributed to data analysis, drafting, and revising of the paper and agreed to be responsible for all the aspects of this work.

CONFLICT OF INTEREST

The authors have approved the article for publication and declare that there is no conflict or potential conflict of interest.

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Scientific research article

Artemia salina bioassay as tool for determination of preliminary *Thunbergia alata* toxicity

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SUMMARY

Introduction: *Thunbergia alata* is considered an invasive species in Colombia and it is responsible for the decrease in native biodiversity and therefore represents a significant threat to flora. Hence, it is considered pertinent to evaluate its toxicological potential and, through the corresponding preliminary phytochemical analysis tests, determine which components are associated with such toxicity, thus conducting a search for possible pharmacological utility of the species under study. **Aim**: Starting at the floral parts of the *Thunbergia alata* species collected in the municipality of Tenjo Cundinamarca, obtain the ethanolic extract. To carry out a preliminary phytochemical analysis and a general lethality bioassay on *Artemia salina* with the extract free of hydroalcoholic solvent in the way to find its toxicity. **Results**: The prepared extract underwent a preliminary phytochemical analysis, where it was found that the floral parts of the *Thunbergia alata* species contain phenols, flavonoids, phenolic antioxidants, tannins, anthraquinones, flavones, and saponins. Finally, the lethality bioassay was conducted on *Artemia salina*, and the toxic potential of the extract of interest was statistically analyzed using Probit analysis, where the curve yielded a lethal concentration 50 (LC_{50}) of 2.036 μg/mL. **Conclusions:** The ethanolic extract of the *Thunbergia alata* species free of solvent has an extreme toxic potential.

Keywords: *Thunbergia alata*; toxicity; *Artemia salina*

RESUMEN

Bioensayo de *Artemia salina* como herramienta para la determinación preliminar de la toxicidad de *Thunbergia alata*

Introducción: *Thunbergia alata* se considera una especie invasora en Colombia, responsable de la disminución de la biodiversidad nativa y, por lo tanto, representa una amenaza significativa para la flora. Por lo tanto, se considera pertinente evaluar su potencial toxicológico y, mediante los análisis fitoquímicos preliminares correspondientes, determinar qué componentes están asociados con dicha toxicidad, buscando así la posible utilidad farmacológica de la especie en estudio. **Objetivo:** A partir de las partes florales de la especie *Thunbergia alata* recolectadas en el municipio de Tenjo, Cundinamarca, se obtuvo el extracto etanólico. Se realizó un análisis fitoquímico preliminar y un bioensayo de letalidad general en *Artemia salina* con el extracto libre de solvente hidroalcohólico para determinar su toxicidad. **Resultados:** El extracto preparado se sometió a un análisis fitoquímico preliminar, donde se encontró que las partes florales de la especie *Thunbergia alata* contienen fenoles, flavonoides, antioxidantes fenólicos, taninos, antraquinonas, flavonas y saponinas. Finalmente, se realizó un bioensayo de letalidad en *Artemia*

salina, y se analizó estadísticamente el potencial tóxico del extracto de interés mediante análisis Probit, donde la curva arrojó una concentración letal 50 (CL50) de 2,036 µg/mL. **Conclusiones:** El extracto etanólico de la especie *Thunbergia alata* libre de solvente presenta un potencial tóxico extremo.

Palabras clave: *Thunbergia alata*; toxicidad; *Artemia salina*

RESUMO

Bioensaio de *Artemia salina* como ferramenta para determinação da toxicidade preliminar de *Thunbergia alata*

Introdução: *Thunbergia alata* é considerada uma espécie invasora na Colômbia e responsável pela diminuição da biodiversidade nativa, representando, portanto, uma ameaça significativa à flora. Portanto, considera-se pertinente avaliar seu potencial toxicológico e, por meio dos correspondentes testes de análise fitoquímica preliminar, determinar quais componentes estão associados a tal toxicidade, buscando assim a possível utilidade farmacológica da espécie em estudo. **Objetivo:** A partir das partes florais da espécie *Thunbergia alata* coletadas no município de Tenjo Cundinamarca, obter o extrato etanólico. Realizar uma análise fitoquímica preliminar e um bioensaio de letalidade geral em *Artemia salina* com o extrato livre de solvente hidroalcoólico, a fim de determinar sua toxicidade. **Resultados:** O extrato preparado foi submetido a uma análise fitoquímica preliminar, onde se constatou que as partes florais da espécie *Thunbergia alata* contêm fenóis, flavonoides, antioxidantes fenólicos, taninos, antraquinonas, flavonas e saponinas. Por fim, o bioensaio de letalidade foi conduzido em *Artemia salina*, e o potencial tóxico do extrato de interesse foi analisado estatisticamente por meio da análise de Probit, onde a curva apresentou uma concentração letal 50 (CL50) de 2,036 µg/mL. **Conclusões:** O extrato etanólico da espécie *Thunbergia alata*, livre de solvente, apresenta um potencial tóxico extremo.

Palavras-chave: *Thunbergia alata*; toxicidade; *Artemia salina*

1. INTRODUCTION

Thunbergia alata is considered an aggressive invasive species [1]. Specifically in Colombia, this species is responsible for the decrease in native biodiversity and therefore represents a significant threat to fauna. Hence, it is considered pertinent to evaluate its toxicological potential and, through the corresponding preliminary phytochemical analysis tests, determine which components are associated with such toxicity, thus conducting a search for possible pharmacological utility of the species under study [2, 3].

Invasive species are considered the second leading cause of biodiversity loss worldwide [4]. It is estimated that 40% of animal extinctions in the last five centuries are attributable to them. The presence of these species can lead to the extinction of native species through predation, competition for resources, or transmission of deadly diseases. Invasive species also have the ability to hybridize with similar native species, altering the genetic heritage and endangering species survival. Their presence can have serious consequences for human activities, thereby affecting the economy of a region or country. They can damage forests, impact fishing and livestock, degrade water quality, and harm industry. Moreover, many agricultural pests, such as weeds and invertebrates, are invasive exotic species that can cause significant damage to production, and their chemical control can have negative effects on other species and human health [5]. The magnitude of environmental and economic impacts resulting from biological invasions makes this issue transcend national borders and become an international concern, implying the need for collaboration between countries to coordinate strategies, control species trafficking, and monitor communication routes [6]. *Thunbergia alata* is a plant species classified

as a highly invasive organism, widely used for ornamentation and demarcation of various areas due to its rapid growth. Its use on a national level in Colombia is significant, to the extent that it has caused a high-risk invasion in various regions. Its harmful impact has been evidenced through ecosystem degradation and alteration, along with the reduction of native biodiversity, affecting biogeochemical cycles and the food chain, and creating significant social and economic problems [2]. This plant is commonly found at forest edges, where it tends to climb over other plants and hinder their growth, sometimes completely smothering them. Such behavior negatively impacts the germination and establishment of native species; it could even be categorized as one of the top ten most challenging invasive species in Colombia.

Due to the high proliferation of this species (*Thunbergia alata*), it is of great importance to determine and quantify the toxic effect of the ethanolic extract from the floral parts of *Thunbergia alata* using the general lethality bioassay in *Artemia salina*. This study aims to assess if the species possesses pharmacological potential that could allow its pharmaceutical use, while also contributing to species control due to its harmful activities in ecosystems [7]. Additionally, it is relevant to note that there is currently no literature specifically addressing toxicity studies regarding the potential toxicity present in the floral parts of this species. Previous studies have only focused on the pharmacological activity found in stems and leaves of *Thunbergia alata*, such as the study conducted by the Pontificia Universidad Javeriana, which aimed to evaluate the chemical and antifungal potential of *Thunbergia alata* in leaves and stems [8].

2. MATERIALS AND METHODS

For the sample collection, 50 grams of plant material, specifically floral parts of the species *Thunbergia alata*, were collected in the region of Cundinamarca, municipality of Tenjo.

For the treatment of the collected plant material, the entire sample was subjected to air drying at room temperature for 10 days. Subsequently, all the material was triturated using an electric grinder until it was completely pulverized, aiming to facilitate the preparation of the desired extract. For the extraction process, 500 milliliters of a hydroalcoholic mixture composed of 65% ethanol and distilled water were used. The mixture was concentrated to a 1:1 ratio using maceration at room temperature as the extraction technique. The obtained extract was then subjected to rotary evaporation until a dry extract was obtained (Figure 1).


(a)


(b)

(c)

Figure 1. Photographic record of methodology: **(a)** Comparison between: fresh sample of floral parts collected from *Thunbergia alata*, dried sample of floral parts collected from *Thunbergia alata and* pulverized sample of floral parts collected from *Thunbergia alata*. **(b)** Extract obtained through filtration. **(c)** Solid residue of the extract obtained after filtration.

After obtaining the extract, preliminary phytochemical screening tests were conducted using a porcelain plate [9]. In this plate, 3 drops of extract were applied, followed by the addition of 4 drops of each corresponding reagent for the proposed tests.

2.1. Alkaloid test

The Mayer's reagent was used for the alkaloid test. The alkaloid test with Mayer's reagent is based on the formation of a yellow or white precipitate when alkaloids react with the reagent. The underlying phytochemical principle involves the formation of insoluble complexes between alkaloids and the reagents present in the solution [10].

2.2. Phenol test

For the phenol test, ferric chloride was used. The phytochemical principle behind this test lies in the ability of phenols to react with ferric ions (Fe^{3+}) present in the reagent, forming colored coordination complexes. [10].

2.3. Saponin test

The foam test was employed for the saponin test. The foam test for saponins is based on the ability of saponins to form stable foam when agitated in the presence of water. The phytochemical principle behind this test lies in the chemical structure of saponins. When a solution containing saponins is vigorously agitated in water, saponins form micelles due to their amphiphilic structure [10].

2.4. Tannin test

The gelatin-salt test and potassium dichromate test were used for the tannin test. The phytochemical principle behind the gelatin-salt test involves the ability of tannins to form precipitated complexes with proteins like gelatin in the presence of metal salts, indicating that the obtained result was a product of the union of cross-links of a biopolymer such as gelatin. Similarly, the tannin test with potassium dichromate reagent is based on the ability of tannins to reduce dichromate ions (VI) to chromium (III) in an acidic medium. The phytochemical principle in this test is the oxidation-reduction reaction between tannins and potassium dichromate [10].

2.5. Phenolic antioxidant test

Additionally, the Folin-Ciocalteu reagent was used for the phenolic antioxidant test. This reagent contains phosphomolybdic and phosphotungstic acids in an aqueous solution. When added to a sample containing phenolic antioxidants, the phenolic groups of the antioxidants can reduce the molybdovanadate (VI) ions present in the reagent to molybdenum (V) ions. This process leads to the formation of a colored complex. The intensity of the formed color is related to the amount of phenolic antioxidants present in the sample [10].

2.6. Flavone test

For the flavone test, sulfuric acid was used. The flavone test with sulfuric acid reagent is based on the ability of flavones to form heterocyclic compounds and the consequent production of characteristic coloration. [10].

2.7. Flavonoid test

Likewise, the Shinoda reagent was used for the flavonoid test. The flavonoid test with Shinoda reagent is based on the formation of intensely colored complexes between flavonoids and Shinoda reagent, which contains a solution of aluminum trichloride and hydrochloric acid. The phytochemical principle behind this test lies in the reaction of hydroxyl groups of flavonoids with aluminum trichloride in an acidic medium [10].

2.8. Anthraquinone test

Finally, the anthraquinone test used hydrochloric acid in conjunction with ammonium hydroxide. Anthraquinones are organic compounds containing a structure consisting of aromatic rings with specific functional groups such as hydroxyl and ketone groups. When anthraquinones are treated with hydrochloric acid and ammonium hydroxide, various reaction products can be produced, including chlorides, condensates, and other derivatives [10].

2.9. Solvent election

For the solvent selection, various aspects were considered that led to the conclusion that hydroalcoholic solutions allow for easier extraction of secondary metabolites and are the most efficient option for extracting active principles from plants. This is reflected in the favorable recovery percentages obtained when using such mixtures, contributing to the establishment of standardized methods for monitoring potential biological activities in plant extracts. Ethanol was highlighted due to its low toxicity compared to methanol, and its environmentally friendly properties. Additionally, it has been found that the best extraction yields are obtained with different concentrations of ethanol (35-90%) in aqueous solution. The concentration percentage of ethanol in the extraction medium significantly affects the recovery yield. Furthermore, the presence of water enhances mass transfer between solid and liquid phases, increasing the permeability of the plant matrix and thereby improving heating efficiency [11]. Hydroalcoholic mixtures are used in plant extraction because they combine the solvent properties of both water and alcohol, thereby enhancing extraction efficiency. Water is effective for polar compounds, similar to ethanol. By combining these solvents in a hydroalcoholic mixture, a wide variety of compounds with different polarities can be extracted, expanding the solvent's utility. Moreover, ethanol in hydroalcoholic mixtures increases the solubility of certain phytochemicals by forming hydrogen bonds with hydroxyl groups present in compounds such as

phenolic compounds. Additionally, the ethanol concentration used in the solvent has antimicrobial effects, which contribute to preserving the extract.

2.10. *Artemia salina* toxicity test

For the hatching of *Artemia* cysts, a container was prepared with 12.5 grams of sea salt in a 500 mL beaker, filled with distilled water to simulate seawater concentration. The water was oxygenated using a pump and maintained under continuous light for 48 hours. After hatching, the nauplii were used, as this is the stage where they mature to an optimal naupliar larval state (Figure 2).

Figure 2. Dark container suitable for incubation of *A. salina* nauplii.

After incubation, a light source was positioned at one side of the container to attract second-stage larvae and separate them from the unhatched eggs. Figure 3. Subsequently, the *Artemia* were transferred to test vials using a Pasteur pipette. The number of second-stage larvae collected and added to each plate was determined using a stereoscopic microscope. Figure 4.

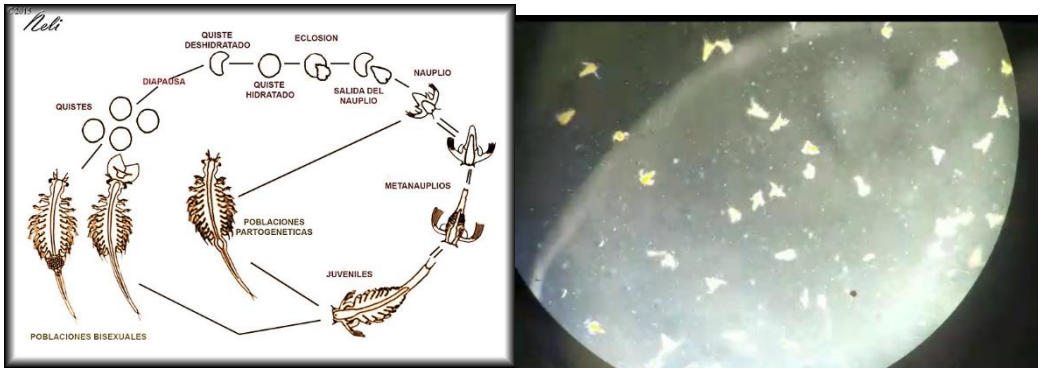


Figure 3. Naupliar stages [2].

The assay involved exposing only nauplius larvae to five increasing concentrations (5, 10, 50, 100, and 500 μg/mL) of the extract from *Thunbergia alata* species for 24 hours at room temperature under continuous light conditions. These five concentrations were chosen to cover a range from moderately to extremely toxic based on the toxicity table reported by CYTED [3]. Figure 5. Three replicates were used for each concentration. Ten nauplius larvae were added to individual vials, and the corresponding amount of concentrated extract was applied to each vial, which was then filled with seawater.

The stock solution of the extract was prepared by adding 50 grams of pulverized plant material to 500 mL of ethanol (at 65% concentration) and concentrating it to achieve a dry extract. From this extract, volumes of 0.5, 0.1, 0.05, 0.01, and 0.005 mL were taken to obtain concentrations of 500, 100, 50, 10, and 5 µg/mL, respectively.

After 24 hours of exposure, the number of dead organisms was counted, and the mortality percentage was calculated. Larvae were considered dead if they did not exhibit movement for 20 seconds of observation. Additionally, strychnine was used as a reference standard, and therefore, the results obtained for the ethanol extract of *Thunbergia alata* were compared with those reported in the literature [12] for strychnine. This comparison aimed to provide a reference framework, verify the sensitivity and reliability of the assay, ensure consistent results, and facilitate interpretation of the findings.

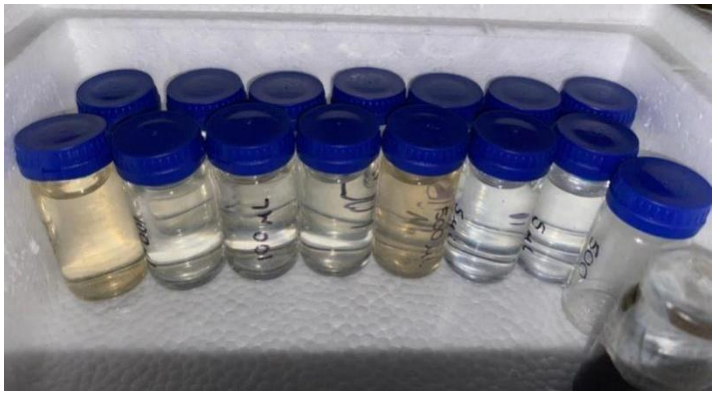


Figure 4. Glass vials used for the lethality bioassay in *Artemia salina.*

The selection of the appropriate method for lethality bioassay in *Artemia salina* was chosen because it has been used in multiple research studies with valid results for over 40 years. *Artemia spp*. larvae have been employed in toxicological and ecotoxicological investigations, and their biology and potential applications in various fields have been extensively studied. It is a cost-effective and practical method for assessing the bioactivity and preliminary toxicity of synthetic compounds and natural products [13]. Additionally, the use of *Artemia* is beneficial for predicting pharmacological and pesticide activities since it responds to a wide range of chemical and pharmacological compounds. This is crucial in research and development programs for new drugs and natural origin pesticides [12]. Furthermore, the potential application of the *Artemia* assay is linked to the particular advantages that bioassays provide in the standardization and quality control of various botanical products. These products may be considered "heterogeneous" due to the presence of combinations of bioactive compounds from the same botanical source or intentionally prepared mixtures.

On the other hand, there are advantages such as not needing to maintain a live colony permanently. Tests can be conducted anywhere and at any time as needed. There is always an adequate number of individuals of the same age and physiological condition available, overcoming ethical issues related to the use of larger animals [14].

Finally, for the execution of the Probit method, the logarithm base 10 was calculated for each of the concentrations used, and subsequently, the Probit values were calculated based on the mortality percentages obtained for each concentration using the table shown below [15]. Table 1.

Table 1. Transformation of mortality percentage into Probit values [15]

%	0	1	2	3	4	5	6	7	8	9
0	-	2.67	2.95	3.12	3.25	3.36	3.45	3.52	3.59	3.66
10	3.72	3.77	3.82	3.87	3.92	3.96	4.01	4.05	4.08	4.12
20	4.16	4.19	4.23	4.26	4.25	4.33	4.36	4.39	4.42	4.45
30	4.48	4.50	4.53	4.56	4.59	4.61	4.64	4.67	4.69	4.72
40	4.75	4.77	4.80	4.82	4.85	4.87	4.90	4.92	4.95	4.97
50	5.00	5.03	5.05	5.08	5.10	5.13	5.15	5.18	5.20	5.23
60	5.25	5.28	5.31	5.33	5.36	5.39	5.41	5.44	5.47	5.50
70	5.52	5.55	5.58	5.61	5.64	5.67	5.71	5.74	5.77	5.81
80	5.84	5.88	5.92	5.95	5.99	6.04	6.08	6.13	6.18	6.23
90	6.28	6.34	6.41	6.48	6.55	6.64	6.75	6.88	7.05	7.33
-	0.0	0.1	0.2	0.3	0.4	0.5	0.6	0.7	0.8	0.9
99	7.33	7.37	7.41	7.46	7.51	7.58	7.65	7.75	7.88	8.09

Finally, the calculated Probit values were plotted together with the logarithms calculated for the concentrations used. For the purpose of calculating the LC_{50}, it was necessary to determine the value corresponding to the intersection of the graph with the trend line on the Y-axis (Probit) at the assigned value of 5 as this corresponds to 50% lethality.

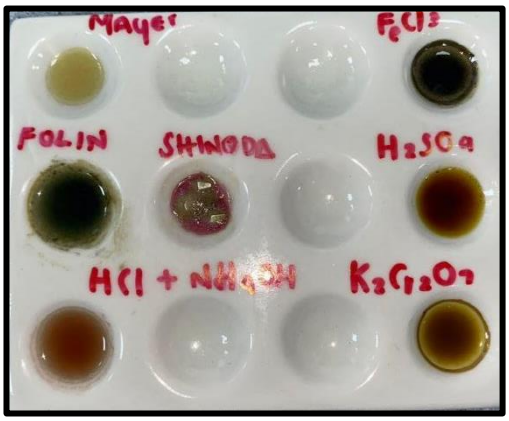
3. RESULTS

3.1. Preliminary phytochemical analysis of floral parts of *Thunbergia alata*

The results of the phytochemical analysis were interpreted as positive or negative based on variations in coloration, precipitation, turbidity, and other indicators in the phytochemical tests for different metabolites as are summarized in Table 2 and can be observed through photographic documentation in Figure 5.

Table 2. Results of the preliminary phytochemical analysis of the floral parts of *Thunbergia alata*

Metabolite	**Test**	**Result**
Alkaloids	Mayer	Negative (-)
Phenols	Ferric chloride	Positive (+)
Saponins	Foam	Positive (+)
Tannins	Gelatin-salt	Positive (+)
Phenolic antioxidants	Potassium dichromate	Positive (+)
Flavones	Folin-Ciocalteou	Positive (+)
Flavonoids	Sulfuric acid	Positive (+)
Anthraquinones	Shinoda	Positive (+)
	Hydrochloric acid + Ammonium hydroxide	



(a)

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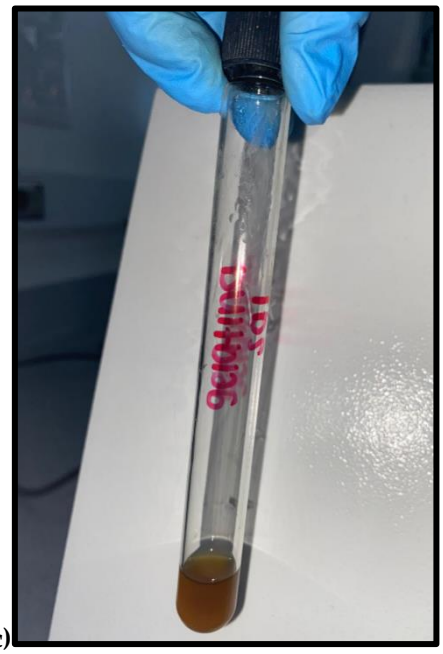

(c)

Figure 5. Results of the preliminary phytochemical analysis of the floral parts of *Thunbergia alata*: **(a)** Porcelain plate showing the color changes observed when applying the respective reagents from the tests described earlier to the ethanolic extract of *Thunbergia alata* floral parts. **(b)** Test tube where the saponin test was performed, showing a positive result with the appearance of foam. **(c)** Test tube where the gelatin-salt test was performed, showing a positive result with the formation of a light brown precipitate.

3.2. Lethality bioassay in *Artemia Salina*

The results of the Lethality Bioassay in *Artemia Salina* were interpreted based on the movement of the nauplii to confirm their survival after the addition of the extract. Each assay used 10 nauplii. It is important to note that the results are expressed as a percentage of mortality, calculated using the following formula:

$$Percentage\ of\ mortality = (Number\ of\ deaths\ /\ Total\ population)\ x\ 100$$

3.2.1. Concentration of 5 µg/mL

At a concentration of 5 µg/mL, 3 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 70%.

3.2.2. Concentration of 10 µg/mL

At a concentration of 10 µg/mL, 2 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 80%.

3.2.3. Concentration of 50 µg/mL

Furthermore, at a concentration of 50 µg/mL, the first replicate resulted in 1 live nauplius, corresponding to a mortality rate of 90%, while the remaining 2 replicates showed 0 live nauplii, resulting in an average mortality rate of 100%.

3.2.4. Concentration of 100 µg/mL

For the concentration of 100 µg/mL, 0 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 100%.

3.2.5. Concentration of 500 µg/mL

Lastly, at a concentration of 500 µg/mL, 0 live nauplii were observed across all 3 replicates, resulting in a mortality rate of 100%.

Similarly, the average percentage of mortality was calculated for the 3 replicates of each concentration, yielding 70% for 5 µg/mL, 80% for 10 µg/mL, 97% for 50 µg/mL, and 100% for both 100 µg/mL and 500 µg/mL. Additionally, standard deviation was calculated, resulting in values of 0 for all replicates of all concentrations except for the 50 µg/mL concentration, where one of the 3 replicates showed a different result compared to the other 2 replicates. The aforementioned results are documented in Table 3.

It is noteworthy that these results do not allow for the calculation of the LD_{50} because the graphical representation of the dose-response relationship, p (Percentage of mortality) vs. d (Concentration of the substance), often shows a parabolic curve that can be difficult to linearly model (as observed in Figure 6). To address this issue, the dose variable, d, is transformed to a logarithmic scale, $X = \log_{10}(d)$. This transformation produces a dose-response relationship with an S-shaped or sigmoidal form, resulting in a distribution of points p vs. X that resembles a normal distribution. Subsequently, using Probit tables, the percentage effect, p, is converted into Probit units. Tables 4 and 5. This involves looking up in a normal distribution table the z-value corresponding to a cumulative probability equal to p. This process generates a distribution of points in a bivariate system that can be analyzed using conventional regression analysis through the method of least squares [16].

The Probit method provides maximum likelihood estimates of regression parameters and natural rates, such as mortality rates, in biological assays. This is achieved by analyzing the percentage effects against the doses within the context of regression, thereby calculating the LD_{50} [16]. All of this is reflected in Figure 7, where the results are graphed and the linear equation is obtained: $y = 1.438X + 4.556$

Table 3. Results of the Lethality Bioassay in *Artemia Salina* for the ethanolic extract of *Thunbergia alata*

Concentration [µg /mL]	Number of nauplii per replicate	Number of live nauplii			Mortality percentage (%)			Average mortality percentage	Standard deviation
		R1	R2	R3	R1	R2	R3		
Blank	10	10	10	10	0	0	0	0	0
5	10	3	3	3	70	70	70	70	0
10	10	2	2	2	80	80	80	80	0
50	10	1	0	0	90	100	100	97	5.8
100	10	0	0	0	100	100	100	100	0
500	10	0	0	0	100	100	100	100	0

Table 4. Data corresponding to the graph Concentration vs. Average Percentage of Mortality.

Concentration	Average mortality percentage
0	0
5	70
10	80
50	97
100	100
500	100

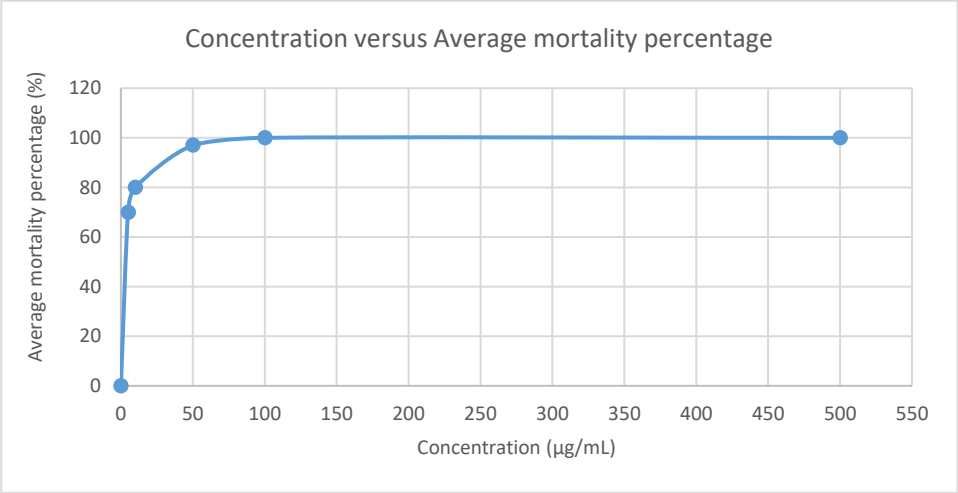

Figure 6. Concentration vs. Average Percentage of Mortality Graph.

Table 5. Data corresponding to the Probit vs. Logarithm10 of Concentration graph.

Logarithm of Concentration	Probit
0	0
0.69897	5.52
1.00000	5.84
1.69897	6.88
2.00000	8.09
2.69897	80.9

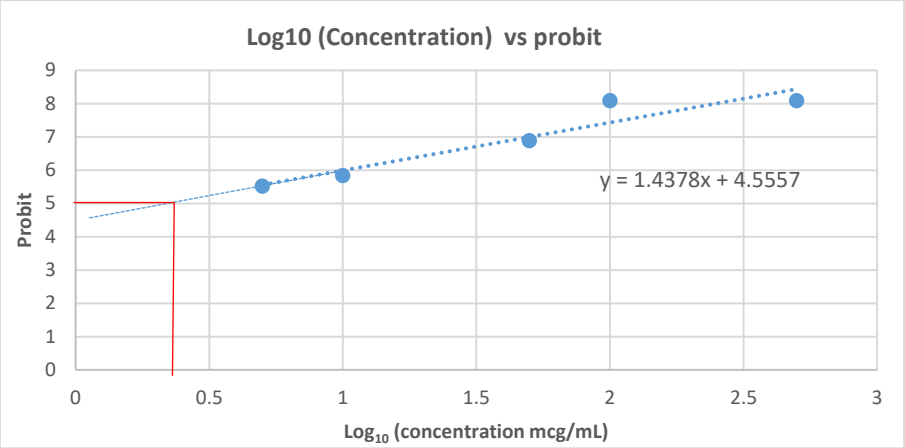

Figure 7. Probit vs. Logarithm10 of Concentration Graph

3.2.6. Calculation of the LD50

$$y = 1.438X + 4.556$$
$$5 = 1.438X + 4.556$$
$$X = (5 - 4.556)/1.438$$
$$X = 0.30876$$
$$LD50 = 10^{x}$$
$$LD50 = 10^{0.30876}$$
$$LD50 = 2.036\ \mu g/mL$$

4. DISCUSSION

Thunbergia alata belongs to the *Acanthaceae* family, within which various biological activities are present, such as antifungal activity [8], anti-inflammatory, antihypertensive, and antioxidant effects [17]. Additionally, several studies indicate the presence of smooth muscle relaxant and calming effects, as well as analgesic and anti-inflammatory properties [18].

It is noteworthy that *Thunbergia alata (Acanthaceae)* has been used in traditional medicine to alleviate various inflammatory conditions, such as fever, cough, and diarrhea, in East African nations like Uganda and Kenya [17]. In India, *Thunbergia alata* has been employed medicinally to treat patients suffering from back and joint pain, ocular inflammation, hemorrhoids, rectal cancer, and gall disease. Some animal studies have demonstrated beneficial biological activities, including improvements in cognitive and emotional deficits in mice using aqueous extracts [19].

Furthermore, the secondary metabolites found in the flowers of *Thunbergia alata* exhibit significant pharmacological interest due to their therapeutic activities. Specifically regarding phenols, Hernandez et al. (2022) suggest that antioxidant activity is closely related to phenolic compounds present in plant extracts [20]. Similarly, concerning saponins, these exhibit a wide range of pharmacological effects from expectorant action to diuretic, antiedematous, and anti-inflammatory effects. The expectorant action is attributed to the stimulation of tracheobronchial secretion through an autonomic reflex originating from gastric mucosa, while the diuretic effect enhances renal blood circulation and glomerular filtration [20].

Regarding tannins, plants containing these metabolites possess astringent, hemostatic, antiseptic, and toning properties. Tannins coagulate tissue and mucosal proteins, forming an isolating and protective layer that relieves skin irritation and pain. Tannin-containing plant products are used to stop minor local bleeding, treat mouth inflammations, bronchitis, burns, skin ulcers, wounds, and other conditions, and also manage diarrhea. However, caution is advised

for individuals with digestive issues due to potential intolerance, as prolonged consumption may inhibit the absorption of certain vitamins and minerals [21].

Additionally, phenolic antioxidants exhibit a wide array of therapeutic activities such as anti-diabetic, anticancer, anti-inflammatory, analgesic, vasodilatory, antidepressant, antihypertensive, antithrombotic, anticoagulant, antimicrobial, anti-aging, anti-allergic, and osteoporosis-fighting effects [22]. Among phenolic antioxidants, flavones are notable for their phytoestrogenic and antimicrobial activity [23]. Many flavonoids have demonstrated antifungal and antiviral activity in addition to their antibiotic, anticancer, antidiarrheal, anti-inflammatory, estrogenic, anti-hepatotoxic, and antispasmodic effects [24]. Finally, another group of metabolites present in *Thunbergia alata*, namely anthraquinones, exhibit interesting antimicrobial and antiviral activities reported through in vitro and/or in vivo studies, making them ideal candidates for exploring new treatments [25].

It is important to note that *Thunbergia alata* (*Acanthaceae*) has been used in traditional medicine to alleviate various inflammatory conditions such as fever, cough, and diarrhea in East African countries like Uganda and Kenya [17]. In India, *Thunbergia alata* has been used medicinally to treat patients with back and joint pain, ocular inflammation, hemorrhoids, rectal cancer, and gall disease. Some animal studies have shown beneficial biological activities, including improvements in cognitive and emotional deficits in mice using aqueous extracts [19].

Furthermore, the secondary metabolites found in the flowers of *Thunbergia alata* are of significant pharmacological interest due to their therapeutic activities. Specifically, phenols are associated with antioxidant activity in plant extracts [20]. Saponins exhibit a wide range of pharmacological effects from expectorant and diuretic properties to anti-inflammatory effects [26]. Tannins found in plants have astringent, hemostatic, antiseptic, and toning properties, while phenolic antioxidants have various therapeutic activities such as anti-diabetic, anticancer, anti-inflammatory, analgesic, vasodilatory, antidepressant, antihypertensive, antithrombotic, anticoagulant, antimicrobial, anti-aging, anti-allergic, and osteoporosis-fighting effects [22].

Justicia secunda Vahl, another member of the *Acanthaceae* family, has been studied extensively, including phytochemical screening and toxicity testing of its extracts. This family is known for its diverse range of herbaceous or erect plants, with a few shrubs or small trees, and includes approximately 230 genera and 4000 species distributed in tropical regions. *Justicia*, the largest genus within this family, comprises about 600 species found in tropical and subtropical regions worldwide. *Justicia secunda* Vahl, also known as "singamochila," "cascajera," or "sanguinaria," is a perennial shrub reaching 0.5 to 1 m in height, with red-violet flowers grouped in corollas of 5 petals. It is common in tropical and temperate regions and widely distributed in several countries [25].

Populations in Latin America have traditionally used *Justicia secunda* Vahl to treat various conditions such as anemia, pain, hypoglycemia, and as an energizer, although its efficacy has not been fully established. It is used in different regions for kidney stones, as an antipyretic, for glucose-related disorders, infections, and even in ethnoveterinary applications for snakebite injuries and dysentery in hunting dogs [25]. Studies have identified tannins, steroids, saponins, anthraquinones, and flavonoids, particularly derivatives of luteolin, in the leaves of *Justicia secunda* Vahl collected from different regions. Sanabria *et al.* [10] have highlighted the presence of these secondary metabolites in plant extracts and their correlation with lethality in *Artemia salina* larvae. This observation could explain the low toxicity observed in the ethanolic leaf extract of *J. secunda* Vahl in this study, as certain secondary metabolites like cumarins were absent [25].

Comparing these findings with *Thunbergia alata*, both species share similar types of secondary metabolites, therapeutic activities, and measured toxicological potential via *Artemia salina* lethality bioassay. Toxicity in both extracts is attributed to saponins and tannins, validating the reliability of the results and their interpretation [25].

To analyze the *Artemia salina* lethality bioassay, it is necessary to clarify the definition of toxicity. A toxin can be defined as any substance capable of causing harmful effects in a biological system [24]. Various studies have evaluated the toxicity of synthetic organic chemicals and plant extracts against *Artemia nauplii* to determine the median lethal concentration (LC_{50}) [27].

The findings on the median lethal concentration are presented in Figure 7, showing the cytotoxic percentage of the plant extract on *Artemia salina*. It was concluded that the toxicity value was 2.036 μg/mL. Figure 7 illustrates the survival and mortality of crustaceans relative to concentration; it is evident that as the concentration in μg/mL increases, so does mortality. This result, consistent with toxicity criteria established by CYTED [3], classifies the plant's toxicity as "extremely toxic," suggesting *Thunbergia alata* may have pharmacological potential. Table 6.

Table 6. Toxicity classification according to CYTED reported in the literature [3]

Toxicity classification according to CYTED	
I Extremely toxic	1 – 10 μg/mL
II Highly toxic	10 – 100 μg/mL
III Moderately toxic	100 – 500 μg/mL
IV Slightly toxic	500 – 1000 μg/mL
V Practically non-toxic	1000 – 1500 μg/mL
VI Relatively harmless	> 1500 μg/mL

It should be noted that when compared with the toxicity standard, which in this case corresponds to Strychnine, which reported a LC_{50} of 50 μg/mL in the reviewed literature [12]. Table 7. This allowed confirming that this organism is highly sensitive to the active principles present in these samples. This supports the utility of the assay in preliminary studies of fraction activity based on toxicity. It is possible to mention that there are several studies indicating that this test is comparable to tests in mammals. It is worth noting that in 1984, a team of scientists from the *Artemia* Reference Center in Belgium developed an acute toxicity test (short-term) with *Artemia salina*. This assay, known as the ARC-TEST, is based on determining the concentration that causes the death of 50% of *Artemia* larvae within 24 hours. The assay was tested in collaboration between research centers in Europe and America. The results were compared with those of other organisms used in toxicology tests, such as *Daphnia* spp. and Brachydanio spp. It was found that the ARC-TEST is as accurate and reproducible as tests with Daphnia spp. and in some cases similar to those with *Brachydanio spp*. Despite the relatively low sensitivity of the assay in the ARC-TEST study conducted in Belgium, the authors concluded that it is a valuable tool for the initial assessment of chemical toxicity [14].

Table 7. Toxicity results obtained for the *Artemia salina* Lethality Bioassay of Strychnine as reported in the literature [12].

Strychnine standard						
[c] μg/mL	Dead	Alive	Lethality	Category	LC_{50} μg/mL Experimental	LC_{50} μg/mL Literature
80	6	4	60%	Highly toxic	50	77.2
70	6	4	60%	Highly toxic		
60	6	4	60%	Highly toxic		
50	5	5	50%	Highly toxic		
Ethanol standard						
80	1	9	10%			

To provide context, it is possible to clarify that the Meyer's toxicity index is a quantitative measure of a substance's lipid solubility, often calculated by dividing the lethal dose 50 (LD_{50}) by the octanol-water partition coefficient (Log *P*) of the substance. The use of this index is related to predicting the potency and toxicity of various chemical compounds based on their ability to dissolve in lipids. In other words, the higher the Meyer's toxicity index, the greater the substance's ability to penetrate the cell membrane of an organism and consequently affect its cellular function, thereby increasing its toxicity. This index allows framing the obtained result within a toxicological classification, as follows [28].

Similarly, upon reviewing Meyer's toxicity index as a theoretical model and contrasting it with the obtained result, it can be stated that the extract is considered toxic since the LC_{50} result is less than 1000 μg/mL (Table 8).

Table 8. Classification of the ethanol extract of *Thunbergia alata* based on Meyer's toxicity index

Extract	**Lethal Concentration 50 (LC_{50})**	**Classification**
Thunbergia alata	2.036 μg/mL	Toxic

[1] Toxicity categories according to Meyer's toxicity index: toxic (LCL_{50} < 1000 μg/mL) and non-toxic (LC_{50} > 1000 μg/mL) [27].

Similar to the previously mentioned Meyer's toxicity index, Clarkson's toxicity index is a measure of acute toxicity that originated from the need to calculate the concentration of multiple heavy metals in blood, determining that higher concentrations of these substances in the human body pose greater toxicity risks. Currently, it is used to quantify and analyze the toxic capacity of various chemical substances when exposed to living organisms. Like Meyer's index, Clarkson's index allows results to be classified according to established guidelines [29]. Additionally, based on Clarkson's toxicity index (Table 9), the result obtained from the *Thunbergia alata* extract is considered highly toxic.

Table 9. Classification of the ethanolic extract of *Thunbergia alata* based on the Clarkson toxicity index

Extract	**Lethal Concentration 50 (LC_{50})**	**Classification**
Thunbergia alata	2.036 μg/mL	Highly toxic

[1] Toxicity categories according to the Clarkson toxicity index: highly toxic (CL_{50} from 0 to 100 μg/mL), moderately toxic (CL_{50} from 100 to 500 μg/mL), slightly toxic (CL_{50} from 500 to 1000 μg/mL), and non-toxic (CL_{50} > 1000 μg/mL) [29].

It is important to consult various sources reporting toxicity indices to conclusively verify the accuracy of the classification of the obtained result, which is why the result was compared with the Clarkson [29], Meyer [28], and CYTED [3] index.

Upon obtaining a result indicating a high degree of toxicity, it is possible to relate this toxicity to the presence of secondary metabolites in *Thunbergia alata*. Specifically, it could be said that saponins play a fundamental role in the toxic nature attributed to the studied extract, as saponins are known to possess toxic properties attributed to their ability to form complexes with sterols. This could interfere with the assimilation of these compounds by the digestive system or lead to the disruption of cell membranes once absorbed into the bloodstream [30].

It is pertinent to mention that there are various toxicity mechanisms shared by the secondary metabolites found in the extract, including interference with cellular signaling pathways, inhibition of metabolic enzymes, oxidative stress, mitochondrial dysfunction, inhibition of cell wall synthesis, activation of enzymes that degrade the cell wall, increased cell membrane permeability, promotion of uncontrolled cell proliferation, and disruption of redox balance. These mechanisms may be related to the toxicological activity of the studied extract, although this was a biological assay and direct confirmation is not feasible [31].

Additionally, it is noteworthy that when secondary metabolites are present together in an extract, they can exhibit synergistic effects. Specifically, tannins and flavonoids have shown synergistic effects in inhibiting fungi such as *R. solani* and *S. rolfsii*, meaning their antifungal capacity is enhanced when these two groups of metabolites are present in the same extract [32]. Similarly, Einhelling and Rasmussen, as well as Williams and Harland, have documented additive or synergistic effects among different phenolic compounds in allelopathic interactions, even at low concentrations. For instance, Quayyum *et al.* (1999) examined how aqueous phenolic extracts of *Myriophyllum verticillatum* Linnaeus, applied at a concentration of 200 mg per 100 g fresh weight, affected germination and root development of *Lactuca sativa*. The results showed significant inhibition, with a 75% reduction in *L. sativa* root length. This underscores the potential impact of phenolic compounds on plant interactions and how even small amounts can have significant effects on the growth and development of other plant species [33-35].

As a recommendation for future phytochemical studies, thorough investigation of the non-polar secondary metabolite components present in *Thunbergia alata* through extractive fractionation using various non-polar solvents is suggested. Additionally, conducting analysis via High-Performance Liquid Chromatography (HPLC) to determine the chromatographic profiles of the secondary metabolites is recommended. Experimental tests such as the MTT assay (cell viability and metabolic activity assay) are advised to elucidate the toxicity mechanisms exerted by the major secondary metabolites present in the extract, thereby expanding the scope and depth of this research. Furthermore, conducting a bioassay-guided study for the isolation of active principles from the species and thorough investigation of their pharmacological activities is recommended.

5. CONCLUSIONS

The toxicological potential detected in the ethanolic extract of the floral parts of *Thunbergia alata* correlates with the presence of secondary metabolites such as saponins and tannins, based on the toxicity mechanisms reported in the literature for these compounds. Additionally, preliminary identification of the secondary metabolites present in the ethanolic extract of *Thunbergia alata* floral parts revealed the presence of generic groups such as phenols, flavonoids,

phenolic antioxidants, tannins, anthraquinones, flavones, and saponins. This reflects the diverse pharmacological potential of the studied extract due to the various therapeutic activities associated with these metabolite groups.

In conclusion, the capacity of the general lethality bioassay in *Artemia salina* was confirmed for evaluating the toxicity of the plant species *Thunbergia alata*, correlating the established concentrations with the responses obtained from the used nauplii. The toxicological potential was categorized as extreme under these conditions, suggesting that this study serves as a crucial starting point for future research endeavors.

CONFLICTS OF INTEREST

The authors declare no conflict of interest. This paper is based on the undergraduate thesis carried out at the Universidad El Bosque (Bogotá-Colombia) as a requirement to qualify for the title of pharmaceutical chemist: (2024-04-30) "Evaluación toxicológica del extracto etanólico de la especie *Thunbergia alata* mediante el bioensayo general de letalidad en *Artemia salina*". Available in: https://hdl.handle.net/20.500.12495/12132 [36].

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Scientific research article

Simplificación de la posología de dolutegravir en coinfección VIH/TB tratada con rifampicina: análisis mediante modelado farmacocinético de base fisiológica

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RESUMEN

Introducción: En pacientes coinfectados con VIH/TB que reciben dolutegravir y rifampicina se recomienda aumentar la dosis diaria de dolutegravir administrando 50 mg cada 12 horas, atendiendo la inducción enzimática provocada por rifampicina y su efecto en la eliminación de dolutegravir. Esto aumenta la complejidad del esquema posológico y disminuye la probabilidad de adherencia. El objetivo de este trabajo fue evaluar simplificaciones al tratamiento utilizando un modelo farmacocinético de base fisiológica (PBPK). **Métodos:** Se desarrolló y validó un modelo PBPK de interacción fármaco-fármaco en PK-Sim®, describiendo las concentraciones plasmáticas de dolutegravir y rifampicina tras distintas dosis. El modelo permitió realizar simulaciones poblacionales de la exposición de dolutegravir en esquemas posológicos simplificados, evaluando la probabilidad de alcanzar el objetivo terapéutico. **Resultados:** Se proyecta que bajo tratamiento con rifampicina 600 mg diarios, los tratamientos con dolutegravir 50 mg o 100 mg en una única toma diaria alcanzarían el objetivo terapéutico en 72,6% y 89% de la población, respectivamente. Estas proyecciones coinciden con resultados de ensayos clínicos previos. **Conclusión:** La administración de dolutegravir 100 mg en una única toma sería una alternativa efectiva para simplificar el tratamiento en pacientes con VIH/TB que reciben rifampicina, favoreciendo la adherencia, mejorando la cobertura y contribuyendo al manejo eficiente de la coinfección VIH/TB.

Palabras clave: dolutegravir; rifampicina; terapia antirretroviral; modelado farmacocinético de base fisiológica.

SUMMARY

Simplification of dolutegravir dosing in HIV/TB patients receiving rifampicin: a physiologically-based pharmacokinetic modeling analysis

Introduction: In HIV/TB coinfected patients receiving dolutegravir (DTG) with rifampicin (RFP), increasing the daily DTG dose to 50 mg every 12 hours is recommended, addressing the enzymatic induction caused by RFP and its effect on DTG elimination. This additional administration results in a more complex dosing regimen with lower probability of patient adherence. The aim of this study was to evaluate simplifications to the recommended posology using a physiologically-based pharmacokinetic model (PBPK). **Methods:** A drug-drug interaction PBPK model was developed and validated in PK-Sim® software, describing plasma concentrations of DTG and RFP administered at different doses, both jointly and independently. The model was used to perform population simulations of DTG exposure resulting from simplified dosing regimens, evaluating the probability of target attainment. **Results:** Under treatment with RFP 600 mg daily, DTG regimens of single daily doses of 50 mg or 100 mg are projected to achieve therapeutic target in 72.6% and 89% of the population, respectively. These projections align with previously reported clinical trial results. **Conclusion:** Administration of DTG as a single daily dose of 100 mg would be an effective alternative to simplify treatment in HIV/TB patients receiving RFP, promoting adherence, improving treatment coverage, and contributing to efficient management of HIV/TB coinfection.

Keywords: dolutegravir; rifampicin; antiretroviral therapy; physiologically based pharmacokinetic modelling.

RESUMO

Simplificação da dosagem de dolutegravir em coinfecção HIV/TB tratada com rifampicina: análise utilizando modelagem farmacocinética de base fisiológica

Introdução: Em pacientes coinfectados por HIV/TB em uso de dolutegravir e rifampicina, recomenda-se aumentar a dose diária de dolutegravir, administrando 50 mg a cada 12 horas, levando em consideração a indução enzimática causada pela rifampicina e seu efeito na eliminação do dolutegravir. Isso aumenta a complexidade do regime posológico e diminui a probabilidade de adesão. O objetivo deste estudo foi avaliar simplificações no tratamento utilizando um modelo farmacocinético de base fisiológica (PBPK). **Métodos:** Um modelo de interação medicamentosa PBPK foi desenvolvido e validado no PK-Sim®, descrevendo as concentrações plasmáticas de dolutegravir e rifampicina após diferentes doses. O modelo permitiu simulações populacionais da exposição ao dolutegravir utilizando regimes posológicos simplificados, avaliando a probabilidade de atingir a meta terapêutica. **Resultados:** Projeta-se que, sob tratamento com 600 mg de rifampicina por dia, os tratamentos com dolutegravir 50 mg ou 100 mg em dose única diária atingiriam a meta terapêutica em 72,6% e 89% da população, respectivamente. Essas projeções são consistentes com os resultados de ensaios clínicos anteriores. **Conclusão:** A administração de dolutegravir 100 mg em dose única seria uma alternativa eficaz para simplificar o tratamento em pacientes com HIV/TB em uso de rifampicina, promovendo a adesão, melhorando a cobertura e contribuindo para o manejo eficiente da coinfecção HIV/TB.

Palavras-chave: dolutegravir; rifampicina; terapia antirretroviral; modelagem farmacocinética com base fisiológica.

1. INTRODUCCIÓN

Dolutegravir (DTG) es un inhibidor de integrasa de segunda generación recomendado por la Organización Mundial de la Salud (OMS) como primera y segunda línea en la terapia antirretroviral (TARV) [1, 2]. Este fármaco es metabolizado por la enzima glucuronosiltransferasa 1A1 (UGT1A1) y en menor medida por citocromo P450 3A4 (CYP3A4) [3-5], lo que da paso a posibles interacciones con otros fármacos, como es el caso de la rifampicina (RFP), antibiótico utilizado como primera línea en el tratamiento antituberculoso.

La tuberculosis (TB) es la enfermedad infecciosa mayormente asociada con VIH y es la principal causa de hospitalizaciones y muertes en personas que viven con este virus; en Uruguay, la prevalencia de coinfección VIH/TB fue de 12 % en 2023 [6], lo que aumenta la probabilidad de recibir de manera concomitante los fármacos mencionados. La interacción entre RFP y DTG ha sido ampliamente documentada, y se caracteriza por una reducción significativa de la exposición a DTG debido al potente efecto inductivo de la RFP sobre las enzimas UGT1A1 y CYP3A4. Esta interacción resulta en una disminución de las concentraciones plasmáticas máximas (Cmax) y mínimas (Cmin) de DTG en aproximadamente 43% y 72%, respectivamente, mientras que el área bajo la curva (AUC) se reduce en cerca del 54% [7]. Es por esta razón que en pacientes coinfectados por VIH/TB con tratamiento antituberculoso basado en RFP se recomienda el ajuste de dosis de DTG a 50 mg dos veces al día (BID), mientras que en pacientes no tratados con RFP la dosis recomendada es de 50 mg DTG una vez al día (OD) [1, 5, 8].

Por otra parte, en Uruguay la cobertura global de la TARV fue del 80% mientras que en pacientes coinfectados con TB apenas alcanzó el 28% en 2023 [9], lo que evidencia dificultades en el manejo de la infección por VIH en esta población pudiéndose asociar a la complejidad del tratamiento; particularmente, la administración de DTG cada 12 horas podría relacionarse con una menor cobertura y adherencia al tratamiento.

En este contexto, se han explorado planes terapéuticos simplificados que contemplan la coadministración de 600 mg de RFP con 50 mg o 100 mg de DTG OD [2, 10, 11]. Griesel y colaboradores reportan que la supresión viral a las 24 semanas para el plan posológico estándar de DTG 50 mg cada 12 horas más RFP es similar a la alcanzada con el régimen terapéutico simplificado de una única dosis diaria de DTG 50 mg más RFP [2]. De la misma manera, en el estudio de Modongo y colaboradores tampoco se encontraron diferencias con significancia estadística en la proporción de pacientes con carga viral suprimida entre los grupos que recibieron los tratamientos mencionados [10]. Asimismo, Wang y colaboradores realizaron un estudio farmacocinético para comparar la administración de DTG 100 mg OD más RFP contra el régimen estándar de DTG 50 mg BID más RFP. Los resultados obtenidos muestran que las concentraciones mínimas con ambos planes de tratamiento se encuentran en niveles de efectividad [11]. Estos reportes indican que la administración de DTG BID podría ser innecesaria y resaltan la importancia de recopilar mayor evidencia sobre la necesidad o no de doblar la dosis de DTG OD en los pacientes con VIH/TB.

El modelado farmacocinético de base fisiológica (PBPK) es un enfoque que permite simular el comportamiento farmacocinético de un principio activo mediante la integración de información cuantitativa específica del organismo, del activo, y del medicamento o formulación administrada. El sistema en estudio se refleja en forma mecanística, permitiendo realizar extrapolaciones con adecuada capacidad predictiva [12]. Una de las principales aplicaciones de los modelos PBPK a nivel regulatorio ha sido el estudio de interacciones fármaco-fármaco

(DDI), describiendo la farmacocinética del fármaco perpetrador (el activo que provoca un cambio) y del fármaco víctima (el activo que sufre el cambio) en forma simultánea. Al considerar los perfiles de concentración plasmática y los parámetros de inducción/inhibición es posible predecir el efecto del perpetrador sobre los procesos de absorción, distribución, metabolismo o excreción y, consecuentemente, sobre la exposición del fármaco víctima, lo que brinda información sobre una posible pérdida de seguridad o efectividad [13]. Los modelos PBPK de DDI consideran la cinética de los procesos mediados por las enzimas o transportadores involucrados y su concentración en los diferentes tejidos del organismo. De esta manera, resultados obtenidos a partir de ensayos *in vitro* e *in vivo* pueden ser integrados al modelo y utilizados para su desarrollo y calibración. Posteriormente, la capacidad predictiva del modelo PBPK desarrollado es evaluada utilizando datos clínicos reportados [14].

La Administración de Alimentos y Medicamentos de los Estados Unidos (FDA) y la Agencia Europea de Medicamentos (EMA) permiten el uso de modelos PBPK como herramienta para evaluar escenarios de DDI, ya sea para explicar de manera cualitativa o cuantitativa los resultados obtenidos en un estudio clínico o para guiar el diseño y desarrollo de estos durante las diferentes etapas. Estas guías orientan sobre la manera en la que diferentes enfoques de modelado pueden ser utilizados para interpretar y trasladar las observaciones *in vitro* en predicciones *in vivo* de posibles interacciones medicamentosas [15-17]. De esta forma, los modelos PBPK son plataformas que informan la toma de decisión a nivel productivo y regulatorio durante el desarrollo de medicamentos.

Desde este enfoque, el objetivo de este trabajo fue evaluar planes de dosificación alternativos de DTG en pacientes coinfectados con VIH/TB a través de un modelo PBPK que refleje adecuadamente la interacción entre RFP y DTG.

2. MÉTODOS

2.1. Software

El modelo PBPK fue desarrollado utilizando el software PK-Sim® versión 11.3 (Open Systems Pharmacology). Se utilizó PlotDigitizer (https://plotdigitizer.com/) para extraer datos de figuras publicadas y convertirlos en formato digital. Se utilizó el entorno R (versión 3.4.1, R Foundation for Statistical Computing; https://www.r-project.org/) y RStudio (Rstudio Team (2024). RStudio: Integrated Development Environment for R. Posit Software, PBC, Boston, MA; http://www.posit.co/) para el análisis estadístico y la creación de gráficos.

2.2. Modelo farmacocinético de base fisiológica

El desarrollo del modelo PBPK se realizó en tres etapas como se esquematiza en la Figura 1.

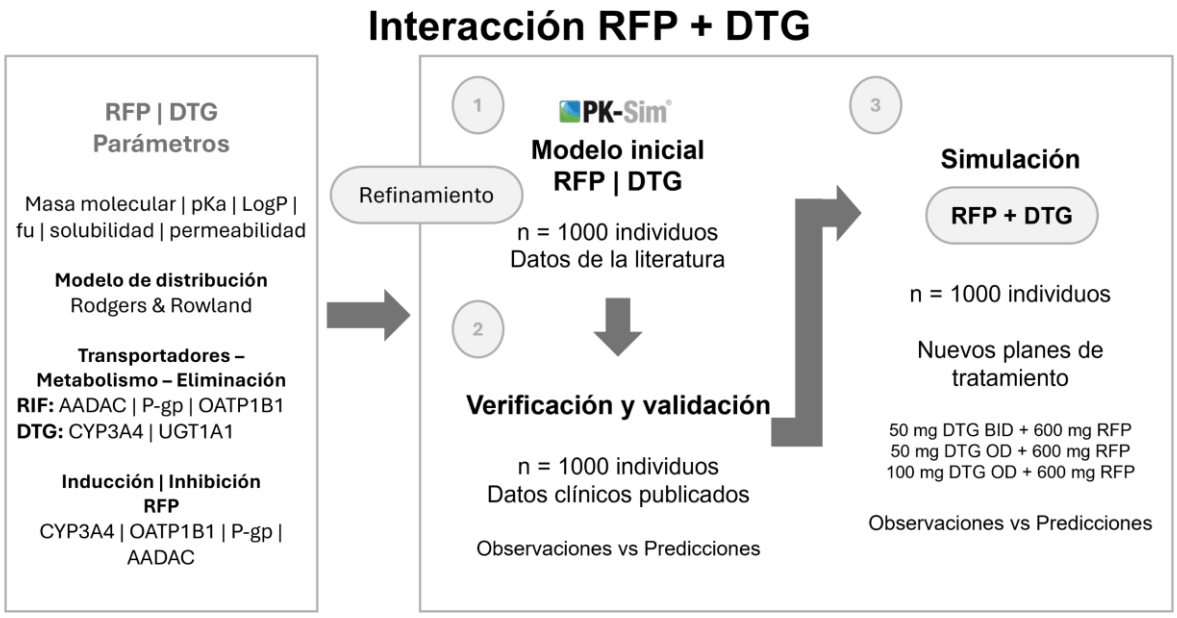

Figura 1. Flujo de trabajo para el desarrollo, validación y aplicación del modelo PBPK de interacción entre RFP y DTG.

2.2.1. Desarrollo del modelo

En la primera etapa, se desarrollaron los modelos independientes de RFP y DTG a partir de información disponible en la literatura sobre las propiedades fisicoquímicas, biofarmacéuticas y farmacocinéticas de ambos fármacos (Tabla 1). Las simulaciones se realizaron para una población conformada por 1000 individuos en el rango de edades entre 18 – 65 años y conformada por hombres y mujeres (50:50).

Para el desarrollo del modelo de RFP se tomó como referencia el modelo PBPK publicado por Berton *et al.* (2023) [18] y el modelo disponible en *Open Systems Pharmacology* (https://github.com/Open-Systems-Pharmacology/Rifampicin-Model), el cual está basado en el trabajo de Hanke *et al.* (2018) [19]. Se incluyó el transporte mediado por el polipéptido transportador de aniones orgánicos (OATP1B1) y por glicoproteína P (P-gp), descrito en ambos casos mediante un proceso saturable con cinética de Michaelis-Menten. El metabolismo de RFP mediado por la enzima arilacetamida desacetilasa (AADAC) también fue incluido como un proceso de capacidad limitada de acuerdo a la siguiente ecuación:

$$Vel\ RFP = \frac{I_{AADAC} \cdot kcat \cdot K_{water,RFP} \cdot [RFP]}{(Km + K_{water/cont} \cdot [RFP])} \quad \text{(Ecuación 1)}$$

donde *Vel RFP* es la velocidad de metabolización de RFP, I_{AADAC} es la expresión enzimática en el tejido correspondiente, $kcat$ es una constante de primer orden correspondiente al número de moléculas de sustrato biotransformadas por unidad de tiempo cuando se trabaja a la máxima actividad enzimática, $K_{water,RFP}$ es el coeficiente de partición para RFP estimado por el software para cada compartimiento, Km es la constante de Michaelis-Menten y $[RFP]$ es la concentración de RFP.

También se incorporó el efecto inductor de RFP (*Eind RFP*) sobre la expresión de OATP1B1, AADAC, P-gp y CYP3A4, como se indica en la Ecuación 2:

$$Eind\ RFP = \frac{k_{deg}\ \cdot\ M_0\ \cdot\ E_{max}\ \cdot K_{water,RFP} \cdot [RFP]_{intracel}}{(EC50\ + K_{water/cont} \cdot [RFP]_{intracel})} \qquad \text{(Ecuación 2)}$$

donde k_{deg} es un coeficiente de degradación de primer orden para la enzima o transportador, M_0 cantidad inicial de enzima o transportador, E_{max} es el efecto máximo, $EC50$ es la concentración que produce la mitad del E_{max} y $[RFP]$ es la concentración de RFP.

El modelo de DTG se basó en los modelos de Berton *et al.* (2023) [18] y Liu *et al.* (2020) [20], se incluyó un proceso de aclaramiento renal y el metabolismo de DTG por UGT1A1 y CYP3A4, como se muestra en la siguiente ecuación:

$$Vel\ DTG = [Enz] \cdot\ CL_{specPerEnzyme}\ \cdot [DTG]\ \cdot K_{water,DTG} \qquad \text{(Ecuación 3)}$$

donde *Vel DTG* es la velocidad de metabolización de DTG $[Enz]$ es la concentración de cada enzima, $CL_{specPerEnzyme}$ es el aclaramiento específico normalizado por la concentración enzimática, $K_{water,DTG}$ es el coeficiente de partición para DTG estimado por el software para cada compartimiento y $[DTG]$ es la concentración de DTG. La variabilidad interindividual en el metabolismo de DTG se consideró al incluir una desviación estándar relativa del 50% en el valor de $CL_{specPerEnzyme}$ para UGT1A1 y CYP3A4, de manera de reflejar la variabilidad interindividual reportada para DTG en Cmin y AUC [8, 11].

Para la calibración del modelo de DTG, se realizó un análisis de sensibilidad de forma de detectar los parámetros del modelo con mayor influencia sobre la exposición del fármaco [21]. De esta manera, se ajustaron la fracción libre y el log$P$ con el objetivo de describir adecuadamente el aclaramiento aparente, la semivida de eliminación y las métricas AUC, Cmax y Cmin de DTG, según los valores reportados en los estudios clínicos utilizados como referencia (Tabla 2).

En el modelo desarrollado, se consideró el efecto de la RFP sobre el metabolismo de DTG al incluir la inducción de RFP sobre la CYP3A4 como se describe en la ecuación 2. Los parámetros que definen la interacción son entonces la concentración de RFP para alcanzar la mitad de la inducción máxima ($EC50$) y el efecto máximo de inducción (E_{max}) de la RFP sobre la CYP3A4. Los valores adjudicados a cada parámetro se indican en la Tabla 1. La magnitud de E_{max} fue optimizada para describir los resultados observados tras la administración del plan de dosificación estándar, correspondiente a 50 mg DTG BID más 600 mg de RFP [8].

2.2.2. Validación del modelo

Como segunda etapa, se realizó la verificación y validación de los modelos para RFP y DTG administrados como monoterapia y en combinación (RFP+DTG). Para ello, se compararon las predicciones con concentraciones plasmáticas reportadas en estudios previamente publicados, los cuales se describen en el Tabla 2. La validación del modelo de RFP se realizó al contrastar las predicciones del modelo con concentraciones plasmáticas reportadas en cinco estudios clínicos en los que se administró una dosis de 600 mg, tres de ellos como dosis única y dos en un régimen de dosis múltiple.

Por otra parte, el modelo de DTG administrado como monoterapia se validó contra cuatro estudios clínicos en los que se administró DTG en dosis de 50 mg y 100 mg OD y 50 mg BID. Finalmente, el modelo para la administración concomitante de DTG y RFP se verificó contra

dos estudios clínicos en los que se administró el tratamiento recomendado por OMS correspondiente a DTG 50 mg BID más RFP 600 mg, además de DTG 50 mg OD + RFP y DTG 100 mg OD + RFP.

El desempeño de los modelos PBPK fue evaluado utilizando diagnósticos gráficos de bondad de ajuste mediante la comparación visual de los perfiles de concentración plasmática-tiempo simulados y los perfiles observados en estudios in *vivo*, también se consideraron los gráficos de los valores predichos vs observados para las métricas farmacocinéticas AUC y Cmax. La capacidad predictiva se evaluó por medio del factor de error absoluto promedio (*absolute average fold-error*, AAFE) (Ecuación 4) y el cociente predicho/observado para AUC y Cmax.

$$AAFE = 10^{\left|\frac{1}{N}\sum log\left(\frac{predicho}{observado}\right)\right|} \qquad \text{(Ecuación 4)}$$

Se consideró como valor aceptable un valor de AAFE menor a 1,5 y un cociente predicho/observado dentro de un rango de error de 1,5 veces respecto a los datos observados (0,67 ≤ Pred/Obs ≤ 1,5) [22].

2.2.3. Simulación de los planes de tratamiento alternativos

En la tercera etapa se realizó la simulación de los planes de tratamiento alternativos en una población de 1000 individuos en el rango de edades entre 18 – 65 años y conformada por 50% hombres. Se reportan las métricas farmacocinéticas AUC, Cmax y Cmin para cada uno de los tratamientos alternativos. Adicionalmente, se reportan la magnitud de la interacción calculada para cada métrica farmacocinética como el cociente entre la administración de DTG con RFP y sin RFP para cada tratamiento y el porcentaje de la población que alcanza valores superiores al límite establecido como objetivo terapéutico, correspondiente a la concentración de fármaco ajustada por proteínas requerida para inhibir el 90% de la replicación viral *in vitro* (PA-IC90).

Tabla 1. Parámetros ingresados al modelo PBPK para RFP y DTG.

	Rifampicina		**Dolutegravir**	
Parámetro	**Valor**	**Referencia**	**Valor**	**Referencia**
Masa molecular	822,94 g/mol	Berton *et al.* (2023) [18]; Hanke *et al.* (2018) [19]; Baneyx *et al.* (2014) [23]	419 g/mol	DrugBank [24]; PubChem [25]; Liu *et al.* (2020) [20]; Rajoli *et al.* (2015) [26]
Log*P*	2,50	Hanke *et al.* (2018) [19]	2,00 (inicial 2,20)	Ajustado por optimización de parámetros
pKa (ácida)	1,70	Berton *et al.* (2023) [18]; Hanke *et al.* (2018) [19]; Baneyx *et al.* (2014) [23]	8,30	Berton *et al.* (2023) [18], Rajoli *et al.* (2015) [26]
pKa (base)	7,90	Berton *et al.* (2023) [18]; Hanke *et al.* (2018) [19]; Baneyx *et al.* (2014) [23]	-	
Fracción libre	0,17	Hanke *et al.* (2018) [19], Baneyx *et al.* (2014) [23]	0,04 (inicial < 0,01)	Ajustado por optimización de parámetros

Proteína de unión principal	Albúmina	Berton *et al.* (2023) [18]; Hanke *et al.* (2018) [19]	Albúmina	Liu *et al.* (2020); Berton *et al.* (2023) [18]
Permeabilidad intestinal específica	1,24E-05 cm/min	Ajustado	0,05 cm/min	Liu *et al.* (2020) [20]
Solubilidad (pH 7.50)	2800 mg/L	Hanke *et al.* (2018) [19]	0,0922 mg/mL	DrugBank [24]
Coeficientes de partición	Rodgers & Rowland		Rodgers & Rowland	
Eliminación y metabolismo				
Fracción TFG	1	Hanke *et al.* (2018) [19]	-	
AADAC K_M AADAC K_{cat}	195,1 µmol/L 9,87 1/min	Nakajima *et al.* (2011) [27]; Hanke *et al.* (2018) [19]	-	
P-gp K_M P-gp K_{cat}	55,0 µmol/L 0,61 1/min	Collett *et al.* (2004) [28]; Hanke *et al.* (2018) [19]	-	
OATP1B1 K_M OATP1B1 K_{cat}	1,50 µmol/L 5,21 1/min	Tirona *et al.* (2003) [29]; Hanke *et al.* (2018) [19]	-	
CYP3A4	-		0,0035 µL/min/pmol	Berton *et al.* (2023) [18]
UGT1A1	-		0,0192 µL/min/pmol	Berton *et al.* (2023) [18]
Aclaramiento renal	-		0,0022 L/h/Kg 0,84 1/min	Berton *et al.* (2023) [18]
Inhibición				
CYP 3A4 Ki	18,5 µmol/L	Kajosaari *et al.* (2005) [30]; Hanke *et al.* (2018) [19]	-	
Inducción				
P-gp EC50 P-gp Emax	0,34 µmol/L 2,50	Greiner *et al.* (1999) [31]; Hanke *et al.* (2018) [19]	-	
CYP 3A4 EC50 CYP 3A4 Emax	0,34 µmol/L 15,0	Ajustado; Templeton *et al.* (2011) [32]; Hanke *et al.* (2018) [19]	-	
OATP1B1 EC50 OATP1B1 Emax	0,34 µmol/L 0,38	Dixit *et al.* (2007) [33]; Hanke *et al.* (2018) [19]	-	
AADAC EC50 AADAC Emax	0,34 µmol/L 0,99	Hanke *et al.* (2018) [19]	-	

TFG: tasa de filtración glomerular.

Tabla 2. Descripción de los estudios utilizados para la validación del modelo PBPK.

Estudio	**Dosis (mg)**	**Vía de administración**	**N (Homb|Muj)**	**Peso (Kg)**	**Edad (años)**
RFP					
Sanofi-Aventis (2013) [34]	600 DU	Inf IV 30 min	12 (12 | 0)	-	-
Peloquin *et al.* (1997) [35]	600 DU	Oral	24 (24 | 0)	76,9 ± 11,0	19 - 45

Peloquin *et al.* (1999) [36]	600 DU	Oral	14 (8 ǀ 6)	79,3 ± 13,2	39,1 ± 7,4
Baneyx *et al.* (2014) [23]	600 DM	Oral	12 (12 ǀ 0)	-	-
Baptista *et al.* (2024) [37]	600 DM	Oral	99 (63 ǀ 36)	64 ± 12 Kg	45 ± 14
DTG					
Dooley *et al.* (2013) [8]	50 OD DM - 50 BID DM	Oral	11 (9 ǀ 2)	63 - 99	31 - 59
Song *et al.* (2013) [38]	50 DU	Oral	8 (5 ǀ 3)	97,15 ± 22,69	57,0 ± 8,72
Weller *et al.* (2014) [39]	50 DU	Oral	8 (5 ǀ 3)	86,59 ± 17,05	56,1 ± 8,32
Oricchio *et al.* (2014) [40]	50 DM	Oral	21 (15 ǀ 6)	69 ± 4	29 - 63
RFP + DTG					
Dooley *et al.* (2013) [8]	50 BID + 600 OD DM	Oral	11 (9 ǀ 2)	63 - 99	31 - 59
Wang *et al.* (2019) [11]	100 OD + 600 OD DM - 50 OD + 600 OD DM	Oral	14 (9 ǀ 5)	-	22 - 55
Dooley *et al.* (2020) [41]	50 OD + 600 OD DM	Oral	69 (39 ǀ 30)	-	18 - 62

DU: dosis única; DM: dosis múltiple; OD: una vez al día; BID: dos veces al día / cada 12 horas.

3. RESULTADOS

3.1. Desarrollo y validación del modelo PBPK

Las simulaciones de concentraciones plasmáticas para RFP y DTG administrados como monoterapia se observan en la Figura 2A y 2B, respectivamente. El 95 % de las predicciones de AUC, Cmax y Cmin para ambos fármacos se encuentran dentro del margen de error de 1,5 veces respecto a los datos clínicos observados (Tabla 3) lo que permite la validación de los modelos de DTG y RFP. Únicamente la Cmax y el AUC correspondientes a la administración por vía intravenosa de RFP superan el límite de 1,5, pero cumplen con el criterio de error de 2 veces respecto a los datos observados.

Adicionalmente, las proyecciones de exposición para RFP en plasma sanguíneo reflejaron adecuadamente las observaciones determinadas en una muestra de 99 pacientes hospitalizados en Uruguay bajo tratamiento anti-TB (Figura 2A). Estos datos derivan de un estudio propio en el que se incluyeron personas mayores de 18 años, con un diagnóstico confirmado de TB o una alta sospecha de la enfermedad con tratamiento antituberculoso iniciado [37]. El protocolo de investigación fue registrado en el Ministerio de Salud Pública de Uruguay (810030) y avalado por los comités de ética institucionales del Hospital de Clínicas "Dr. Manuel Quintela", Hospital Español "Dr. J.J. Crottogini", y Hospital Pasteur.

De la misma manera, la simulación de las concentraciones plasmáticas de DTG representa de manera adecuada las observaciones reportadas por Oricchio y colaboradores (2024) [40] en un estudio clínico observacional realizado en una muestra de 21 pacientes diagnosticados con VIH en Uruguay (Figura 2B).

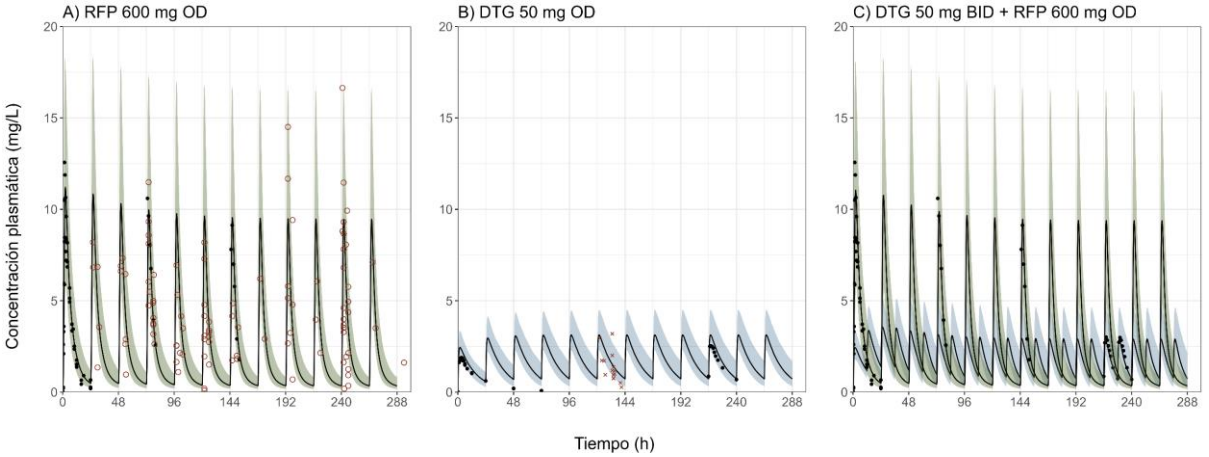

Figura 2. Concentraciones plasmáticas simuladas de RFP y DTG en los planes de tratamiento A) RFP 600 mg OD, B) DTG 50 mg OD, C) RFP 600 mg OD y DTG 50 mg BID utilizando el modelo descrito en la Tabla 1. La línea sólida representa la mediana de la concentración plasmática, el área sombreada representa los percentiles 5 y 95, los puntos negros corresponden a datos observados de la literatura, los círculos rojos corresponden a datos observados en el estudio reportado por Baptista *et al.* (2024) [37] y las cruces rojas corresponden a los datos observados de Oricchio *et al.* (2024) [40].

En la Figura 3A y 3B se observan los gráficos de bondad de ajuste de los valores predichos vs observados y el cociente entre predicciones y observaciones de AUC y Cmax para los tratamientos administrados como monoterapia (Figura 3C), los resultados obtenidos son indicadores de un buen desempeño y una buena capacidad predictiva del modelo.

Los resultados obtenidos tras la administración concomitante de DTG y RFP se verificaron mediante la comparación de los perfiles plasmáticos simulados y observados para el tratamiento estándar correspondiente a DTG 50 mg BID más RFP 600 mg OD. Según lo observado en la Figura 2C el modelo tiene la capacidad de describir las concentraciones plasmáticas observadas de RFP y DTG.

Las métricas farmacocinéticas AUC, Cmax y Cmin obtenidas tras la simulación del tratamiento estándar, así como la simulación de DTG 50 mg OD + RFP y DTG 100 mg OD + RFP, fueron comparadas con los valores reportados en la literatura. Los resultados muestran que las predicciones se encuentran dentro del margen de error aceptable de 1,5 veces respecto a los datos clínicos observados (Tabla 3). La Figura 3D presenta el cociente entre las predicciones y las observaciones para los planes de tratamiento combinados.

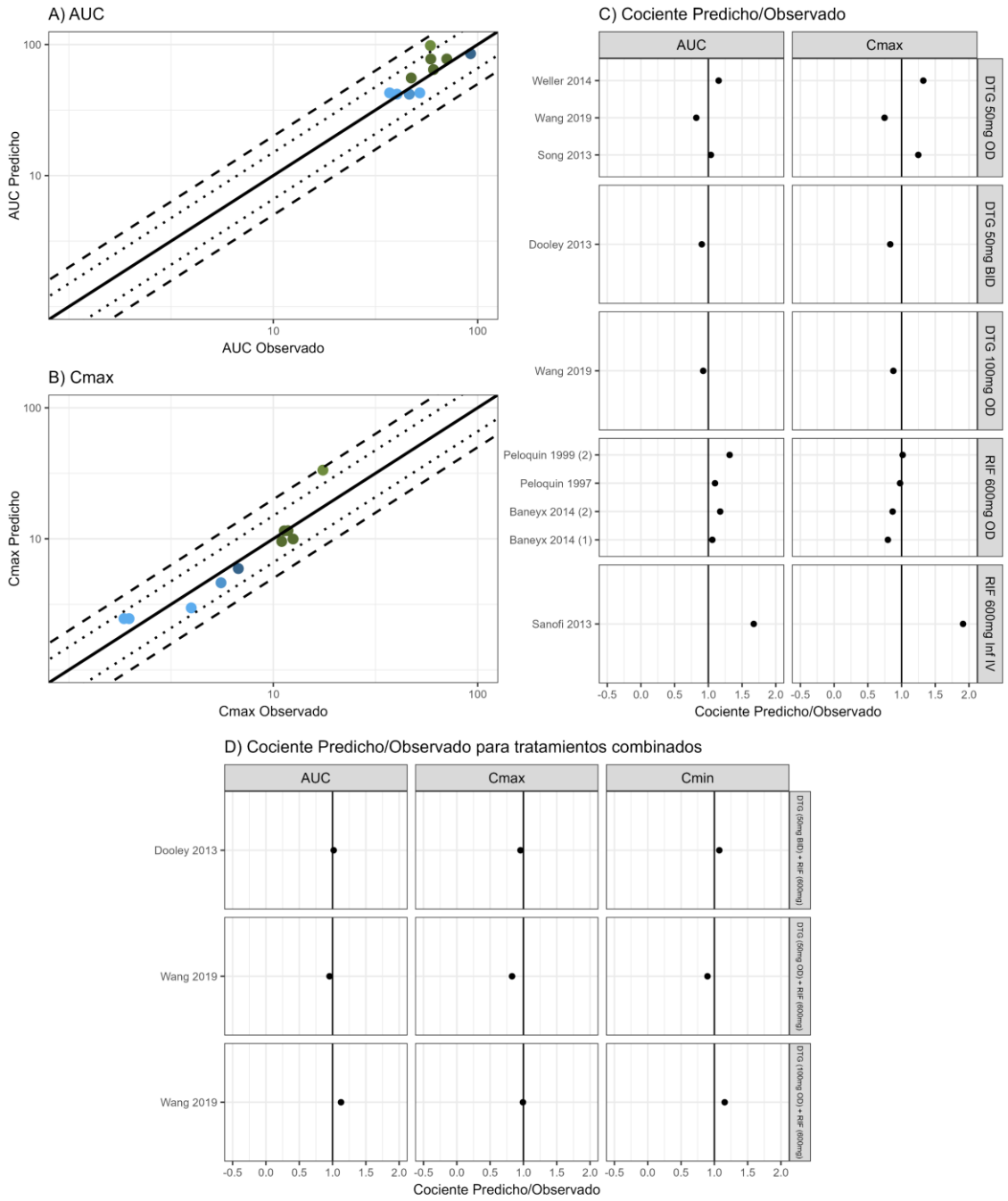

Figura 3. Desempeño del modelo PBPK de DTG y RFP. Gráfico de bondad de ajuste de los valores predichos vs observados para A) AUC y B) Cmax. La línea sólida indica la línea de identidad, la línea punteada indica un desvío de 1,5 veces y las líneas discontinuas un desvío de dos veces. C) Cociente predicho/observado para la administración de DTG y RFP como monoterapia. D) Cociente predicho/observado para los diferentes esquemas de tratamiento combinados. La línea sólida representa un cociente predicho/observado igual a 1 y el cociente predicho/observado para cada estudio clínico se representa con un punto.

Tabla 3. Evaluación del ajuste del modelo para DTG y RFP.

Tratamiento	Observaciones			Predicciones			AAFE			Referencia
	AUC (mgh/L)	Cmax (mg/L)	Cmin (mg/L)	AUC (mgh/L)	Cmax (mg/L)	Cmin (mg/L)	AUC	Cmax	Cmin	
RFP 600 mg Inf IV	58,70	17,50	-	98,20	33,46	-	1,67	1,91	-	Sanofi-Aventis (2013) [34]
RFP 600 mg OD	70,62	11,80	-	77,57	11,54	-	1,10	1,02	-	Peloquin (1997) [35]
	57,15	10,54	-	77,57	11,54	-	1,36	1,09	-	Peloquin *et al.* (1999) [36]
	58,98	11,32	-	77,57	11,54	-	1,32	1,02	-	Peloquin *et al.* (1999) [36]
RFP 600 mg OD – DM	60,80	12,50	-	64,37	9,97	-	1,06	1,25	-	Baneyx *et al.* (2014) [23]
	47,30	11,00	-	55,58	9,55	-	1,18	1,15	-	Baneyx *et al.* (2014) [23]
DTG 50 mg OD	40,30	1,97	-	41,81	2,46	-	1,04	1,25	-	Song *et al.* (2013) [38]
	37,10	1,86	-	42,75	2,46	-	1,15	1,32	-	Weller *et al.* (2014) [39]
DTG 50 mg OD - DM	32,10	2,65	0,55	41,84	3,16	0,73	1,30	1,19	1,33	Dooley *et al.* (2013) [41]
	52,10	3,969	1,06	42,77	2,97	0,80	1,22	1,34	1,33	Wang (2019) [11]
	-	-	0,85	-	-	0,80	-	-	1,07	Dooley *et al.* (2020) [41]
	-	-	0,88	-	-	0,80	-	-	1,10	Dooley *et al.* (2020) [41]
DTG 50 mg BID - DM	46,30	5,55	2,41	41,78	4,62	2,34	1,11	1,20	1,03	Dooley *et al.* (2013) [8]
DTG 100 mg OD – DM	92,31	6,75	2,02	85,24	5,93	1,60	1,08	1,14	1,26	Wang *et al.* (2019) [11]
DTG (50 mg BID) + RFP (600 mg)	21,30	3,13	0,67	21,67	2,99	0,72	1,02	1,05	1,07	Dooley *et al.* (2013) [8]
	-	-	0,87	-	-	0,72	-	-	1,21	Dooley *et al.* (2020) [41]
	-	-	0,96	-	-	0,72	-	-	1,33	Dooley *et al.* (2020) [41]
DTG (50 mg OD) + RFP (600 mg)	22,75	2,57	0,16	21,72	2,13	0,14	1,05	1,21	1,12	Wang *et al.* (2019) [11]
DTG (100 mg OD) + RFP (600 mg)	38,73	4,31	0,25	43,66	4,28	0,29	1,13	1,01	1,16	Wang *et al.* (2019) [11]

OD: una vez al día; DM: dosis múltiple; AUC: área bajo la curva (OD: AUC_0^{inf}; DM: AUC_0^{τ}); Cmax: concentración máxima; Cmin: concentración al final del intervalo de dosificación; Las observaciones y predicciones de Wang *et al.* (2019) [11] corresponden a una administración con alimentos.

3.2. Simulación de planes de tratamiento alternativos

En la Figura 4 se observan las concentraciones plasmáticas de DTG obtenidas tras la simulación de los tratamientos combinados en diferentes planes posológicos. El perfil de concentraciones de DTG tras el esquema de dosificación estándar con y sin RFP (Figura 4A y 4B) muestra

que las concentraciones plasmáticas alcanzadas se encuentran mayoritariamente sobre el IC90, lo mismo se observa para el plan alternativo que contempla la administración de DTG 100 mg OD más RFP (Figura 4D).

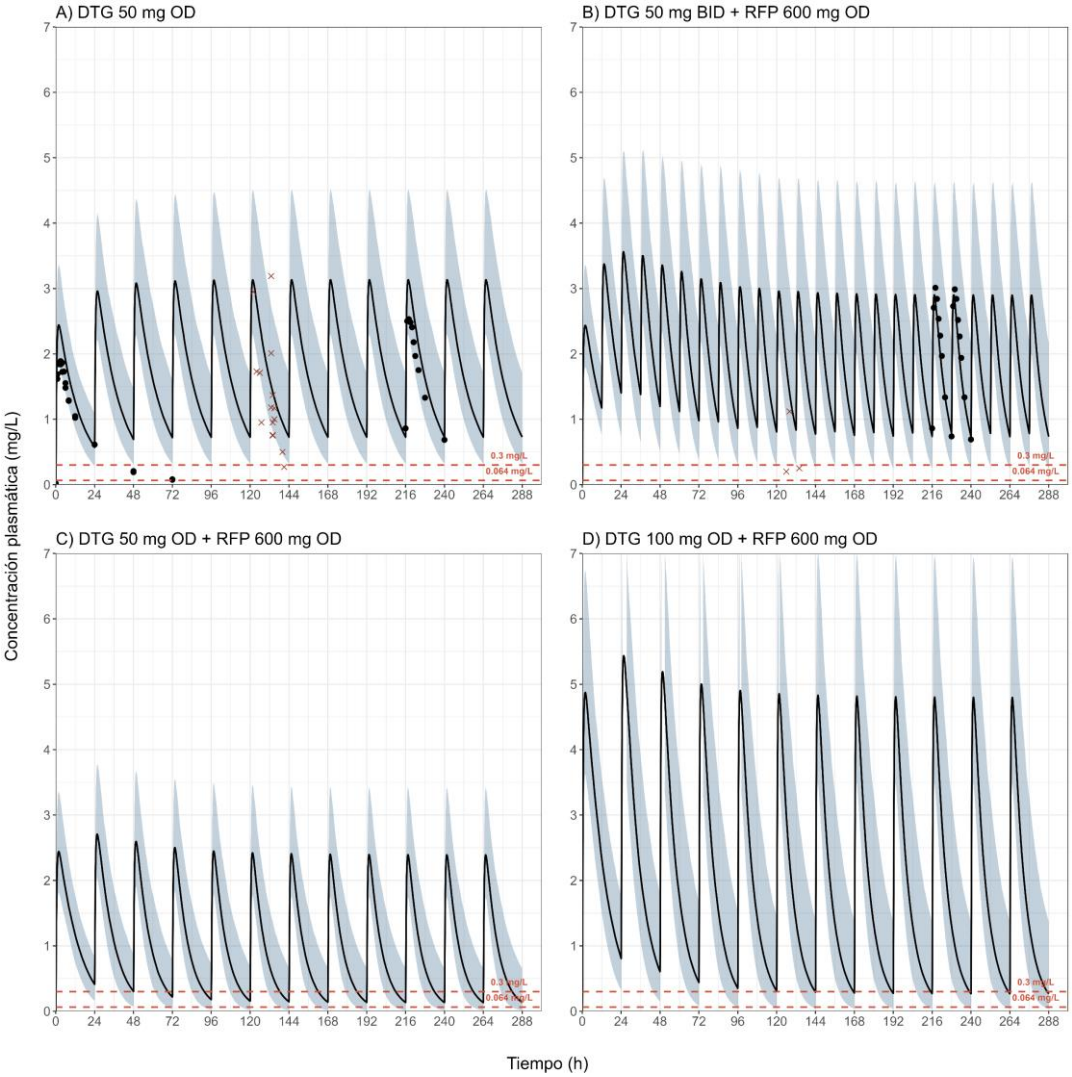

Figura 4. Concentraciones plasmáticas simuladas de DTG en los planes de tratamiento A) 50 mg OD, B) 50 mg BID con RFP, C) 50 mg OD con RFP y D) 100 mg OD con RFP en condiciones de ayuno, utilizando el modelo descrito en la Tabla 1. La línea sólida representa la mediana de la concentración plasmática, el área sombreada representa los percentiles 5 y 95, los puntos negros corresponden a datos observados de la literatura, las cruces rojas corresponden a los datos observados de Oricchio *et al.* (2024) [40] y las líneas rojas punteadas corresponden a los límites IC90 y EC90.

En la Tabla 4 se presenta la magnitud de la interacción fármaco-fármaco como un indicador del cambio en AUC, Cmax y Cmin tras la administración de los diferentes planes posológicos con y sin RFP. El modelo desarrollado predice que la RFP reduce el AUC de DTG en aproximadamente un 50%, mientras que las concentraciones, máxima y mínima, disminuyen en más de un 64% y 16%, respectivamente. En la Figura 5 se observa la bondad de ajuste entre las magnitudes de interacción predichas y observadas evidenciando que las predicciones se encuentran en el margen error de 1,5 veces respecto a los valores de magnitud de interacción observados.

En la Figura 6 y Tabla 5 se presenta la evaluación de estos esquemas de tratamiento, se observó que los tratamientos estándar permiten alcanzar concentraciones por encima de 0,064 mg/L para más del 99% de la población, mientras que en la evaluación del régimen alternativo de DTG 100 mg OD más RFP, el porcentaje de individuos que supera este límite es de 89%. Por otra parte, tras la simulación de la administración de DTG 50 mg OD combinado con RFP, las concentraciones plasmáticas mínimas alcanzan valores inferiores al 0,064 mg/L en un 27,4% de los individuos.

Tabla 4. Magnitud de la interacción fármaco-fármaco (DDI) en los planes de dosificación evaluados.

Tratamiento	**DDI observado**			**DDI predicho**		
	AUC	Cmax	Cmin	AUC	Cmax	Cmin
DTG 50 mg BID (con RIF / sin RFP)*	0,46 (0,38-0,55)	0,56 (0,49-0,65)	0,28 (0,23-0,34)	0,51 (0,49-0,52)	0,64 (0,62-0,65)	0,30 (0,29-0,32)
DTG 50 mg OD (con RIF / sin RFP)†	0,44 (0,37-0,52)	0,65 (0,55-0,75)	0,15 (0,13-0,17)	0,51 (0,49-0,52)	0,76 (0,74-0,77)	0,16 (0,15-0,17)
DTG 100 mg OD (con RIF / sin RFP)†	0,42 (0,35-0,50)	0,64 (0,55-0,74)	0,12 (0,10-0,15)	0,52 (0,50-0,53)	0,76 (0,75-0,78)	0,17 (0,16-0,18)

Los resultados se reportan como el cociente de medias geométricas entre la administración de DTG con RFP y sin RFP para cada esquema de tratamiento y su intervalo de confianza 90%. * Tomado de Dooley *et al.* (2013) [8]. † Tomado de Wang *et al.* (2019) [11].

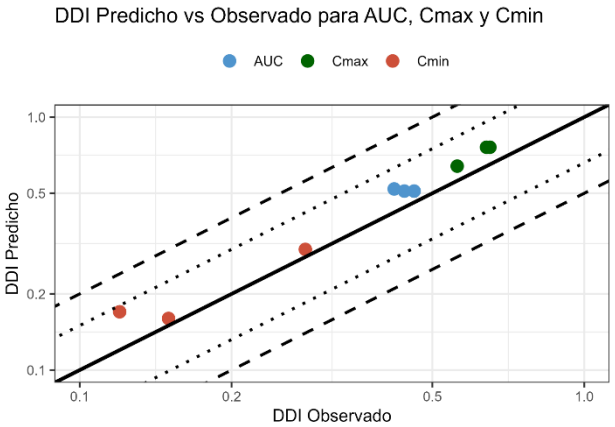

Figura 5. Gráfico de bondad de ajuste de la magnitud de interacción predicha vs observada para AUC, Cmax y Cmin. La línea sólida indica la línea de identidad, la línea punteada indica un desvío de 1,5 veces y las líneas discontinuas un desvío de dos veces.

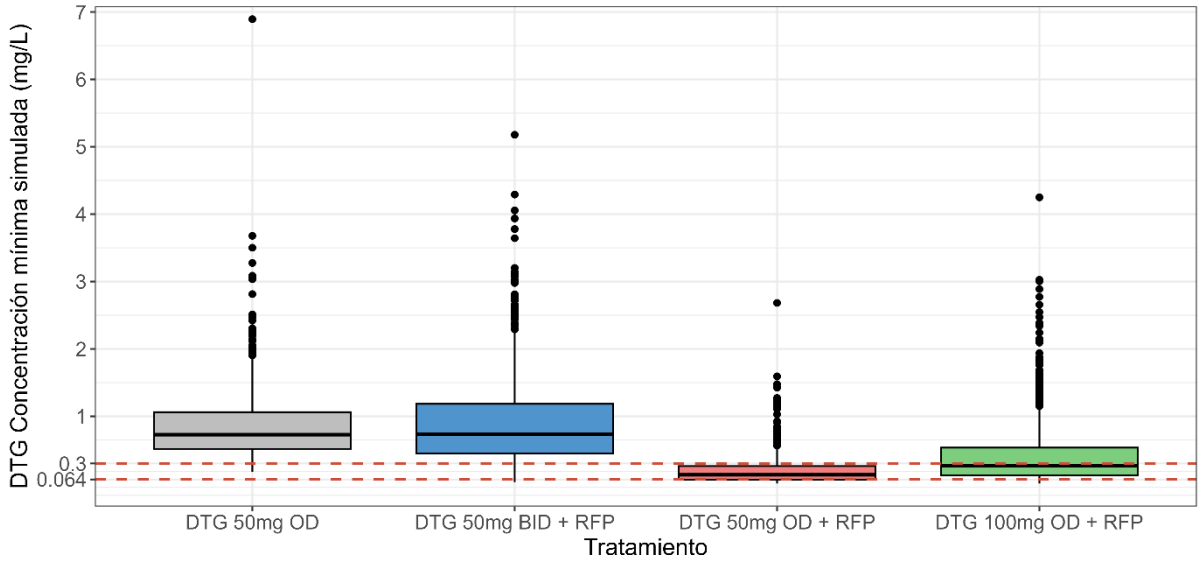

Figura 6. Boxplot de las concentraciones mínimas simuladas de DTG para los planes de tratamiento (de izquierda a derecha) a 50 mg OD, 50 mg BID con RFP, 50 mg OD con RFP y 100 mg OD con RFP, utilizando el modelo descrito en la Tabla 1. Las cajas representan los percentiles 25, 50 y 75, mientras que los "bigotes" muestran los percentiles 5 y 95, las líneas rojas punteadas corresponden a los límites IC90 y EC90.

Tabla 5. Resultados de la simulación para los planes de tratamiento alternativo.

Tratamiento	**Cmin (mg/L)***	**Porcentaje de la población que supera IC90 – EC90**	
		> 0,064 mg/L IC90	**> 0,3 mg/L EC90**
DTG (50 mg OD)	0,74 (59)	>99%	96,1%
DTG (50 mg BID) + RFP (600 mg)	0,72 (70)	>99%	90,6%
DTG (50 mg OD) + RFP (600 mg)	0,12 (115)	72,6%	20,5%
DTG (100 mg OD) + RFP (600 mg)	0,25 (110)	89,1%	45,6%

* Los valores de concentración plasmática mínima se reportan como la media geométrica con su coeficiente de variación (%CV), calculado como [(desvío estándar/media)×100].

4. DISCUSIÓN

En el presente trabajo se desarrolló un modelo PBPK que permite la simulación de concentraciones plasmáticas de DTG al ser administrado con o sin RFP bajo diferentes esquemas de tratamiento. La interacción entre DTG y RFP se incorporó considerando el efecto inductor de la RFP sobre CYP3A4. Las simulaciones mostraron una disminución en las concentraciones plasmáticas de DTG debido al aumento en su aclaramiento, consistente con observaciones clínicas (Figura 4).

Para evaluar la efectividad de los distintos regímenes posológicos para DTG es necesario contar con un objetivo farmacocinético, existiendo dos reportes en la literatura. Cottrell *et al.* (2013) reportan que la concentración de fármaco ajustada por fracción libre requerida para inhibir el 90% de la replicación viral *in vitro* (PA-IC90) del VIH es 0,064 mg/L, por lo que un tratamiento efectivo debería asegurar concentraciones plasmáticas mínimas superiores a este valor [42]. Por otro lado, Van Luzen *et al.* (2012) establecieron un límite de concentración más

estricto con un valor mínimo de DTG igual a 0,3 mg/L según un estudio fase II en el cual se desarrolló un modelo de Emax a partir de la administración de diferentes dosis (10, 25 y 50 mg) de DTG [43]. Este segundo criterio requiere una interpretación cautelosa por múltiples razones. Primero, el objetivo farmacocinético fue derivado de la media geométrica de concentraciones mínimas observadas tras la administración de 10 mg, sin realizar una evaluación de exposición-respuesta completa. Los autores no encontraron una relación dosis dependiente en la supresión viral, y no estudiaron el efecto con dosis inferiores a 10 mg [5, 43]. Asimismo, en la práctica clínica, el DTG se coadministra con otros antirretrovirales en pacientes con VIH, sugiriendo que concentraciones menores a 0,3 mg/L podrían ser efectivas.

Las simulaciones del tratamiento recomendado por OMS para pacientes con VIH/TB (DTG 50 mg BID con 600 mg RFP) resultan en que más de un 99% de la población supere el límite de PA-IC90, correspondiente a una concentración de DTG igual a 0,064 mg/L. Un porcentaje favorable (89%) también se observó tras la coadministración de DTG 100 mg OD más RFP, lo que indica que esta podría ser una opción de tratamiento que permite alcanzar los niveles de exposición adecuados sin necesidad de recurrir a la administración de DTG dos veces al día. Esto supone una mayor adherencia al tratamiento y mayor probabilidad de lograr una terapia exitosa. Además, se hipotetiza que, al ser una estrategia para la simplificación de la terapia, podría contribuir a ampliar la cobertura de la TARV. De manera general, los resultados obtenidos con este régimen de tratamiento están acorde a lo reportado por Wang y colaboradores en el estudio RADIO, que corresponde a un estudio fase I realizado en individuos sanos donde se evaluó la farmacocinética de los esquemas de tratamiento simulados en un grupo de 14 voluntarios [11]. Según las proyecciones para este esquema de tratamiento (DTG 100 mg OD más RFP), un 11 % de la población alcanzaría concentraciones inferiores al objetivo terapéutico. Si bien esta proyección no condice con lo observado por Modongo *et al.* [10], y Griesel *et al.* [2], en donde no se detectaron problemas de efectividad con DTG 50 mg OD, debe considerarse que en el escenario clínico el DTG es coadministrado con otros agentes antirretrovirales. El seguimiento del tratamiento mediante la carga viral continúa siendo clave para evaluar la efectividad de la terapia antirretroviral.

Los resultados obtenidos en este trabajo están en concordancia con los reportes de otros modelos. Kawuma *et al.* [5] desarrollaron un modelo farmacocinético poblacional para caracterizar la farmacocinética de DTG y RFP. Este predice que las concentraciones de DTG obtenidas al administrar 100 mg DTG más RFP, superan el valor límite de 0,064 mg/L en un 91% de la población [5]. Asimismo, en el modelo desarrollado por Barcelo y colaboradores, se predice que, bajo este plan de tratamiento alternativo, el 60% de la población alcanza concentraciones mínimas superiores al valor límite [3].

Los estudios clínicos publicados por Griesel *et al.* [2], así como Modongo *et al.* [10] presentan evidencia que respalda la administración de DTG una vez al día para pacientes bajo tratamiento antibuberculoso basado en RFP. En ambos casos se observó que no hay diferencias en la supresión viral tras la administración del tratamiento estándar (50 mg DTG BID) y la administración de una única dosis de DTG de 50 mg [2, 10]. Las simulaciones realizadas con el modelo PBPK desarrollado en este trabajo predicen que bajo este régimen de tratamiento (DTG 50 mg OD más RFP) un 72,6% de la población alcanza concentraciones mínimas que superan el PA-IC90.

Las observaciones anteriores sugieren que la suplementación de la TARV con una dosis extra de DTG podría no ser necesaria, pero se requiere de mayor evidencia clínica para llegar a resultados concluyentes. Los resultados de las predicciones realizadas, así como los reportes de estudios clínicos previos y las predicciones de otros modelos farmacocinéticos publicados,

sugieren que una única administración diaria de 50 mg o 100 mg de DTG es efectiva para pacientes con VIH/TB en tratamiento con RFP. En ambos casos se plantea como ventaja la simplificación de la TARV al sustituir la administración cada 12 horas por una única toma diaria, lo que se relaciona con mayor adherencia a la terapia y, por ende, mayor probabilidad de lograr los objetivos terapéuticos, que en el caso del DTG, es la supresión de la replicación viral. Dado lo anterior, este trabajo podría ser considerado para apoyar el diseño de un estudio clínico con el objetivo de evaluar los esquemas de tratamiento mencionados.

5. CONCLUSIONES

Los resultados de este estudio aportan evidencia para el uso de dos regímenes alternativos de dosificación de DTG en combinación con RFP en pacientes coinfectados con VIH/TB. La administración de DTG a dosis de 50 mg y 100 mg en una sola dosis diaria durante el tratamiento antituberculoso basado en RFP podría ser una opción de tratamiento seguro y efectivo. Estos esquemas de dosificación optimizan el uso de medicamentos mediante la simplificación de la TARV, mejoran la adherencia y la cobertura terapéutica, lo cual contribuye a un manejo más eficiente de la coinfección VIH/TB.

CONFLICTO DE INTERESES

Los autores declaran no tener conflicto de intereses.

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Scientific research article

Anatomical traits and phytochemical screening of *Curcuma newmanii* Škorničk.

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SUMMARY

Introduction: *Curcuma newmanii* Škorničk. has been recently described as a new species to science which the type specimens collected from Vietnam. **Aim:** The present study firstly described the pharmacological properties of this species, including micro-morphological features and phytochemical screening. **Methodology:** the iodine green-carmine double staining method was used to provide the details of anatomical features whereas the qualitative and quantitative assays were used to perform the phytochemical screening of this plant. **Results:** the micro-morphological characteristics of the different organs of *C. newmanii*, including the petiole, root, root tuber, leaf, leaf sheath, and rhizome were firstly provided. Additionally, the ethanolic extracts obtained from leaf, flower, and rhizome of *C. newmanii* consisted of various bioactive components, including coumarin, terpenoid, steroid, flavonoid, saponin, alkaloid, phenolic, and tannin. Furthermore, the rhizome extract possessed the highest contents of the triterpene, flavonoid, and polyphenol with the contents of 18.57 mg OAE/g DW, 44.84 mg QE/g DW, and 8.38 mg GAE/g DW, followed by the leaf extract (3.08 mg OAE/g DW, 28.78 mg QE/g DW, and 5.48 mg GAE/g DW), and the flower extract (0.39 mg OAE/g DW, 16.85 mg QE/g DW, and 3.71 mg GAE/g DW). **Conclusion:** the pharmacological properties of *C. newmanii* obtained from this study hopefully provide the potential application of this plant the pharmacological fields in the future.

Keywords: *Curcuma newmanii*; micro-morphological features; qualitative and quantitative assays.

RESUMEN

Características anatómicas y análisis fitoquímico de Curcuma newmanii Škorničk.

Introducción: *Curcuma newmanii* Škorničk. ha sido descrita recientemente como una nueva especie para la ciencia, cuyos especímenes tipo se recolectaron en Vietnam. **Objetivo:** El presente estudio describió inicialmente las propiedades farmacológicas de esta especie, incluyendo las características micromorfológicas y el análisis fitoquímico. **Metodología:** Se utilizó el método de doble tinción con verde de yodo-carmín para obtener los detalles de las características anatómicas, mientras que los ensayos cualitativos y cuantitativos se emplearon para realizar el análisis fitoquímico de esta planta. **Resultados:** En primer lugar, se proporcionaron las características micromorfológicas de los diferentes órganos de *C. newmanii*, incluyendo el pecíolo, la raíz, el tubérculo radicular, la hoja, la vaina foliar y el rizoma. Además, los extractos etanólicos obtenidos de hoja, flor y rizoma de *C. newmanii* consistieron en varios componentes bioactivos, incluyendo cumarina, terpenoide, esteroide, flavonoide, saponina, alcaloide, fenólico y tanino. Además, el extracto de rizoma poseía los contenidos más altos del triterpeno, flavonoide y polifenol con los contenidos de 18,57 mg OAE/g PS, 44,84 mg QE/g PS y 8,38 mg GAE/g PS, seguido por el extracto de hoja (3,08 mg OAE/g PS, 28,78 mg QE/g PS y 5,48 mg GAE/g PS), y el extracto de flor (0,39 mg OAE/g PS, 16,85 mg QE/g PS y 3,71 mg GAE/g PS). **Conclusión:** Se espera que las propiedades farmacológicas de *C. newmanii* obtenidas en este estudio permitan su posible aplicación en el campo farmacológico en el futuro.

Palabras clave: *Curcuma newmanii*; características micromorfológicas; ensayos cualitativos y cuantitativos.

RESUMO

Características anatômicas e triagem fitoquímica de *Curcuma newmanii* Škorničk.

Introdução: *Curcuma newmanii* Škorničk. foi recentemente descrita como uma nova espécie para a ciência, a partir de espécimes-tipo coletados no Vietnã. **Objetivo:** O presente estudo descreveu inicialmente as propriedades farmacológicas desta espécie, incluindo características micromorfológicas e triagem fitoquímica. **Metodologia:** O método de dupla coloração com verde-iodo-carmim foi utilizado para fornecer os detalhes das características anatômicas, enquanto ensaios qualitativos e quantitativos foram utilizados para realizar a triagem fitoquímica desta planta. **Resultados:** As características micromorfológicas dos diferentes órgãos de *C. newmanii*, incluindo pecíolo, raiz, tubérculo radicular, folha, bainha foliar e rizoma, foram inicialmente fornecidas. Além disso, os extratos etanólicos obtidos de folhas, flores e rizomas de *C. newmanii* consistiram em vários componentes bioativos, incluindo cumarina, terpenoide, esteroide, flavonoide, saponina, alcaloide, fenólico e tanino. Além disso, o extrato do rizoma possuía os maiores teores de triterpeno, flavonoide e polifenol com os teores de 18,57 mg OAE/g DW, 44,84 mg QE/g DW e 8,38 mg GAE/g DW, seguido pelo extrato da folha (3,08 mg OAE/g DW, 28,78 mg QE/g DW e 5,48 mg GAE/g DW) e o extrato da flor (0,39 mg OAE/g DW, 16,85 mg QE/g DW e 3,71 mg GAE/g DW). **Conclusão:** as propriedades farmacológicas de *C. newmanii* obtidas neste estudo proporcionam potencial aplicação desta planta em áreas farmacológicas no futuro.

Palavras-chave: *Curcuma newmanii*; características micromorfológicas; ensaios qualitativos e quantitativos.

1. INTRODUCTION

Curcuma L., the third largest genus belonging to the family Zingiberaceae, comprises about 130 species widely found in islands of the South Pacific, South East Asia, and northern Australia [1]. In herbal remedies, species of this genus have been used as the medicinal plants to treat many diseases such as bronchial complaints, diarrhea, insect bites, leucorrhea, pneumonia, abscesses, and infectious wounds [2]. In addition, a large number of the *Curcuma* species have been reported to consist of bioactive compounds as well as pharmaceutical properties [2].

Currently, accurate classification of plant species, especially medicinal plants, is very important in their research and application. Studies provided that *Curcuma* is one of the genera of the family Zingiberaceae with high diversity due to morphological and genetic variation as well as the wide hybridization and polyploidization [1]. The annual flowering cycle of species in this genus is short. Moreover, the vegetative organs are usually have similar morphological characteristics among species, leading to difficulty in classifying species [3].

To solve the difficulties in taxonomy using the comparative morphological method, many supporting methods have been applied, of which the micro-morphological characteristics are considered to play an important role in classification and standardization of medicinal plants [4]. It is necessary to study on the micro-morphological features to provide taxonomic information as well as bioactive compounds of newly discovered species in the *Curcuma* genus. In 2013, Leong-Škorničková and Tran were firstly discovered and described *Curcuma newmanii* Škorničk. as a new species to science which the type samples were collected from Ban Don village, Dak Lak province, Vietnam [5]. The present study, therefore, firstly investigated the micro-morphological features and phytochemical screening of *C. newmanii*.

2. MATERIALS AND METHODS

2.1. Plants

The specimens of the *C. newmanii* were collected from Krong Na commune, Ban Don District, Daklak province, Vietnam, at the altitude of 190-195 m, coordinates of 12°54'43.4''N/107°43'58.2''E. The voucher sample (NPN-DK-025) was deposited at the Herbarium of University of Science, Vietnam National University-HCMC (PHH).

2.1.1. Anatomical characteristics

The petiole, root, root tuber, leaf, leaf sheath, and rhizome of *C. newmanii* were cut into the thin slices of using razor blade. The Javel solution was used to soak these thin slices to remove the undesirable constituents in the tissue. The microscopic specimens were stained using the iodine green-carmine double staining method. These specimens were washed by the distilled water and then, the 10% glycerol was used to preserve them [6]. The studied samples were observed and taken the picture with the Olympus BX53 Digital Upright Microscope.

2.1.2. Extraction procedures

The fresh leaf, flower, and rhizome of *C. newmanii* were washed and dried at 50 °C using drying cabinet. The samples were then grinded into the powder. Ethanol solution was used to soak five grams of studied powder according to the ratio of 1 sample: 30 ethanol (w/w) for 8 hours and then it was filtered using whatman paper. This extraction was repeated twice more

with the residue. The supernatant was and all filtrate fractions were combined to collect the final extract.

2.1.3. Qualitative phytochemistry of C. newmanii

The qualitative phytochemistry of *C. newmanii* were determined using the methods as follows, (1) the phenolic and tannin: 2 mL of studied extract were taken into the tube, 2 mL of H_2O and 2-3 drops of $FeCl_3$ (5%) were added; if the blue-black or brown-green precipitate appeared after completion of the reactions, it was considered as positive [7]; (2) Alkaloids: 2 mL of studied extract and 3-4 drops of Wagner reagent were taken; if the red brown precipitate appeared after completion of the reactions, it was positive [8]; (3) Flavonoids: 2 mL studied extract and 2 mL 10% $Pb(COOH)_2$ were taken; if the yellow precipitate appeared after completion of the reactions, it was positive [9]; (4) Saponin: 2 mL studied extract were taken into the tube, 10 mL of distilled water were added and boiled for 2 minutes; if the mixture formed foam, it was positive [10]; (5) Terpenoids and steroids: 5 mL of studied extract were taken, 2 mL chloroform and 3 mL concentrated H_2SO_4 were added; if the reddish brown colour appeared, it was positive [11]; (6) Coumarin: 2 mL of studied extract were taken and 3 mL NaOH (10%) were added; if the yellow colour appeared, it was positive [12].

2.2. Quantitative phytochemistry of *C. newmanii*

2.2.1. Total triterpene content (TTC)

1.0 mL of the studied extract was taken into a tube, then 5% acetic acid (0.2 mL) and perchloric acid (1.2 mL) were added, this mixture was shaken slightly, incubated at 70 °C for 15 minutes, and cooled quickly within 2 minutes. 2.6 mL of ethyl acetate was added the mixture to make 5.0 mL and then it was photometrically measured at 550 nm wavelength. Acetic acid (5%) was used as a control agent. The total triterpene concentration (milligrams of oleanoic acid equivalent-OAE) in the studied extract was identified based on the photometric results and standard curve graph [13]. The TTC was calculated according to the formula:

$$\text{TTC (mg OAE/g DW)} = C_x \times \frac{V}{10^3} \times \frac{100}{a \times (100 - W)} \times K$$

Where, Cx: the total triterpenoid content in the studied extract obtained from the standard curve (ppm); a: initial sample mass (g); V: sample volume (mL); K: dilution factor; W: humidity (%); 10^3: conversion factor.

2.2.2. Total flavonoid content (TFC)

1.0 mL of the studied extract and 0.3 mL of $NaNO_2$ 5% were added into a tube, this mixture was shaken slightly. The tube was kept at room temperature for 5 minutes and then, 0.3 mL of $AlCl_3$ solution 10% were added, then the tube was well shaken and leaved at room temperature for 5 minutes. 2.0 mL of 1 M NaOH solution was added and shaken, then, 6.4 mL of distilled water were also added to make 10.0 mL. The mixture was measured photometrically at wavelength λ= 510 nm. Distilled water was used as a control agent. The total flavonoid content (milligrams of quercetin acid equivalent-QE) in the studied extract was identified based on the photometric results and standard curve graph [14]. The TFC was calculated according to the formula:

$$\text{TFC (mg QE/g DW)} = C_x \times \frac{V}{10^3} \times \frac{100}{a \times (100 - W)} \times K$$

Where, Cx: the total flavonoid content in the studied extract obtained from the standard curve (ppm); a: initial sample mass (g); V: sample volume (mL); K: dilution factor; W: humidity (%); 10^3: conversion factor.

2.2.3. Total polyphenol content (TPC)

A volume of 0.1 mL of the studied extract and 1.8 mL of Folin-Ciocalteu solution were added into a tube, this mixture was shaken slightly and the tube was kept at room temperature for 5 minutes. Then, 1.2 mL of 15% Na_2CO_3 solution were added, and finally 8.7 mL of distilled water were added to make 10 mL. The tube was covered, shaken, and incubated at room temperature and dark conditions for 90 minutes. The mixture was measured photometrically at wavelength λ= 734 nm. Distilled water was used as a control agent. The total polyphenol content (milligrams of gallic acid equivalent-GAE) in the studied extract was identified based on the photometric results and standard curve graph [15]. The TPC was calculated according to the formula:

$$\text{TPC (mg GAE/g DW)} = C_x \times \frac{V}{10^3} \times \frac{100}{a \times (100 - W)} \times K$$

Where, Cx: the total polyphenol content in the studied extract obtained from the standard curve (ppm); a: initial sample mass (g); V: sample volume (mL); K: dilution factor; W: humidity (%); 10^3: conversion factor.

3. RESULTS

Photos of all parts of studied *Curcuma newmanii* Škorničk. samples are shown in Fig. 1.

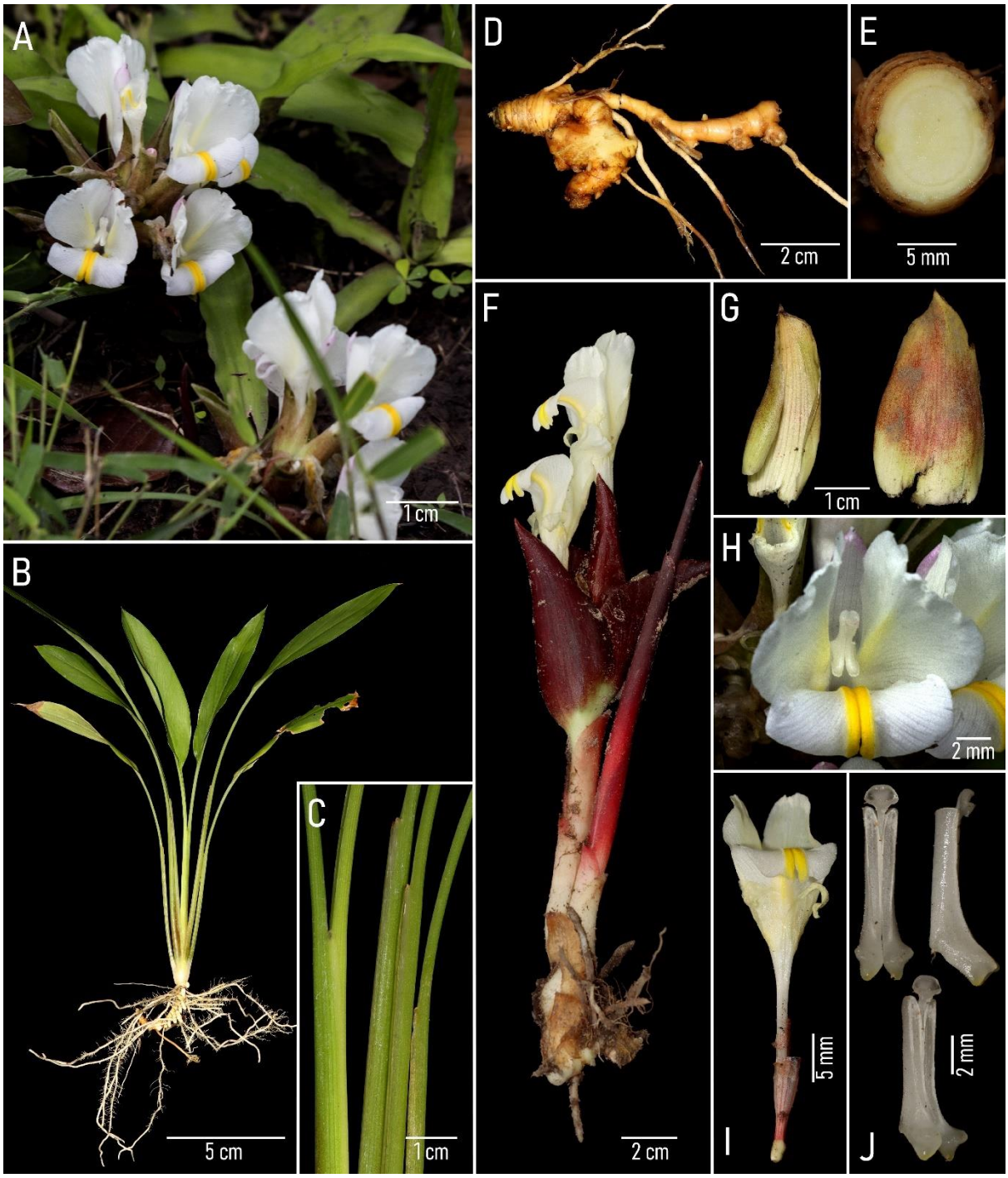

Figure 1. *Curcuma newmanii*. A. Habit (flowering stage). B. Whole plant (after flowering). C. Petioles and ligules. D. Rhizome and roots. E. Cross section of rhizome. F. Inflorescence with flower. G. Bracts. H. Flower (front view). I. A flower in detail. J. Anthers in different views. Photos: Diep Quang Dinh: A-I, Nga Nguyen-Phi: J.

3.1. Anatomical characteristics of *C. newmanii*

3.1.1. Root

The cross-section of root is nearly circular, divided into 2 regions, the cortical area includes 3/4 of the radius of cross-section while ¼ of the remaining region is stele. The piliferous layer consists of a layer of polygonal or rectangular cells, the walls are impregnated with phellem, approximately equal size, closely arranged, and many root hairs. The exodermis consists of 2-

3 layers of rectangular or square cells, walls impregnated with phellem, radially arranged. The cortical parenchyma is divided into 2 regions, outer parenchyma consists of 8-10 layers of nearly polygonal round cells, cellulose walls, irregular size, haphazardly arranged, leaving quite large intercellular spaces (spongy parenchyma); inner parenchyma includes 5-6 layers of rectangular or square cells with cellulose walls, gradually smaller in size towards the center, arranged in concentric rings and radial rows to create small intercellular spaces. There are many cells containing yellow-brown secretions in the cortical parenchyma. The endodermis with Casparian *strip* comprises 1 layer of rectangular cells, quite regular. The pericycle consists of a single layer of polygonal cells with cellulose walls, alternately arranged with endodermis. The vascular system consists of 18 - 22 primary phloem bundles, alternately arranged with 18 - 22 primary xylem bundles on a ring close to the pericycle. The primary phloem has 3-5 layers of polygonal cells, cellulose walls, irregular, radially differentiated. The protoxylem bundle consists of 2–4 vessels, polygonal shape, lignin-impregnated walls, radially differentiated. There are 12-14 metaxylem veins located in a ring under primary phloem and protoxylem bundle. The metaxylem veins are polygonal in shape, slightly rounded, with cellulose or lignin-impregnated walls, large size. The medullary parenchyma is divided into 2 regions, the outer medullary parenchyma consists of 6-7 layers of polygonal cells, lignin-impregnated walls, tightly packed together; Inner medullary parenchyma includes of 2-4 layers of polygonal cells, cellulose walls, irregular, with small intercellular spaces.

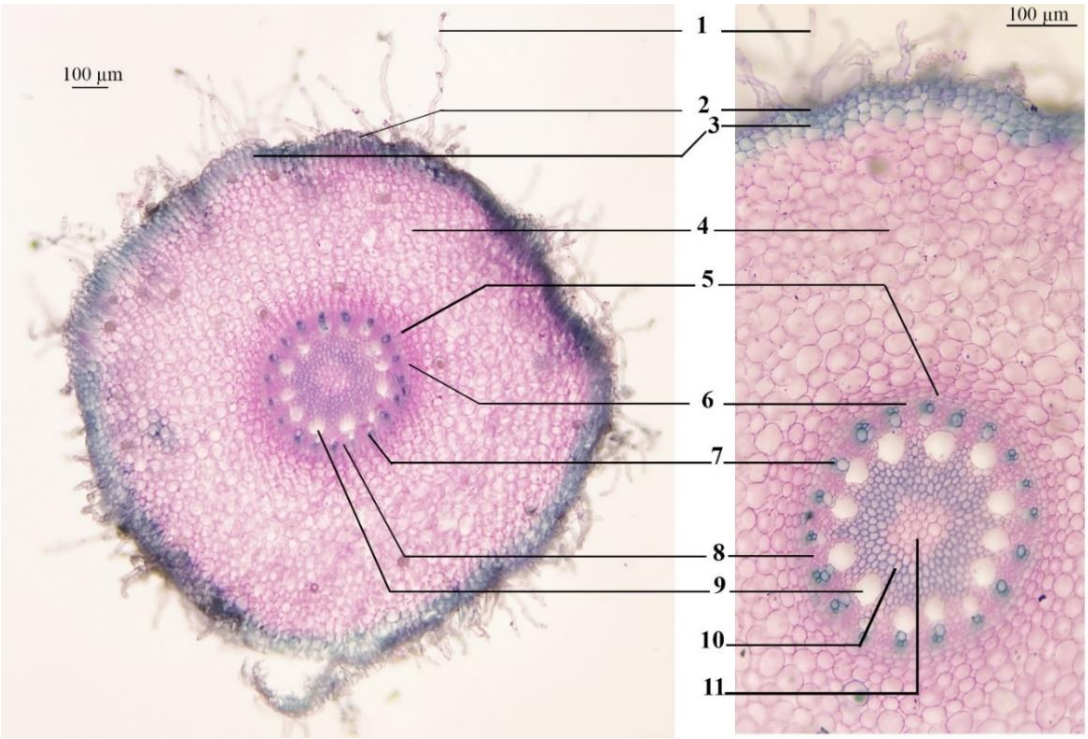

Figure 2. The cross-section of root. 1: root hair; 2: piliferous layer; 3: exodermis; 4: cortical parenchyma; 5: endodermis with Casparian strip; 6: pericycle; 7: primary xylem; 8: primary phloem; 9: metaxylem; 10: sclerenchymatous conjunctive tissues; 11: parenchymatous pith.

3.1.2. Root tuber

The cross-section is often distorted, rarely round, divided into 2 regions, the cortex is very thick and grows according to the growth of tuber, the stele is thin. The piliferous layer includes a layer of rectangular, distorted, very flat, small cells, and scattered with hairs. The phellem

has 3-4 layers of rectangular, flat cells, walls impregnated with cork, radially arranged. The phelloderm consists of 4-6 layers of polygonal cells with curved cellulose walls, large size, radially arranged. The cortical parenchyma is a type of spongy parenchyma with polygonal or nearly round cells, cellulose walls; there are many cells containing yellow secretions in the cortical parenchyma. The endodermis with Casparian *strip occurs. The* pericycle has 1 layer of polygonal, regular cells with cellulose walls. There are 14-18 protoxylem bundles alternately arranged with 14-18 primary phloem bundles per a ring. The protoxylem bundle has 2-4 polygonal xylem vessels, walls impregnated with lignin, and radially differentiated. The primary phloem bundle consists of 3-5 layers of polygonal cells, cellulose walls, haphazardly arranged. There are 14-16 polygonal metaxylem vessels with walls impregnated with lignin, usually located close to the protoxylem bundle. The medullary parenchyma is a dense tissue with polygonal cells, cellulose walls, closely arranged.

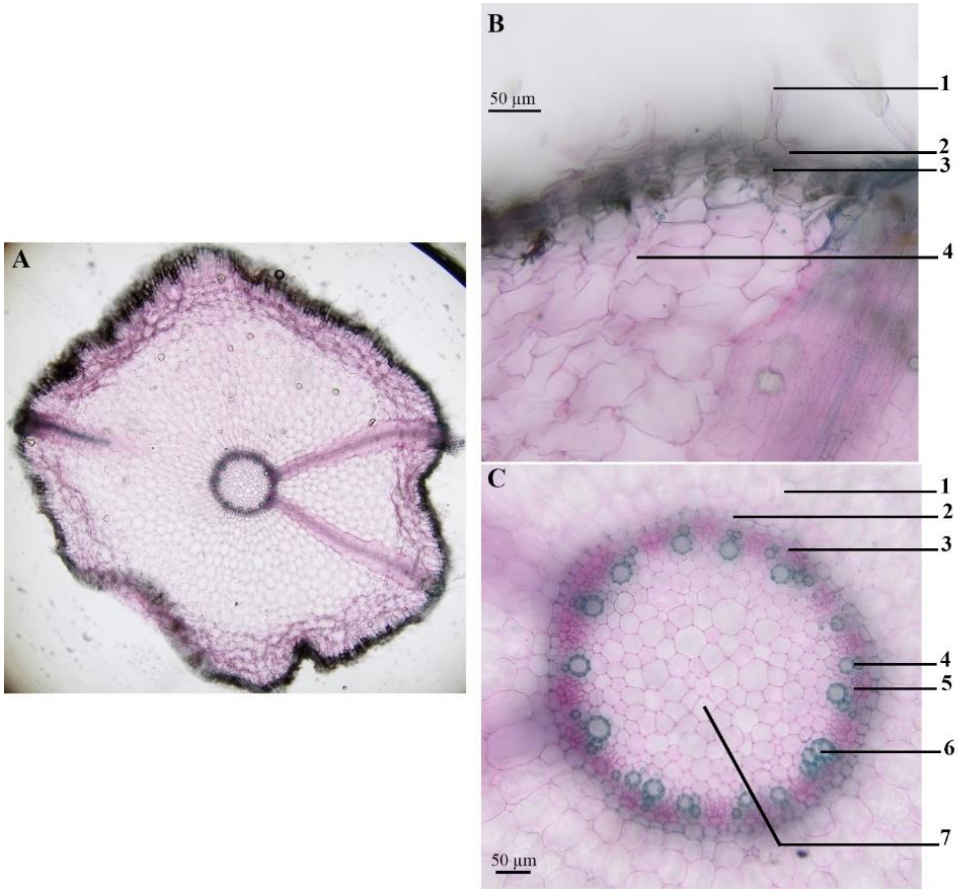

Figure 3. The cross-section of root tuber. A. the whole wiew of cross-section. B. Cortex (1: root hair, 2: piliferous layer, 3: phellem, 4: phelloderm). C. Stele (1: cortical parenchyma, 2: endodermis with Casparian strip, 3: pericycle, 4: primary xylem, 5: primary phloem, 6: metaxylem, 7: medullary parenchyma)

3.1.3. Rhizome

The cross-section is often distorted, rarely round. The epidermis includes 1 layer of rectangular cells, very flat, and small. The phellem has 3-8 layers of distorted rectangular cells, 1-2 layers of phellem under the epidermis are large size, the lower cell layers are very flat rectangular, the walls are impregnated with phellem, radially arranged. The phelloderm consists of 2-3 layers of rectangular cells, with cellulose walls. The cortical parenchyma belongs to the medullary parenchyma type, consisting of round or nearly round polygonal cells, scattered with

primary vascular bundle with xylem inside and phloem outside. The endodermis with Casparian *strip occurs.* The pericycle consists of a single layer of cellulose wall cells, many locations of which are unclear. Many vascular bundles are arranged in an orderly manner from the endodermis into the medullary parenchyma; the differentiation of the xylem bundles is unclear. The medullary parenchyma includes nearly round or polygonal cells and cellulose walls. There are many cells containing yellow secretions scattered throughout the cortical and medullary parenchyma.

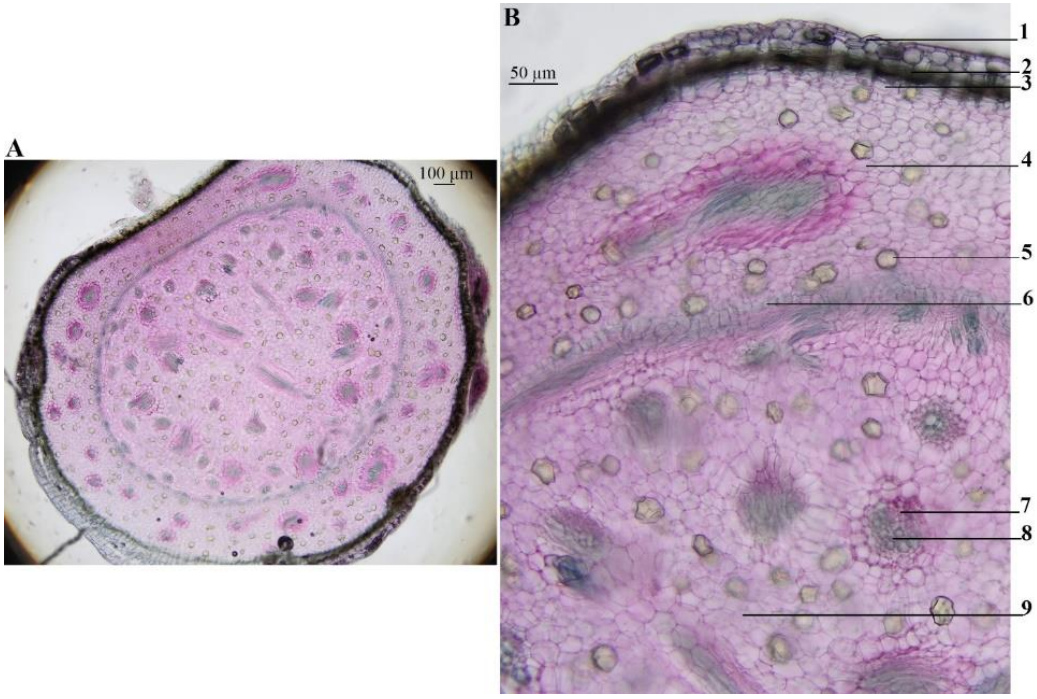

Figure 4. The cross-section of rhizome. A. The whole view of cross section. B. The details of cross section (1: epidermis, 2: phellem, 3: phelloderm, 4: cortical parenchyma, 5: secretory cell, 6: endodermis with Casparian strip, 7: primary phloem, 8: primary xylem, 9: medullary parenchyma).

3.1.4. Leaves (Figure 5)

3.1.4.1. Midrib: The upper surface is concave, the lower surface is convex. The epidermis consists of closely rectangular or polygonal cells, sometimes with unicellular protective hairs. The angular collenchyma consists of 1-2 layers of cells located below the concave part of the upper epidermis. The parenchyma has polygonal cells, sometimes distorted and irregular size. There are vascular bundles arranged in rows above the lower epidermis with xylem located above the phloem, there are large air cavities among these vascular bundles. The xylem includes: 1-3 metaxylem vessels, 2-6 small protoxylem vessels located below the metaxylem; under the phloem, horseshoe-shaped sclerenchyma cluster includes polygonal cells and very thick walls impregnated with lignin. In the middle of the parenchyma, there are 4-5 vascular bundles, arranged in rows; below xylem and above phloem, there are 2 layers of sclerenchyma cells with thick walls by cellulose or impregnated with lignin. ***Lamina:*** The upper and lower epidermis consist of a layer of rectangular cells, cellulose walls, and stomata scattered in both epidermis. The hypodermis is 1 layer of polygonal cells, cellulose wall, located below the epidermis on both sides. The vascular bundles are the same structure as those in the midrib. The

spongy parenchyma comprise round or oval cells, near the upper epidermis, there are 2-3 layers of slightly elongated cells arranged in rows like the chlorenchyma, containing many chloroplasts.

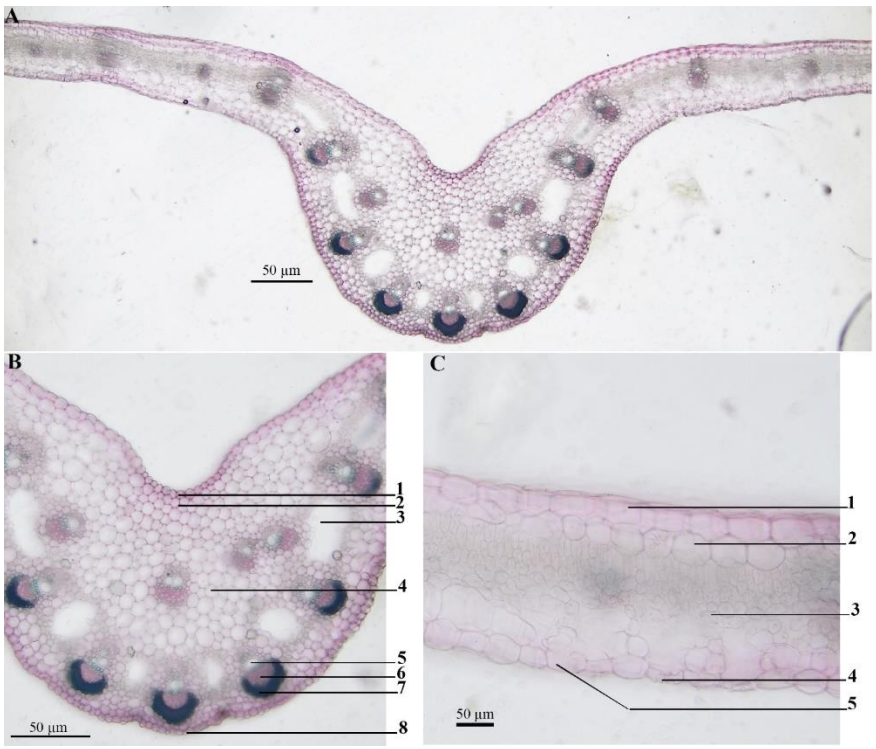

Figure 5. The cross-section of leaf. A. The whole view of cross-section. B. Midrib (1: upper epidermis, 2: angular collenchyma, 3: air cavity, 4: parenchyma, 5: xylem, 6: phloem, 7: sclerenchyma, 8: lower epidermis). C. Lamina (1: upper epidermis, 2: hypodermis, 3: spongy parenchyma, 4: stomata, 5: lower epidermis).

3.1.5. Petiole (Figure 6)

The outline is open 'U' shaped. The cross section has a concave at upper surface and a convex at lower surface, with the same structure as those in the lamina. The epidermis on the petiole has many unicellular protective hairs.

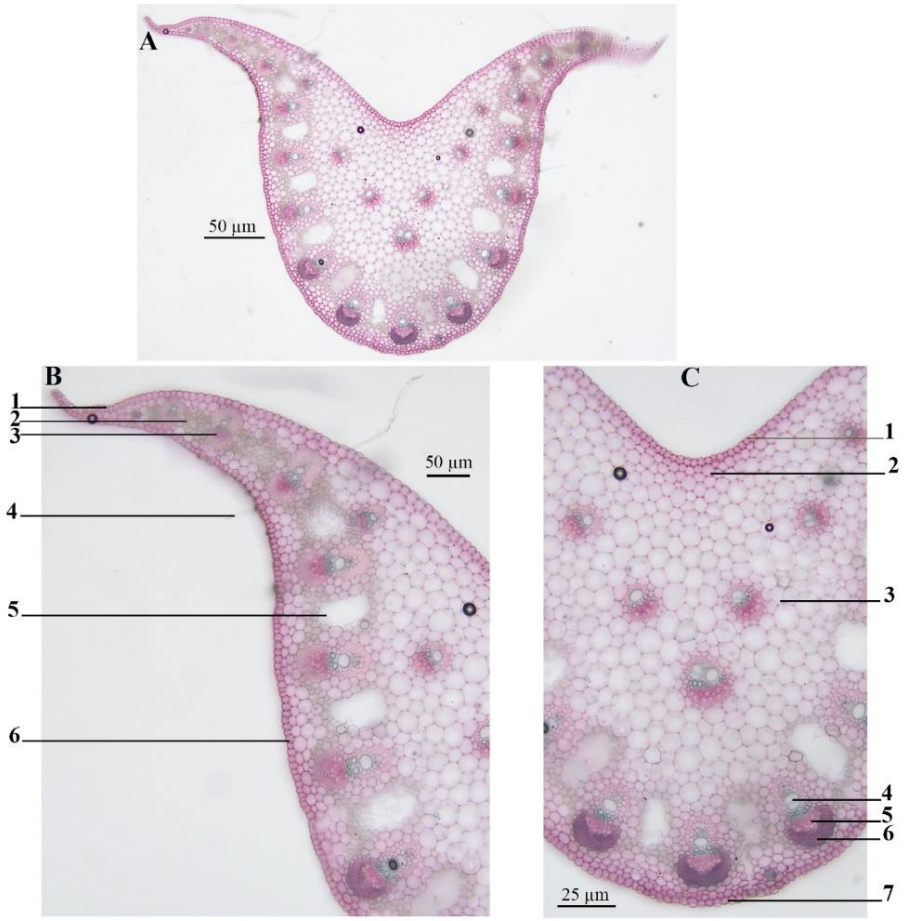

Figure 6. The cross-section of petiole. A. The whole view of cross section. B. Side view of cross section (1: upper epidermis, 2: spongy parenchyma, 3: vascular bundle, 4: unicellular protective hair, 5: air cavity, 6: lower epidermis). C. The middle of the cross section (1: upper epidermis, 2: angular collenchyma, 3: parenchyma, 4: xylem, 5: phloem, 6: sclerenchyma, 7: lower epidermis).

3.1.6. Leaf sheath (Figure 7)

The cross section is curved with a deeply concave at upper surface, thick in the middle and gradually thinner on both sides. The upper and lower epidermis are the same, consisting of 1 layer of rectangular cells, the upper epidermal cells are larger than the lower epidermis. The parenchyma includes polygonal cells. On the lower epidermis, there are large and small vascular bundles arranged in alternating rows; in the middle, there are the large vascular bundles with large air cavities. The vascular bundle includes xylem above, phloem below. Xylem comprises 1-2 metaxylem vessels, 3-5 protoxylem vessels; sclerenchyma clusters located above xylem and below phloem, 2-3 layers of sclerenchyma cells above the xylem have thin cellulose walls; 5 - 6 layers of sclerenchyma cells under the phloem have thick walls, lignin-impregnated walls. There are small vascular bundles also arranged in rows in the middle of the parenchyma area with a similar structure to the large vascular bundles. The vascular bundles gradually become smaller in size and are only arranged in a row towards the thin parts on both sides of the leaf sheath.

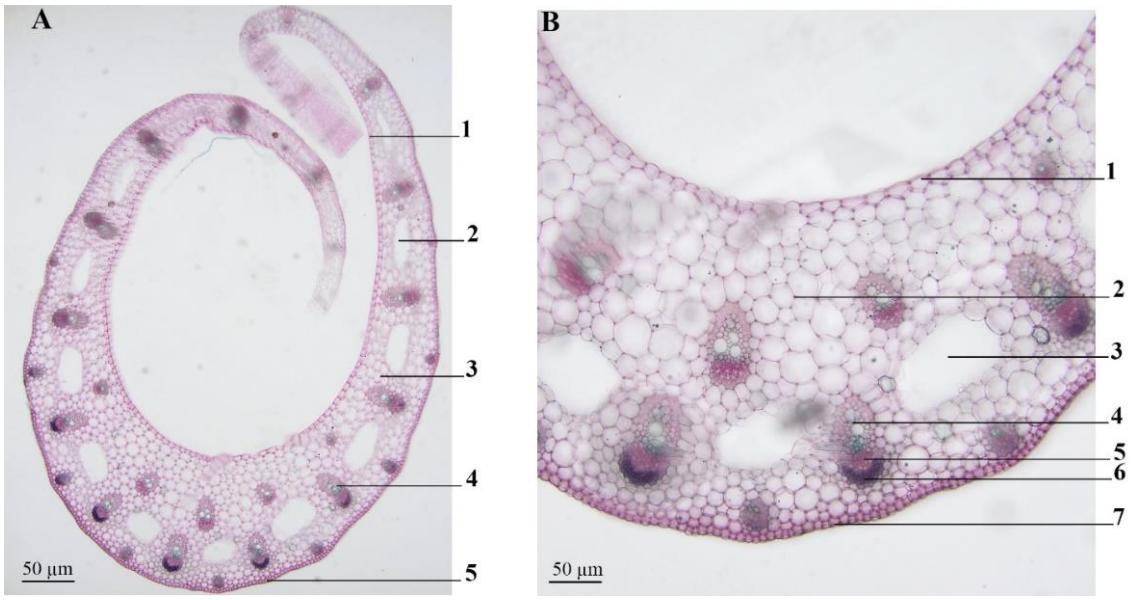

Figure 7. The cross-section of leaf sheath. A. The whole view of cross section (1: upper epidermis, 2: air cavity, 3: parenchyma, 4: vascular bundle, 5: lower epidermis). B. the middle of the cross section (1: upper epidermis, 2: parenchyma, 3: air cavity, 4: xylem, 5: phloem, 6: sclerenchyma, 7: lower epidermis).

3.2. Qualitative phytochemistry of *C. newmanii*

The results in Tab. 1 demonstrated that the ethanolic extracts obtained from leaf, flower, and rhizome of *C. newmanii* consisted of various bioactive components, including coumarin, terpenoid, steroid, flavonoid, saponin, alkaloid, phenolic, and tannin.

Table 1. Preliminary phytochemistry of *C. newmanii*

Compounds	Leaf	Flower	Rhizome
Phenolic	+++	++	+++
Tannin	+++	++	+++
Alkaloid	+	++	++
Flavonoid	++	+	+++
Saponin	+	+	+
Terpenoid	+++	+++	+++
Steroid	+++	+++	+++
Coumarin	++	++	++

Note: (+) Less, (++) Medium, (+++) Very abundant.

3.3. Quantitative phytochemical content of *C. newmanii*

The data in Tab. 2 showed the total of triterpene, flavonoid, and polyphenol contents of the ethanolic extracts from *C. newmanii*. Accordingly, the rhizome extract possessed the highest contents of the triterpene, flavonoid, and polyphenol with the contents of 18.57 mg OAE/g DW, 44.84 mg QE/g DW, and 8.38 mg GAE/g DW, followed by the leaf extract (3.08 mg OAE/g DW, 28.78 mg QE/g DW, and 5.48 mg GAE/g DW), and the flower extract (0.39 mg OAE/g DW, 16.85 mg QE/g DW, and 3.71 mg GAE/g DW).

Table 2. The triterpene, flavonoid, and polyphenol contents of *C. newmanii*

Phytochemical contents	Leaf	Flower	Rhizome
TTC (mg OAE/g DW)	3.08 ± 0.30	0.39 ± 0.30	18.57 ± 0.34
TFC (mg QE/g DW)	28.78 ± 0.23	16.85 ± 0.26	44.84 ± 0.60
TPC (mg GAE/g DW)	5.48 ± 0.70	3.71 ± 0.10	8.38 ± 0.40

Note: TTC: total triterpene content, TFC: total flavonoid content, TPC: total polyphenol content.

4. DISCUSSION

The anatomical traits of some *Curcuma* species have been also provided by prior studies. Overall, micro-morphological features of *C. newmanii* are relative to other *Curcuma* species in having: open 'U'shaped petiole; vascular bundles distributed close collateral alternating with air cavities in the leaf, petiole, and leaf sheath; thick layer of cortical parenchyma in root etc. However, the studied species can be distinguished from *C. singularis* in having: (1) the cortical parenchyma of *C. newmanii* root mostly consists of polygonal or nearly round cells while the cortical parenchyma of *C. singularis* root usually contained elongated oval cells in a radial direction, the metaxylem vessel of *C. singularis* root has thick walls that are almost always impregnated with lignin while the metaxylem vessel of *C. newmanii* root has walls that are usually made of cellulose; (2) in the *C. newmanii* rhizome, the vascular bundles in the cortical region are often larger than that in the stele, the direction of differentiation of phloem and xelem is unclear whereas the vascular bundles in the cortical region and stele of *C. singularis* rhizome are quite uniform in size with the phloem overlapping the xylem, the xylem is centrifugally differentiated, this can be explained by the fact that the *C. singularis* rhizome has less branching, so the structure and differentiation of the conductive bundles are clear; (3) the *C. singularis* leaf blade has many protective hairs on the lower epidermis while the *C. newmanii* leaf blade has very few or almost no protective hairs [16].

Anu and Dan (2020) {17] provided the anatomical traits of the petiole of 12 different *Curcuma* species. Accordingly, the outline shape of these plants were divided into 5 groups, including horseshoeshaped (*C. longa*), V shaped (*C. oliganth* and *C. aurantiaca*), open V shaped (*C. aromatica*, *C. zedoaria* and *C. vamana*), U shaped (*C. haritha* and *C. caesia*), and open U shaped (*C. pseudomontana, C. aeruginosa*, *C. zanthorrhiza,* and *C. amada*). According to Figure 6A, the outline shape of the micro-morphological features of the *C. newmanii* petiole has open U shaped like that of *C. pseudomontana, C. aeruginosa*, *C. zanthorrhiza,* and *C. amada* [17]. In addition, the shape of epidermal cells of the *C. newmanii* petiole are rectangular which are similar to that of *C. aeruginosa*, *C. amada, C. aurantiaca, C. aurantiaca, C. haritha, C. longa,* and *C. vamana* [17].

The phytochemical screening of the *Curcuma* species have been reported by prior studies. For instance, Akter *et al.* provided the total phenolic and flavonoid contents as well as antioxidant effects of the methanol extracts isolated from the rhizomes of six *Curcuma* samples from Japan, including *C. longa* (collected from 2 regions, Ryudai Gold and Okinawa), *C. xanthorrhiza, C. aromatica, C. amada*, and C. *zedoaria*. Accordingly, the total phenolic content from these samples were 154.4, 59.2, 38.5, 37.9, 48.7, and 43.7 mg GAE/g, respectively while 797.5, 310.7, 89.3, and 15.3 GAE/g, respectively were the total flavonoid content towards the same species [18]. Rajamma *et al.* showed that the total phenol content isolated from the rhizome dichloromethane extracts of 7 *Curcuma* species such as *C. aeruginosa, C. amada, C. aromatica, C. brog, C. caesia, C. malabarica,* and *C. rakthakanta* collected from Kerala, India, were 34.0, 23.0, 69.0, 40.0, 63.0, 46.0, and 46.0 mg GAE/g [19]. Similarly, the total phenol contents of the various solvent

extracts obtained from the fresh and dried rhizome of *C. caesia* grown in India were also investigated. Accordingly, the hexane, petroleum ether, benzene, chloroform, ethyl acetate, methanol, and water extracts isolated from fresh and dried rhizome were found to contain the total phenol contents of 50.44 and 53.44, 38.42 and 26.43, 56.64 and 96.68, 57.53 and 109.41, 45.48 and 86.29, 32.58 and 28.33, 34.39 and 48.49 mg GAE/g [20].

The qualitative phytochemicals of the distilled water and methanol extract from the rhizome of *C. aromatica* and *C. xanthorrhiza* grown in Kerala, India showed that the distilled water extracts of these plants contained flavonoid, tannin, saponin, carbohydrate, terpenoids, sterols, protein, and phenols. The methanol extract from *C. aromatica* consisted of 5 later compounds while the same components and flavonoid were presented in the *C. xanthorrhiza* methanol extract. Also, the quantitative identification of curcumin provided that *C. aromatica* and *C. xanthorrhiza* contained curcumin with the contents of 0.0175 g/100 g and 1.0863 g/100 g [21]. The ethanol extract of *C. zedoaria* rhizomes grown in Savar, Bangladesh, contained some phytochemical components, including steroids, carbohydrates, terpinoids, alkaloids, saponins, flavonoids, and tannins [22]. Joshi *et al.* provided the qualitative phytochemicals of the ethanol and methanol extracts from *C. longa* and *C. aromatica* rhizome collected from India. The results showed that both extracts from these plants contained flavonoid and alkaloid while tannin was found in the *C. longa* ethanol and methanol extracts [23].

The phytochemical screening of the various extracts, including water, ethanol, methanol, acetone, ethyl acetate, chloroform, and petroleum ether of the *C. caesia* rhizome collected from Raipur, India have been also investigated. Experiments were carried out in a total of 13 compounds, including alkaloids, cardiac glycosides, carbohydrates, flavonoids, phenols, phlobatannins, proteins, saponins, sterols, tannins, terpenoids, quinones, and oxalates. The results showed that the methanol is the best solvent which contained 11 out of 13 compounds (except phlobatannins and oxalates) [24]. In addition, the ethanol extracts isolated from *C. xanthorrhiza* and *C. domestica* grown in Indonesia have been also reported of which saponin, steroid, alkaloid, and flavonoid were found in the extract of the first plant while the later species contained steroid and flavonoid [25]. The different extracts of *C. sahuynhensis* rhizomes and inflorescences collected from Quang Ngai province, Vietnam, were also investigated using 13 tests for the phytochemical screening, including fats, carbohydrates, essential oil, carotenoids, alkaloids, amino acids, cardiac glycosides, coumarins, flavonoids, saponins, tannins, triterpenoid, and polyuronides. Accordingly, the ether and ethanol extracts of the rhizome comprised 7 out of 13 compounds, the water extract of this organ consisted of 3 out of 13 constituents. Meanwhile, the water, ether, and ethanol extracts of inflorescences contained 3, 4, 5, respectively out of 13 components [26].

5. CONCLUSION

The present study firstly provided the details of micro-morphological features of the different organs of *C. newmanii*, including the petiole, root, root tuber, leaf, leaf sheath, and rhizome. In addition, the ethanolic extracts obtained from leaf, flower, and rhizome of *C. newmanii* consisted of various bioactive components, including coumarin, terpenoid, steroid, flavonoid, saponin, alkaloid, phenolic, and tannin. Also, the studied plant consisted of the significant amounts of triterpene, flavonoid, and polyphenol contents. Like many valuable species of the genus *Curcuma,* the results from this study are expected to be a scientific basis for the application of this species in the pharmaceutical field in the future.

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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Review article

Bacterial immobilization matrices: a scientometric review

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SUMMARY

Introduction: This study analyzes the evolution of research on bacterial immobilization matrices using a scientometric approach, identifying trends in scientific production, materials used, characterization methodologies and emerging biotechnological applications. **Methodology:** To this end, a scientometric review based on PRISMA was carried out, with the search of articles in Scopus and PubMed using terms such as "cell immobilization", "bacterial" and "matrix", combined with "AND". A total of 1,232 publications were identified, of which 94 were selected for analysis after applying filters of relevance and originality. Bibliometric tools were used to assess annual production, international collaboration, and key terms in publications. **Results:** The results show that scientific output experienced fluctuations between 2019 and 2024, with a drop in 2023, followed by a rebound in 2024. China, India and the United States lead research in this field. Biopolymers such as alginate, chitosan and polyvinyl alcohol are the most researched materials, while lignocellulosic waste is emerging as more sustainable alternatives. In terms of methodologies, the most commonly used include scanning electron microscopy (SEM) and infrared spectroscopy (FTIR). **Conclusions:** Bacterial immobilization continues to expand, with promising applications in bioremediation and biocatalysis. Diversification in materials and methodologies suggests that multidisciplinary approaches will be critical in the future, so it is recommended to strengthen international collaboration and increase funding in biotechnology to maximize the impact of these technologies.

Keywords: Bacterial immobilization; matrices for immobilization; scientometrics.

RESUMEN

Matrices de inmovilización bacteriana: una revisión cienciométrica

Introducción: Este estudio analiza la evolución de la investigación sobre matrices de inmovilización bacteriana mediante un enfoque cienciométrico, identificando tendencias en producción científica, materiales utilizados, metodologías de caracterización y aplicaciones biotecnológicas emergentes. **Metodología:** Para ello, se llevó a cabo una revisión cienciométrica basada en la metodología PRISMA, con la búsqueda de artículos en Scopus y PubMed usando términos como "cell immobilization", "bacterial" y "matrix", combinados con "AND". Se identificaron 1,232 publicaciones, de las cuales 94 fueron seleccionadas para el análisis tras aplicar filtros de pertinencia y originalidad. Se utilizaron herramientas bibliométricas para evaluar la producción anual, la colaboración internacional y los términos clave en

las publicaciones. **Resultados:** Los resultados muestran que la producción científica experimentó fluctuaciones entre 2019 y 2024, con una caída en 2023, seguida de un repunte en 2024. China, India y EE.UU. lideran las investigaciones en este campo. Los biopolímeros como alginato, quitosano y polivinil alcohol son los materiales más investigados, mientras que los residuos lignocelulósicos están emergiendo como alternativas más sostenibles. En cuanto a las técnicas, las más utilizadas incluyen microscopía electrónica de barrido (SEM) y espectroscopía infrarroja (FTIR). **Conclusiones:** La inmovilización bacteriana sigue en expansión, con aplicaciones prometedoras en bioremediación y biocatálisis. La diversificación en materiales y metodologías sugiere que los enfoques multidisciplinarios serán fundamentales en el futuro, por lo que se recomienda fortalecer la colaboración internacional y aumentar el financiamiento en biotecnología para maximizar el impacto de estas tecnologías.

Palabras clave: Inmovilización bacteriana, matrices para inmovilización; cienciometría.

RESUMO

Matrizes de imobilização bacteriana: uma revisão cienciométrica

Introdução: Este estudo analisa a evolução das pesquisas sobre matrizes de imobilização bacteriana utilizando uma abordagem cienciométrica, identificando tendências na produção científica, materiais utilizados, metodologias de caracterização e aplicações biotecnológicas emergentes. **Metodologia:** Para tanto, foi realizada uma revisão cienciométrica baseada no PRISMA, com a busca de artigos na Scopus e PubMed utilizando termos como “imobilização celular”, “bacteriana” e “matriz”, combinados com "AND". Foram identificadas 1.232 publicações, das quais 94 foram selecionadas para análise após aplicação de filtros de pertinência e originalidade. Ferramentas bibliométricas foram utilizadas para avaliar a produção anual, a colaboração internacional e os termos-chave nas publicações. **Resultados:** Os resultados mostram que a produção científica experimentou flutuações entre 2019 e 2024, com queda em 2023, seguida de recuperação em 2024. China, Índia e Estados Unidos lideram pesquisas neste campo. Biopolímeros como alginato, quitosana e álcool polivinílico são os materiais mais pesquisados, enquanto os resíduos lignocelulósicos estão surgindo como alternativas mais sustentáveis. Em termos de metodologias, as mais comumente utilizadas incluem microscopia eletrônica de varredura (MEV) e espectroscopia de infravermelho (FTIR). **Conclusão**: A imobilização bacteriana continua a se expandir, com aplicações promissoras em biorremediação e biocatálise. A diversificação de materiais e metodologias sugere que abordagens multidisciplinares serão críticas no futuro, por isso é recomendável fortalecer a colaboração internacional e aumentar o financiamento em biotecnologia para maximizar o impacto dessas tecnologias.

Palavras-chave: Imobilização bacteriana; matrizes para imobilização, cienciometria.

1. INTRODUCTION

Cell immobilization consists of the confinement of intact cells within a material or device known as a matrix or support, with the aim of maintaining their biological functionality. This process can be carried out by various methods, such as physical adsorption, encapsulation, entrapment, or self-aggregation [1]

The matrix, as a key component in these systems, acts as a three-dimensional support that provides a suitable structural and functional environment for the fixation of living cells, enzymes or other bioactive elements. In addition to providing physical stability, these matrices protect the immobilized components against adverse conditions, allowing them to maintain their viability and functionality for prolonged periods in different biological and chemical processes [2]. This ability to preserve the integrity and biological activity of cells or enzymes has

been essential to maintain their viability, maximize the efficiency of biotechnological processes and guarantee their reproducibility [3].

These technologies have proven to be highly versatile and applicable in various fields, including medicine, where they are used in tissue regeneration, controlled drug release, and the production of therapeutic compounds [4]. In the agricultural field, its implementation has allowed the development of sustainable solutions such as bioinoculants, biofertilizers, and biopesticides, optimizing crop growth and reducing environmental impact [5]. Likewise, in the food industry, these technologies improve fermentation and enzymatic processes, while in the environmental sector they contribute to bioremediation and waste treatment [6-8].

The wide range of matrices used in cell immobilization encompasses both inorganic and organic materials. Among the most commonly used inorganic matrices are clays, silicates, crystals, ceramics, diatomaceous earth, polyurethane foam and porous volcanic stones, as well as systems based on the sol-gel technique. These matrices have been widely studied due to their high chemical and thermal stability, as well as their specific properties that favor the efficient immobilization of microorganisms. Recent research has validated its application in various biotechnological and industrial processes [9-15].

Among the most commonly used organic matrices are polymers such as polyacrylamide, agarose, polyvinyl alcohol and alginates, which provide adequate structural support and preserve the biological functionality of the immobilized bacteria. In addition, natural materials such as nylon fiber, cellulose, agar, κ-carrageenan, chitin and collagen have been used, which stand out for their biocompatibility and favorable mechanical properties [16, 17].

Agricultural by-products and lignocellulosic waste have emerged as innovative alternatives in cell immobilization, aligning with circular economy principles. Materials such as sugarcane bagasse, green coffee residues, sugarcane leaves, fibers *Opuntia* spp. y *Agave* spp., biochar and micronized plant residues of coffee have proven to be economical and effective for the immobilization of microorganisms. Other innovative options include the *Luffa cylindrica*, coconut fibers and biological layers of straw, peat and soil, which offer sustainable solutions with specific applications in bioremediation and industrial biotechnology [18-27].

To understand the impact and evolution of matrices used in bacterial immobilization, it is essential to analyze scientific production in this field. Bibliometrics and scientometrics allow us to evaluate trends, identify patterns of collaboration and highlight the technological advances that have driven the development of new cell immobilization strategies. In this context, the present research employs a scientometric approach to examine the scientific literature on bacterial immobilization matrices, providing a comprehensive view of its application in different sectors.

2. METHODOLOGY

This review was developed under a scientometric approach, following the guidelines of the PRISMA 2020 protocol (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) [28]. The objective was to identify, analyze, and synthesize scientific publications focused on bacterial immobilization matrices, with emphasis on the types of materials used, characterization methodologies, and their applications in environmental and industrial biotechnology.

2.1. Search Strategy

The bibliographic search was conducted in December 2024 in two scientific databases of high coverage and relevance: Scopus and PubMed. The search terms used were:

"cell immobilization" AND "bacterial" AND "matrix"

The results were limited to articles written in English, without restrictions by year of publication, in order to ensure broad temporal coverage. A total of 1,232 records were retrieved: 662 from Scopus and 570 from PubMed.

Before the screening process, the following were removed:

- Records older than five years: 491 from Scopus and 468 from PubMed.
- Duplicate records between both databases: 53 records eliminated using Mendeley.

After this initial filtering, 220 records were retained for evaluation.

2.2. Inclusion and Exclusion Criteria

The following inclusion criteria were applied:

- Original research articles (reviews, editorials, and conference abstracts were excluded).
- Studies specifically focused on bacterial cell immobilization using natural, synthetic, or composite matrices.
- Studies describing practical biotechnological applications, such as biocatalysis, biofertilization, or bioremediation.

During the evaluation process, the following were excluded:

- 28 records that were not original research articles (mainly reviews or short communications).
- 29 articles that were not available in full text.
- 69 articles that did not meet the thematic eligibility criteria, categorized as follows:
  - Not related to cell immobilization ($n = 25$): studies on general microbial processes or enzyme immobilization without bacterial involvement.
  - Focused on chemical matrices without biological interaction ($n = 29$).
  - Lacking relevance to biotechnology ($n = 15$): purely physical or theoretical studies without direct application.

Ultimately, 94 articles were selected that met all the established criteria.

The full process of identification, screening, exclusion, and eligibility is summarized in Figure 1 (PRISMA flow diagram).

2.3. Data Processing and Analysis

From each of the 94 selected articles, the following information was systematically extracted:

- Bibliographic data: title, authors, year of publication, country of affiliation, journal, and citation count.
- Technical content: type of matrix (biopolymers, lignocellulosic materials, inorganic materials), immobilization technique, bacterial strains used, characterization methods (SEM, FTIR), and biotechnological application.
- Keywords: for co-occurrence analysis and thematic trend evaluation.

The data were organized in Excel spreadsheets and categorized by type of matrix, method, application, and analytical technique. The analysis was performed using the following tools:

- Bibliometrix (in RStudio): to assess publication trends, keyword co-occurrence, and scientific output by country.
- Power BI: to generate the word cloud (Figure 6), based on the frequency of the most used terms.
- Python was used to create: Figure 2: annual scientific production graph (2019–2024). Figure 5: hierarchical clustering of terms.

- LaTeX was used to design the collaborative network diagrams (Figures 3a and 3b), distinguishing studies indexed in Scopus and PubMed.
- Excel was used to construct the geographic distribution map of scientific production (Figure 4) and to generate complementary statistical charts.
- To analyze thematic trends in the field of bacterial immobilization matrices, a hierarchical clustering analysis was performed using co-occurring author keywords extracted from the Scopus database. The most frequent terms were selected and processed applying Euclidean distance as the similarity measure and Ward's method for agglomerative linkage.

This methodological framework enabled a detailed and reproducible characterization of current trends, predominant topics, and knowledge gaps in the scientific literature on bacterial immobilization matrices.

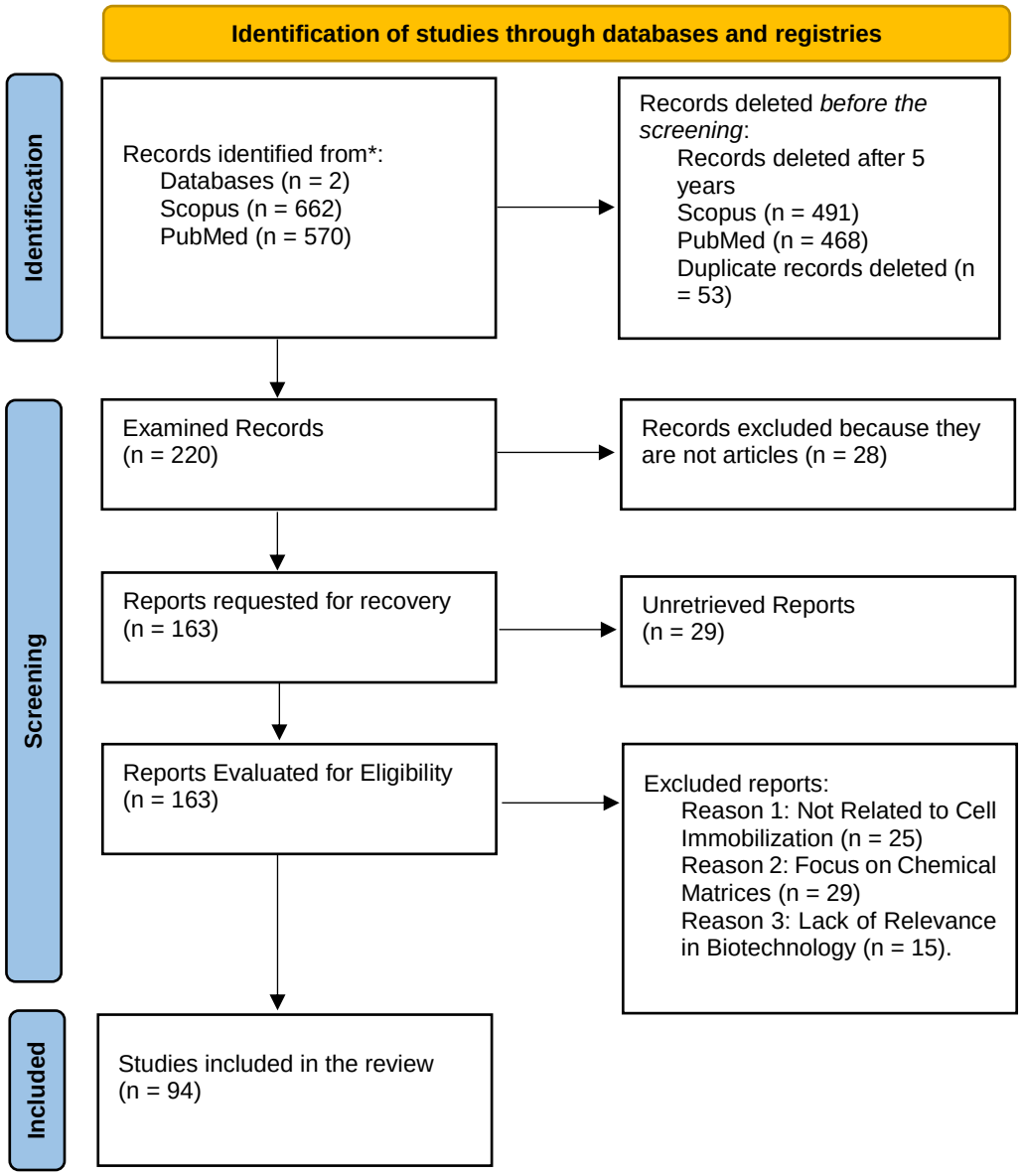

Figure 1. Prism diagram of the systematic review and reference selection process.

3. RESULTS

3.1. Annual Scientific Production

The analysis of the annual scientific production on cell immobilization matrices reveals a fluctuating trend in the number of publications throughout the period 2019-2024. A progressive decrease in the number of articles published from 2019 (19 articles) to 2023 (12 articles) is observed, suggesting a decrease in interest in or funding for research in this area during that period. However, in 2024, there is a rebound in production with a total of 19 publications, indicating a possible reactivation of the field or an increase in the relevance of research related to bacterial immobilization in biotechnology.

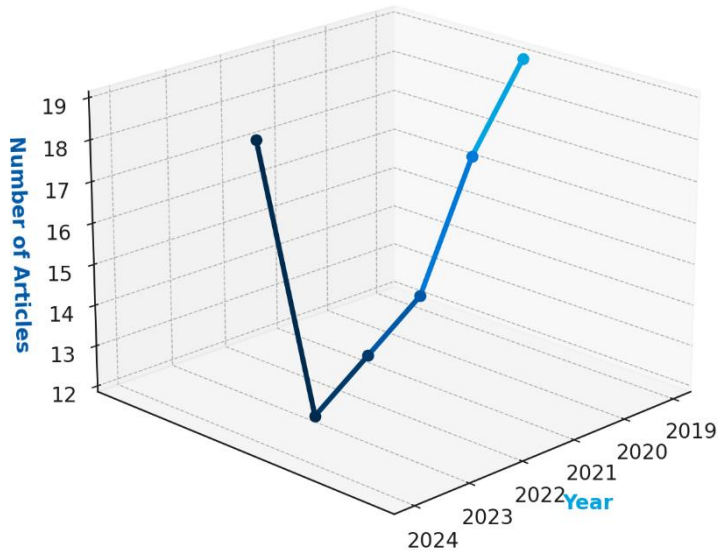

Figure 2. Annual scientific production.

The pattern of scientific production suggests that research on cell immobilization arrays has experienced periods of stabilization and decline, possibly due to changes in scientific priorities, the availability of resources, or the consolidation of previous knowledge. The low recorded in 2023 (12 articles) could be related to transitions in research strategies or the emergence of new areas of application that have diversified the focus of biotechnology studies. However, the increase in 2024 could be associated with advances in immobilization methodologies, new applications in industrial bioprocesses, and greater integration of these systems into emerging sectors such as bioremediation and environmental biotechnology.

These results highlight the importance of continuing to monitor scientific production in this field, as they reflect the impact and evolution of cell immobilization strategies in biotechnology. The upturn observed in 2024 suggests a possible consolidation of new lines of research or greater interdisciplinary collaboration in the development of innovative materials for the immobilization of microorganisms. This underscores the need to strengthen scientific collaboration networks and funding strategies to ensure sustained growth in this area of study.

3.2. Collaborative network

The analysis of the collaboration network in scientific production related to bacterial immobilization matrices (Figures 3a and 3b) reveals the structure and intensity of co-authorships within this field. The visualizations, generated from Scopus data, highlight the presence of

different well-defined research groups, each led by central authors with a large volume of publications and collaborative links.

In Figure 3a, a dense group of authors, such as Tarasov Se, Arlyapov Va, and Machulin Av, exhibits strong internal connectivity, suggesting the existence of a consolidated research group working intensively on bacterial immobilization technologies. The pattern observed in this network points to an institutional or regional concentration, with limited external links to other groups. On the other hand, Figure 3b illustrates a more diversified and internationally distributed network, with prominent nodes including Muñoz B, Chen W and Andres A. These authors appear to act as bridges between different groups, fostering transnational and multi-disciplinary collaboration

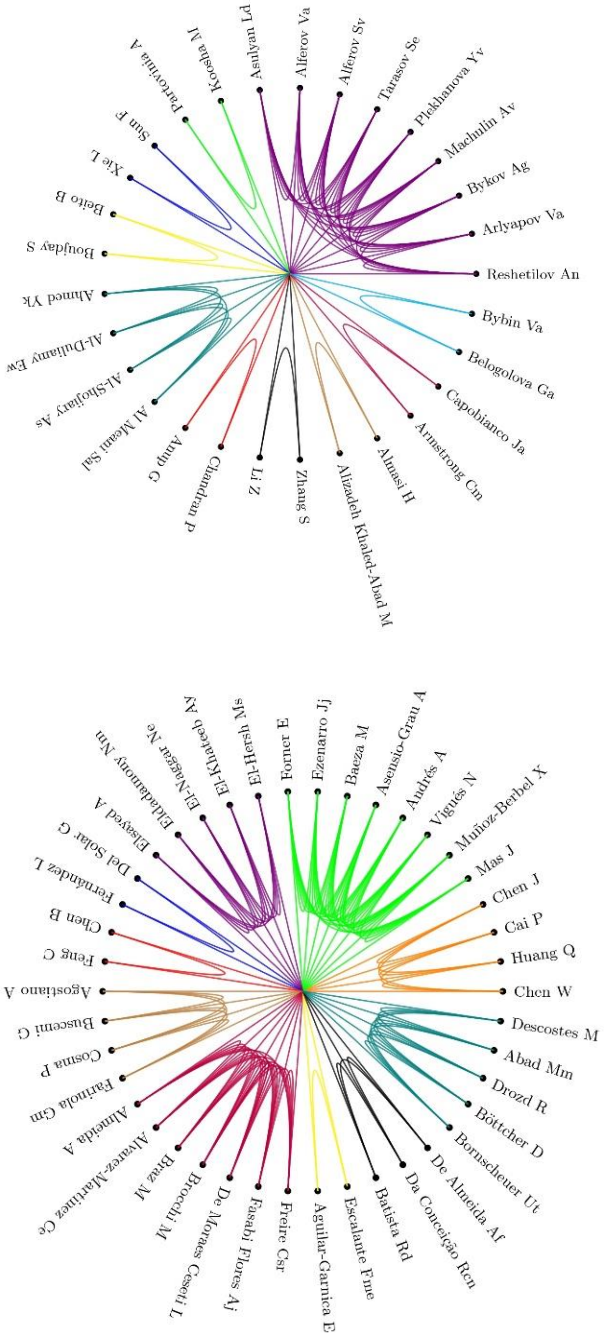

Figure 3a (*Scopus*) 3b (*PubMed*). Collaboration network between authors in research on bacterial immobilization matrices.

In Figure 3a, a dense group of authors, such as Tarasov Se, Arlyapov Va, and Machulin Av, exhibits strong internal connectivity, suggesting the existence of a consolidated research group working intensively on bacterial immobilization technologies. The pattern observed in this network points to an institutional or regional concentration, with limited external links to other groups. On the other hand, Figure 3b illustrates a more diversified and internationally distributed network, with prominent nodes including Muñoz B, Chen W and Andres A. These authors appear to act as bridges between different groups, fostering transnational and multidisciplinary collaboration.

Both networks show a significant presence of recurring authors, reflecting consistent academic contributions and the development of long-term lines of research. The connections observed between multiple institutions indicate a growing trend towards cooperative strategies and the integration of knowledge to address the complex challenges involved in microbial immobilization systems. This highlights not only the scientific maturity of the field but also the potential for greater global collaboration and innovation

3.3. Production by country

The analysis of scientific production by country in the field of bacterial immobilization, based on the Scopus and PubMed databases, Figure 1 reveals a heterogeneous geographical distribution with a strong concentration of publications in certain regions. China leads production with 26% of total publications, consolidating itself as the main contributor in this area. India, with 8.2%, and the United States, with 5.7%, also stand out as key actors in the generation of knowledge on the subject. These countries have extensive collaboration networks and a high impact on research applied to biotechnology and microbiology.

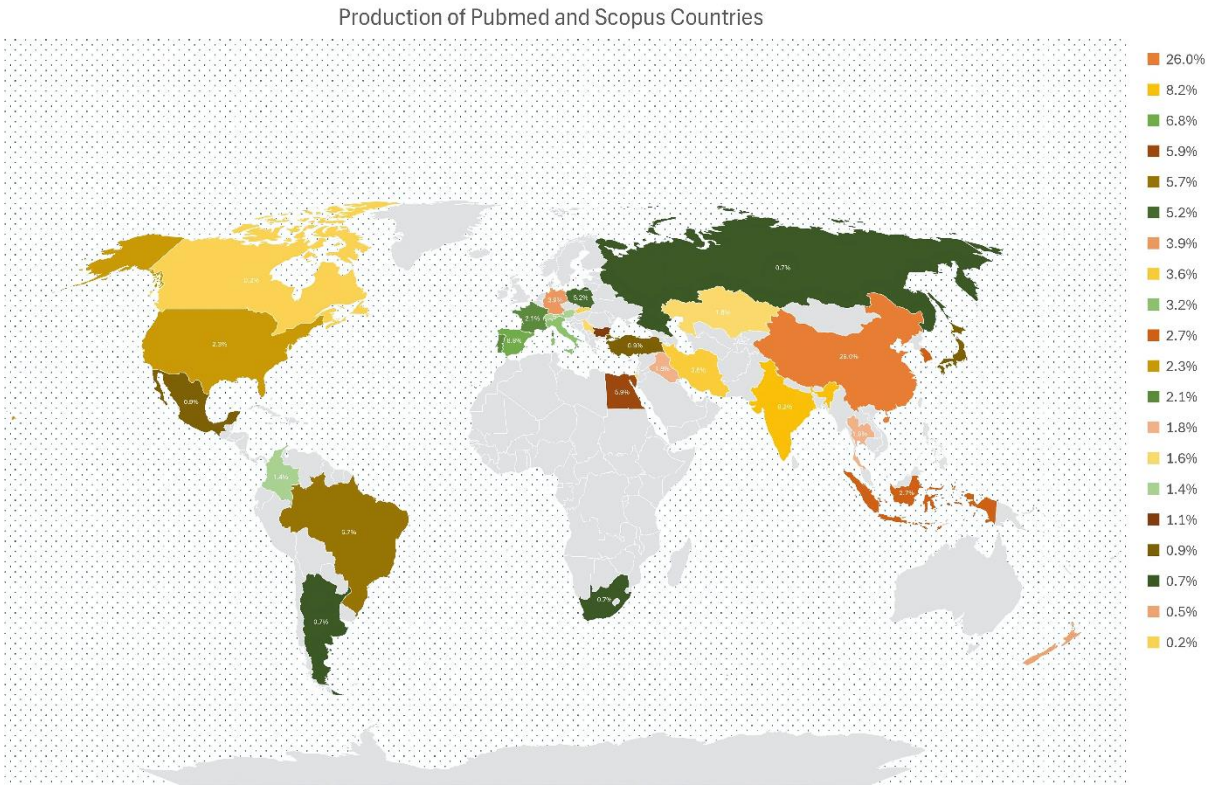

Figure 4. Geographical distribution of scientific production on bacterial immobilization matrices

In Latin America, Brazil is the country with the highest scientific production in this field, with 5.7% of total publications, followed by Argentina (0.7%) and Mexico (1.4%), reflecting a growth in biotechnology research in the region. In Europe, Germany (2.3%) and Italy (3.6%) appear as leaders in scientific contributions, followed by countries with lower production such as Spain and France. On the other hand, in the Middle East and Central Asia, Turkey (5.9%) and Russia (0.7%) have a relevant participation, while other nations show a lower representation in the development of research on bacterial immobilization matrices.

These results show a correlation between investment in biotechnology and scientific production, with highly industrialized countries and research funding policies leading the field. However, the presence of emerging countries on the list indicates an expansion of interest in bacterial cell immobilization in regions with biotechnological development potential. International cooperation and access to research infrastructures influence scientific production capacity, highlighting the importance of strengthening global collaboration networks to advance the biotechnological application of these technologies

3.4. Hierarchical grouping of terms in research on cell immobilization matrices

Hierarchical clustering analysis applied to the most relevant terms in cell immobilization matrix research (Figure 5) reveals interconnectivity and thematic trends in the field. The dendrogram shows the relationship between keywords, establishing groups based on Euclidean distance. It is observed that terms related to matrix types, such as *chitosan*, *sodium alginate*, and *polyvinyl alcohol*, are grouped into specific clusters, indicating their frequent use and their importance in scientific literature. These polymers have been extensively studied for their ability to provide an optimal environment for bacterial immobilization, reinforcing their relevance in biotechnology.

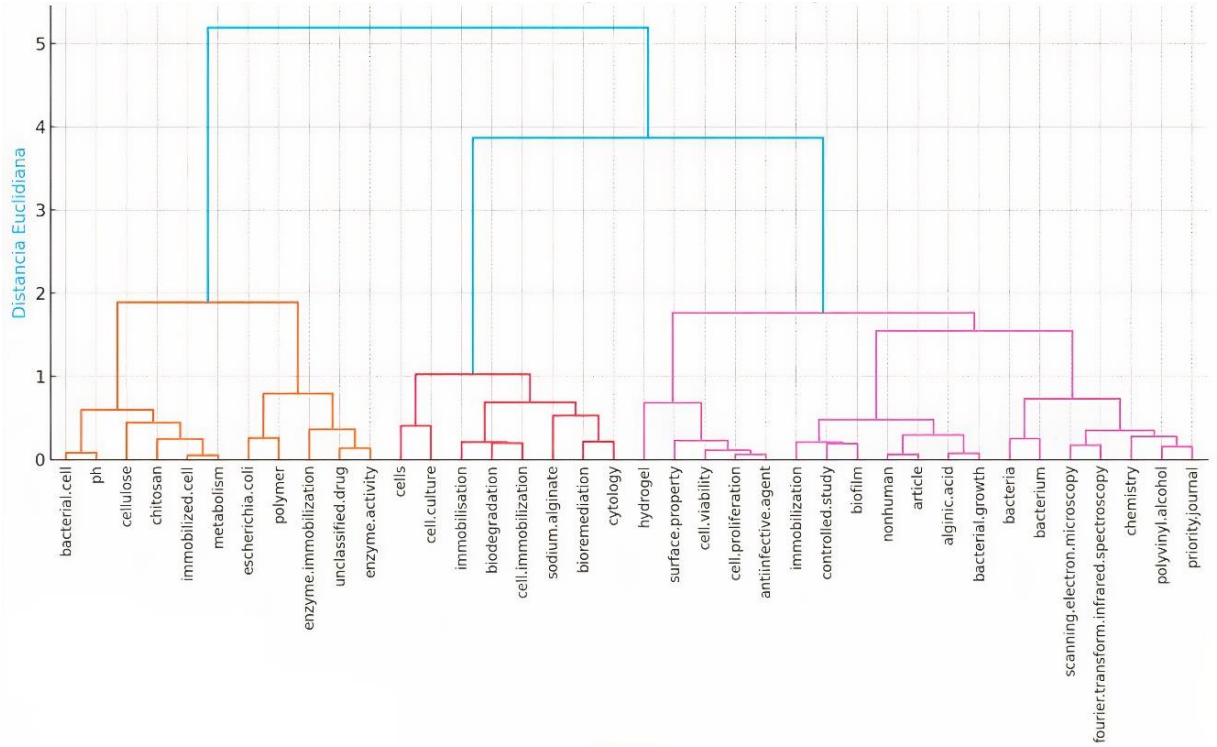

Figure 5. Analysis of hierarchical grouping of terms in research on cell immobilization matrices.

Another significant group of terms relates to the functionality and applications of immobilization matrices. Concepts such as *bioremediation*, *enzyme activity*, *bacterial growth* and *biofilm* appear in groupings that reflect the applied approach of research in this field. The strong relationship between cell immobilization and biocatalysis, evidenced by the proximity of terms such as *enzyme immobilization* and *metabolism*, suggests a continued interest in the use of immobilized systems to optimize enzymatic reactions and improve the efficiency of biotechnological processes.

Likewise, the dendrogram highlights the link between methodological and characterization terms, such as *scanning electron microscopy* and *Fourier transform infrared spectroscopy*, suggesting that these analytical methods are essential for the structural and functional evaluation of immobilization matrices. The presence of these terms in a separate group indicates that their application is transversal to different approaches within the study of cell immobilization, from the physicochemical characterization of supports to the evaluation of cell viability in immobilized systems.

In general, the clustering analysis suggests that research in cell immobilization is segmented into three main areas: the materials used, biotechnological applications and characterization methods. This classification allows for a structured visualization of trends in scientific production and suggests the need to continue exploring new combinations of matrices and methodologies to improve the efficiency of biotechnological processes that depend on cell immobilization.

3.5. Word Cloud

Figure 5 reveals that the most frequent term is immobilization, indicating that research in this field focuses on the use of methods to fix microorganisms in various matrices in order to improve their stability and efficiency. Along with this term, controlled study and bioremediation appear, suggesting that a large number of studies in this area are developed under controlled conditions and are aimed at environmental applications, such as the remediation of contaminated soils and waters.

Figure 6. Word cloud based on a scientometric approach to trends in bacterial immobilization research.

In addition, the prominent presence of terms such as biodegradation, adsorption, and cellulose, indicates that the degradation of compounds by microbial processes is a key focus in the literature analyzed. The inclusion of Escherichia coli, Staphylococcus aureus, and "Pseudomonas", along with words related to bacterium and bacterial cell, demonstrates that multiple bacterial genera have been studied in these investigations, due to their ability to produce biopolymers or degrade toxic compounds.

The appearance of terms such as polyvinyl alcohol, alginate, chitosan, and bentonite, suggests that the materials used for immobilization include natural and synthetic polymers. This reflects a trend towards the use of biopolymers and materials with adsorption and retention properties, which is crucial to improve the effectiveness of microorganisms in biotechnological and environmental applications

4. DISCUSSION

The scientometric analysis carried out on bacterial immobilization matrices has allowed the identification of key trends in research in this field, as well as the main associated biotechnological applications. The results reflect a constant development in this area, with variations in annual scientific production that may be influenced by the focus of research [29]. The identification of the most commonly used materials, emerging applications, and most commonly employed methodological approaches provides a comprehensive view of the state of the art in bacterial immobilization [30].

Bacterial immobilization has proven to be a versatile strategy in various biotechnology sectors, including bioremediation, biomolecule production, and biocatalysis [31]. The high frequency of terms such as immobilization, bioremediation and biodegradation in word cloud analysis suggests that the main efforts in this field are aimed at the application of immobilized microorganisms for the degradation of polluting compounds and wastewater treatment [32]. The consolidation of these approaches has been supported by the growing evidence that cell immobilization not only improves the stability and viability of microorganisms, but also increases the efficiency of biotechnological processes by providing a controlled microenvironment that favors their metabolic activity [33].

Analysis of the matrices used in bacterial immobilization shows that natural and synthetic polymers play a central role in this technology. Materials such as alginates, chitosan and polyvinyl alcohol have been widely used due to their biocompatible properties, their ability to form structured gels and their chemical stability [34]. The prominent presence of terms such as polyvinyl alcohol, alginate and chitosan in scientometric analysis reinforces the relevance of these materials in scientific literature and their potential for industrial and environmental applications [35]. In addition, the use of lignocellulosic residues and agricultural by-products as support for bacterial immobilization aligns with the principles of the circular economy, providing sustainable and low-cost alternatives that have shown promising results in bioremediation processes and bioproduct production [36].

From a methodological point of view, the characterization of matrices and immobilized bacteria has evolved significantly in recent years. The hierarchical grouping of terms has shown that analytical techniques such as scanning electron microscopy (SEM) and Fourier transform infrared spectroscopy (FTIR) are fundamental tools for assessing the morphology and chemical composition of immobilized systems [37]. The importance of these methods in the literature suggests that the structural and functional characterization of the supports is a key aspect to optimize their performance in biotechnological applications [38].

On the other hand, the geographical distribution of scientific output in this field indicates that China, India, and the United States are leading research on bacterial immobilization matrices, which can be attributed to their investment in biotechnology and the number of research institutions specializing in microbiology and materials science [39]. In Latin America, Brazil is positioned as the country with the highest production in this area, reflecting a growth in the development of biotechnological technologies with applications in agriculture and the environment [40]. However, the lower participation of other countries in this area suggests the need to strengthen international cooperation and investment in research infrastructure to consolidate innovation in immobilization bacterial.

The analysis of collaborative networks reveals that studies in this area tend to be grouped around certain authors and institutions with a strong interconnection in Scopus, while in PubMed a lower density of interactions is observed. This may indicate differences in the focus of the publications indexed in each database, with Scopus being more inclined towards applied and multidisciplinary studies, while PubMed contains research with a more biomedical focus [41]. The presence of well-established collaborative networks in the literature suggests that bacterial immobilization is a field that benefits from interdisciplinary cooperation, integrating knowledge from microbiology, materials science, chemistry, and biotechnology [42].

5. CONCLUSION

The scientometric analysis carried out in this study has made it possible to identify the key trends in research on bacterial immobilization matrices, highlighting their relevance in various biotechnological applications, such as bioremediation, biodegradation and biocatalysis. The assessment of scientific output suggests that while there have been fluctuations in the number of publications in recent years, the growing interest in innovative materials and advanced immobilization techniques could drive new research in the field. The strong presence of natural and synthetic polymers such as alginates, chitosan and polyvinyl alcohol in the reviewed literature highlights the importance of these materials in the development of efficient systems for the stabilization and control of microbial activity in industrial and environmental environments. Likewise, the analysis of collaboration networks and geographical distribution of scientific production shows that research in this field is highly cooperative and dominated by countries with a strong investment in biotechnology, which underlines the need to strengthen global cooperation networks in this area.

The results of this study also show the increasing diversification of methodologies used to evaluate the efficacy of immobilization matrices, including electron microscopy techniques, infrared spectroscopy, and biocompatibility analysis. This trend suggests that the integration of multidisciplinary approaches will be key to the optimization of these systems in the future. Given the potential of bacterial immobilization in emerging applications, such as bioenergy and biomaterials production, it is critical to continue monitoring the development of new strategies and assessing their long-term impact on industrial and environmental biotechnology. Finally, it is recommended to promote greater collaboration between research groups from different countries and disciplines, as well as to strengthen financing policies in biotechnology, in order to promote innovation and maximize the benefits of these technologies in different productive sectors.

CONFLICT OF INTEREST

The authors do not declare a conflict of interest.

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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, δ, 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.

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