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Chemical characterization of Copaiba essential oil and study of its cellular cytotoxicity

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Summary

Introduction: copaifera is a genus of plants that comprises several species that produce copaiba oil, which is widely used for various purposes, such as healing, anti-inflammatory, antimicrobial, wound antiseptic, anti-tumor, among other functions. There are very few chemical studies to characterize copaiba oil. **Aim:** To characterize 3 different species of copaiba oil Subsequently, the effects of oils were estimated on neoplastic cells in a human glioma protocol (U251). **Methods**: 2 methods of analysis were used for the chemical characterization, GC-MS and ESI-MS. **Results:**

Through these analytical techniques, 20 types of components were found in the oils. Obtaining the cell viability and cytotoxicity of the oil we performed two methods, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and Neutral Red (NR), both assays were quantified by spectrophotometry. Concentrations of 1 to 10^{-5} μg/mL were used for 3 different species of copaiba oil and doxorubicin hydrochloride was used as a positive control. The 3 different species of copaiba oil from Pernambuco (oil 1) and Manaus (oils 2 and 3) were cytotoxic in U251, the IC_{50} value obtained was $6.171 \cdot 10^{-2}$ μg/mL, $8.344 \cdot 10^{-2}$ μg/mL and $1.385 \cdot 10^{-4}$ μg/mL, respectively. They also presented cytotoxic effect, with IC_{50}, $2.4 \cdot 10^{-1}$ μg/mL, $3.7 \cdot 10^{-2}$ μg/mL and $4.6 \cdot 10^{-2}$ μg/mL, respectively. A high correlation was evidenced between the MTT and Neutral Red studies for the three different species of copaiba oil, with α=0.05 and r values above 0.9, using the Pearson correlation coefficient. All types of resin oils positively affected cell proliferation of *in vitro* studies with a correspondence between concentration and effect.

Keywords: Copaiba oil, cytotoxicity and cell viability, human glioma.

Resumo

Caracterização química do óleo essencial de copaíba e estudo de sua citotoxicidade celular

Introdução: copaifera é um gênero de plantas que compreende diversas espécies produtoras de óleo de copaíba, que é amplamente utilizado para diversas finalidades, como cicatrizante, anti-inflamatório, antimicrobiano, antisséptico de feridas, antitumoral, entre outras funções. Existem poucos estudos químicos para caracterizar o óleo de copaíba. **Objetivo:** caracterizar 3 espécies diferentes de óleo de copaíba foram caracterizadas neste trabalho. Posteriormente, os efeitos dos óleos foram estimados em células neoplásicas em um protocolo de glioma humano (U251). **Métodos:** utilizamos 2 métodos de análise para a caracterização química, GC-MS e ESI-MS. **Resultados:** por meio dessas técnicas analíticas, 20 tipos de componentes foram encontrados nos óleos. Obtendo a viabilidade celular e a citotoxicidade do óleo realizamos dois métodos, o MTT (brometo de 3-(4,5-dimetiltiazol-2-il)-2,-5-difeniltetrazólio) e o Vermelho Neutro (NR), ambos os ensaios foram quantificados por espectrofotometria. Concentrações de 1 a 10^{-5} μg/mL foram usadas para 3 espécies diferentes de óleo de copaíba e cloridrato de doxorrubicina foi usado como controle positivo. As 3 espécies diferentes de óleo de copaíba de Pernambuco

(óleo 1) e Manaus (óleos 2 e 3) foram citotóxicas em U251, o valor de IC_{50} obtido foi de $6{,}171 \cdot 10^{-2}$ µg/mL, $8{,}344 \cdot 10^{-2}$ µg/mL e $1{,}385 \cdot 10^{-4}$ µg/mL, respectivamente. Também apresentaram efeito citotóxico, com IC_{50}, $2{,}4 \cdot 10^{-1}$ µg/mL, $3{,}7 \cdot 10^{-2}$ µg/mL e $4{,}6 \cdot 10^{-2}$ µg/mL, respectivamente. Foi evidenciada uma alta correlação entre os estudos de MTT e Vermelho Neutro para as 3 diferentes espécies de óleo de copaíba, com valores de α=0,05 e r acima de 0,9, utilizando o coeficiente de correlação de Pearson. Todos os tipos de óleos de resina afetaram positivamente a proliferação celular de estudos in vitro com uma correspondência entre concentração e efeito.

Palavras-chave: Óleo de copaíba, citotoxicidade e viabilidade celular, glioma humano.

Resumen

Caracterización química del aceite esencial de Copaiba y estudio de su citotoxicidad celular

Introducción: copaifera es un género de plantas que comprende varias especies que producen aceite de copaiba, el cual es ampliamente utilizado para diversos fines, como cicatrizante, antiinflamatorio, antimicrobiano, antiséptico de heridas, antitumoral, entre otras funciones. Hay muy pocos estudios químicos para caracterizar el aceite de copaiba. **Objetivo**: caracterizar 3 especies diferentes de aceite de copaiba. Posteriormente, se estimaron los efectos de los aceites sobre las células neoplásicas en un protocolo de glioma humano (U251). **Métodos**: utilizamos 2 métodos de análisis para la caracterización química, GC-MS y ESI-MS. **Resultados**: a través de estas técnicas analíticas, se encontraron 20 tipos de componentes en los aceites. Para la obtención de la viabilidad celular y citotoxicidad del aceite se realizaron dos métodos, el MTT (3-(4,5-dimetiltiazol-2-il)-2,5-difeniltetrazolio bromuro) y el Rojo Neutro (NR), ambos ensayos fueron cuantificados por espectrofotometría. Se utilizaron concentraciones de 1 a 10^{-5} µg/mL para 3 especies diferentes de aceite de copaiba y se utilizó clorhidrato de doxorrubicina como control positivo. Las 3 especies diferentes de aceite de copaiba de Pernambuco (aceite 1) y Manaus (aceites 2 y 3) fueron citotóxicas en U251, el valor de IC_{50} obtenido fue de $6{,}171 \cdot 10^{-2}$ µg/mL, $8{,}344 \cdot 10^{-2}$ µg/mL y $1{,}385 \cdot 10^{-4}$ µg/mL, respectivamente. También presentaron efecto citotóxico, con IC_{50}, $2{,}4 \cdot 10^{-1}$ µg/mL, $3{,}7 \cdot 10^{-2}$ µg/mL y $4{,}6 \cdot 10^{-2}$ µg/mL, respectivamente. Se evidenció una alta correlación entre los estudios MTT y Neutral Red para las 3 diferentes especies de aceite de copaiba, con α=0,05 y valores de r superiores a

0,9, utilizando el coeficiente de correlación de Pearson. Todos los tipos de aceites de resina afectaron positivamente la proliferación celular de estudios in vitro con una correspondencia entre concentración y efecto.

Palabras clave: Aceite de copaiba, citotoxicidad y viabilidad celular, glioma humano.

Introduction

The study of medicinal plants began a long time ago, going through generations until nowadays, due to its easy access, low cost and compatibility with popular traditions. The commercialization of medicines of plant origin has increased due to scientific advances in the chemical, pharmacological and toxicological areas [1]. Given this context, copaiba oils are widely used due to their many properties, such as medicinal, cosmetic and industrial [2].

The copaiba plants belong to the family Leguminosae, subfamily Caesalpinoideae, genus Copaifera [3, 4]. They are common to Latin America and West Africa, and usually live about 400 years, reaching heights between 25 and 40 meters [5, 6]. In Brazil, they are in the Southeast, Central-West and Northern regions, mainly covering the states of Amazonas, Pará and Ceará [7-9]. There are 72 described species of Copaifera, more than 20 of them are in Brazil, among which 17 are endemic. The main species are: *Copaifera officinalis L.*, *Copaifera reticulata Ducke*, *Copaifera multijuga Hayne*, *Copaifera confertiflora*, *Copaifera langsdorffii*, *Copaifera cariacea* and *Copaifera cearensis Huber ex Ducke* [10, 11].

The oil from these trees — copaiba oil or balm — varies in yellow gold to brown coloring, depending on the species. The oil is extracted by making an incision in the trunk of the tree, it is composed of a solid, non-volatile resinous part formed by diterpene acids, corresponding to 55 to 60% of it, diluted in the other part, an essential oil, composed of sesquiterpenes [12-15]. This oil composition varies depending on the concentration and nature of the diterpenes and sesquiterpenes present, according to species variation, biological factors and abiotic factors [13-15]. Some of the active components in these oils are β-bisabolol and β-caryophyllene, both with anti-inflammatory action, in addition to bactericidal action in the second [16].

Indigenous populations of Brazil's Northern and Northeast regions have used the oil to treat various diseases since the 16th century [17]. Nowadays, it is popularly used as a wound healer, anti-inflammatory, antiseptic in wounds, antitumoral, eczemas, psoriasis and urticaria, anti-inflammatory of the urinary tract, in pulmonary affections (coughs,

pneumonia, bronchitis, antiasthmatic, colds and flu), sinusitis, scarring of small irritations of the scalp, dysentery, urinary incontinence, cystitis, leucorrhoea, analgesic and antitetanic [18-22].

Despite being used to treat various diseases, there are no studies on the effects of copaiba oil on gliomas. Gliomas are part of the group of neuroepithelial tumors that correspond to 31% of primary tumors (neuroepithelial, meninges, cranial and paraspinal nerves, germinative, sellar region and hematopoietic) and 80% of malignant tumors on the central nervous system (CNS) [23] Among 1% of all neoplasms, it can be seen that this type of tumor in adults is responsible for about 2% of deaths attributed to malignant gliomas. In glioblastoma multiforme, the more aggressive type, the average survival time of patients is approximately one year after diagnosis, regardless of the strategy used in the treatment [24].

This study analyzed and characterized 3 types of oil from different species of copaiba tree from different regions of Brazil through gas chromatography with mass spectrometry detector (GC-MS) and easy ambient sonic-spray ionization with mass spectrometry detection mass spectrometry (ESI-MS). Evaluating cell viability and oil cytotoxicity in human glioma cells (U251).

Materials and methods

We studied 3 different species of copaiba, one from the region of Pernambuco (1), and two others from the region of Manaus (2 and 3), all from Brazil. The Research Ethics Committee of the State University of Campinas (UNICAMP) approved this study through protocol number 2414-1.

Chemical Analysis

We used two methods to perform the GC-MS and ESI-MS.

Esi-ms

For this study, the spectra were obtained in the negative mode, using a mass spectrometer with Orbitrap mass analyzer, Q Exactive™ Hybrid Quadrupole-Orbitrap equipped with an ESI source (Thermo Scientific, San Jose, USA). The conditions of analysis were resolution (140,000); spray voltage (3.5 kV); 3 micro-scans per spectrum; capillary temperature (275 °C). The Xcalibur software was used to process the mass spectra (version 2.0, Service Release 2, Thermo Electron Corporation).

CG-MS

GC-MS analyzes were performed using a gas chromatograph (HP6890) with a mass spectrometry detector (HP5975) equipped with a HP5-MS fused silica capillary column (30 m × 0.25 mm × 0.25 μm). The carrier gas was helium with flow rate of 1 mL per minute. The injector and detector temperatures were 220 and 290 °C, respectively. The injection volume was 1 μL and the samples were at a concentration of 15 mg/mL. The compounds were identified from their retention index in the NIST library database, using a homologous series of n-alkanes (C8-C22).

Cytotoxicity and Cell Viability Assay

We performed two tests to determine the cytotoxicity and cell viability of the 3 samples of copaiba oils in the glioma cell line (U251), the MTT assay and Neutral Red. The experiments were performed six times and the results are presented as mean ± standard deviation. The positive control drug was doxorubicin hydrochloride.

Cultivation of the Cell Line, U251

The U251 human cell line (glioma) was kindly given by the CPQBA - UNICAMP. We cultured the samples of this strain in cell culture flasks (75 cm^3) containing RPMI 1640 (R6504, Sigma) supplemented with 10% fetal bovine serum (16000-044, Gibco), 2% Glutamax (35050-061, Gibco), 1% sodium pyruvate (S8636, Sigma) and 0.2% Pen Strep (15070-073, Gibco). The cells were incubated at an initial concentration of 3-10×10^5 cells/mL and maintained at 37 °C in a humified atmosphere of 5% CO_2. When they reached 70-80% confluency, the cells were used for cytotoxicity and cell viability assays. The cell lines were stored in N_2.

MTT

Protocol [24]. The U251 cells (9×10^5 cells/well) were distributed in 96-well plates. The cell lines were treated with copaiba oil using 10^{-5} μg/mL concentrations. The cells were incubated for 72 hours with copaiba oil, then the plates were centrifuged at 2000 rpm for 5 minutes. The supernatant was removed, and MTT (Sigma, M5665) 5 mg/mL dissolved in PBS buffer (Sigma P4417) was added. The plates were incubated for 4 hours at 37 °C in a humified atmosphere of 5% CO_2. The plates were centrifuged again after this period, the supernatant was removed, and the formazan crystals were dissolved in 150 μL of isopropyl alcohol. The absorbance values were obtained using a Synergy ELISA plate reader (Bio Tek Instruments, Highland Park, Winooski, USA) at 570 nm. We expected 100% cell viability in the negative control.

Neutral Red

Protocol [25]. The U251 cells were distributed in 96-well plates as described on the MTT protocol. The cells were incubated for 72 hours with copaiba oil, the plates were also centrifuged at 2000 rpm for 5 minutes. The supernatant was removed, and the 10% NR (Sigma, N7005) dissolved in culture medium was added. The plates were incubated for 2 hours at 37 °C in a humified atmosphere of 5% CO_2. After this procedure, the plates were centrifuged again, the supernatant was removed, and the cells were subjected to a developing solution prepared with 50% ethanol, 1% acetic acid and distilled water. The absorbance values were obtained using a Synergy ELISA plate reader (Bio Tek Instruments, Highland Park, Winooski, USA) at 540 nm. We expected 100% cell viability in the negative control.

Figure 1 shows both assays described above for a better understanding:

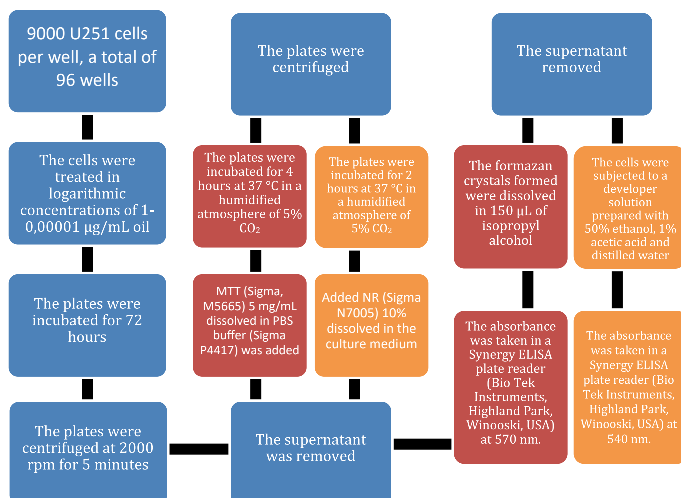

Figure 1. Scheme of tests: steps common for both are in blue, the steps of MTT are in red and the steps of Neutral Red are in orange [24, 25].

Results

Chemical analysis

The ESI-MS spectra below present the chemical fingerprint of all 3 oils in the negative mode (Figure 2).

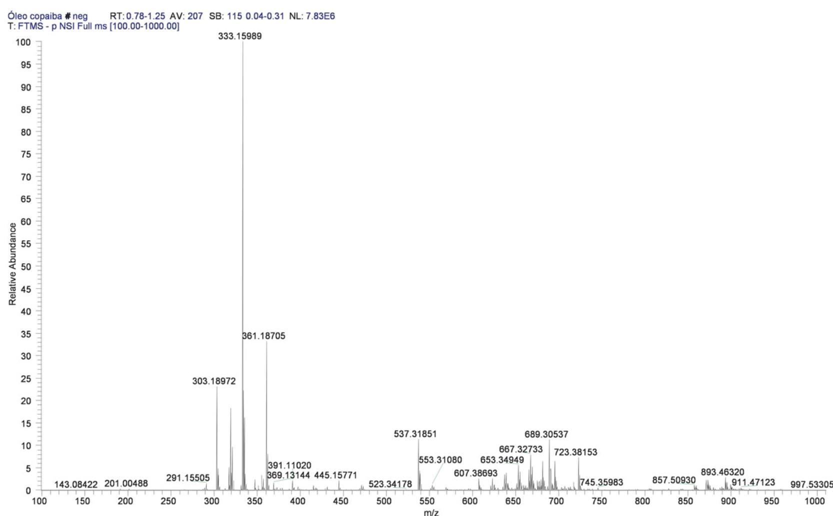

Figure 2. Spectrum of all 3 oils.

Our analysis of the spectra was based on a review study and tables 1 and 2 show the compounds identified in the oils through ESI-MS and GC-MS methods [21]:

Table 1. Compounds found in copaiba oils by the ESI-MS method.

Initials	Compounds	m/z	Oil 1	Oil 2	Oil 3
1	3-clerodane-15, 18-dioecious acid	335.17398	X		X
2	13-clerodane-15, 16-ylide-18-oic acid	333.15989		X	
3	clerodane-15, 16- dioecious acid	337.24000			X
4	*ent*-15, 16-epoxy-3, 13 (16), 14-clerodatriene-18-oic (Hardwickiic acid)	315.15045	X		
5	15, 16-epoxy-7β-acetoxy-3, 13 (16), 14-clerodatriene-18-oic acid	373.12381	X	X	
6	*ent*-15,16-epoxy-8 (17), 13 (16), 14-labdatriene-18-oic acid (Polyaltic acid)	315.15045	X		
7	18-hydroxy-8 (17), 13-labdadiene-15-oic acid (Copaiferolic acid)	319.18406		X	

(*Continued*)

Initials	Compounds	m/z	Oil 1	Oil 2	Oil 3
8	*ent*-11-hydroxy-labda-8 (17), 13-diene-15-oic acid (11-hydroxy-copalic acid)	319.18406		X	
9	*ent*-3-hydroxy-labda-8 (17), 13-diene-15-oic acid	319.18406		X	
10	*ent*-8 (17), 13-labdadiene-15, 19- dioecious acid (*ent*-agatic acid)	333.15989		X	X
11	*ent*-8 (17)-labdeno-15,18-dioecious acid (eperu-8 (20)-15, 18- dioecious acid)	335.17398	X		X
12	*ent*-8 (17)-13E-labdadiene-15-oic acid (Copalic acid)	303.23109		X	X
13	*ent*-11-acetoxy-8 (17)-13E-labdadiene-15-oic (11-acetoxy-copalic acid)	361.18884		X	X
14	Kaur-16-en-18-oic acid	302.17212	X		

Table 2. Compounds found in copaiba oils by the GC-MS method.

Initials	Compounds	MM	Oil 1	Oil 2	Oil 3
1	Kaur-16-ene	272	X		
2	Cativic acid	306	X		
3	Polyaltic acid	316	X		
4	3-acetoxy-copaiferic acid	362		X	X
5	Copalic acid	304		X	X
6	16-beta-Kauran-18-oic acid	304	X		
7	Kaur-16-en-18-oic acid	302	X		
8	Pinifolic acid	336	X		
9	Agathic acid	334		X	X

Cytotoxicity and Cell Viability

The mean and standard deviation were calculated with the values found in the experiment, and from them we obtained the cell viability graphic and the IC_{50} value, which represents the required concentration of each oil to cause the death of 50% of the treated cells, as shown in the Figures 3 and 4 and Table 3.

MTT

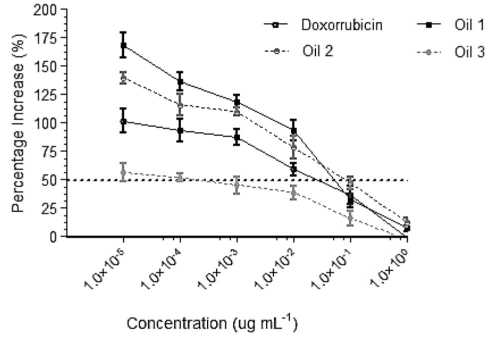

Figure 3. Graph of cell viability after 72 hours of treatment using Copaifera spp oil in a human glioma cell line (U251) for the MTT assay.

Neutral Red

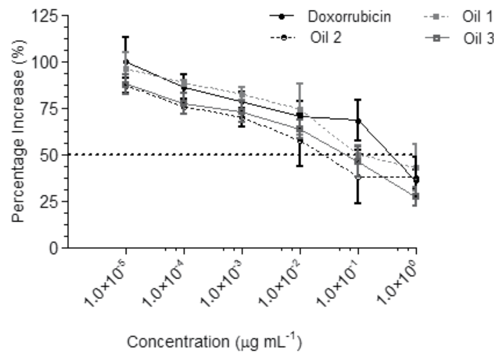

Figure 4. Graph of cell viability after 72 hours of treatment using Copaifera spp oil in a human glioma cell line (U251) for the NR test.

Table 3. IC_{50} values of each copaiba oil sample after 72 hours.

Assay	IC_{50} (μg/mL)			
	Oil 1	**Oil2**	**Oil 3**	**Doxorubicin**
MTT	0.06171	0.08344	0.0001385	0.02266
Neutral Red	0.2396	0.03769	0.04624	0.3426

Correlation between MTT and Neutral Red Test

There was a significant correlation between the MMT and Neutral Red assays (Figure 5). We used the Pearson correlation coefficient to establish the correlation (Table 4).

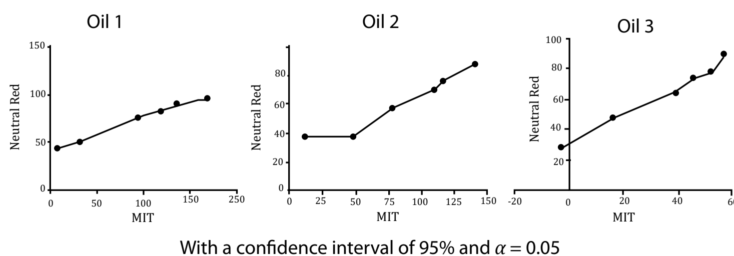

Figure 5. Graphs of the MTT x Neutral Red assays for oils 1, 2, and 3.

Table 4. Pearson correlation coefficients for Oil 1, Oil 2, and Oil 3.

Coefficients	Oil 1 value	Oil 2 value	Oil 3 value
Pearson r	0.9972	0.9723	0.9934
Confidence Interval (95%)	0.9735 to 0.9997	0.7621 to 0.9971	0.9379 to 0.9993
p-value (two-tailed)	p<0.0001	0.0011	p<0.0001

The results show α = 0.05 and r values greater than 0.9, which indicates a strong correlation between both methods used. Despite being used to assess the viability/ cytotoxicity of cells, each method we used needs different processes to measure this characteristic.

Photomicrographs

The photomicrographs below show the effects of all oils on the U251 cells at a concentration of 10^{-1} μg.mL^{-1} after 72 hours of treatment (Figure 6).

We can observe that the treated cells suffered intense nuclear pleomorphism, show little cohesiveness, nuclear pyknosis, and sometimes scant cytoplasm, dense chromatin, and nuclear atrophy. Cell death can also be observed in photomicrographs.

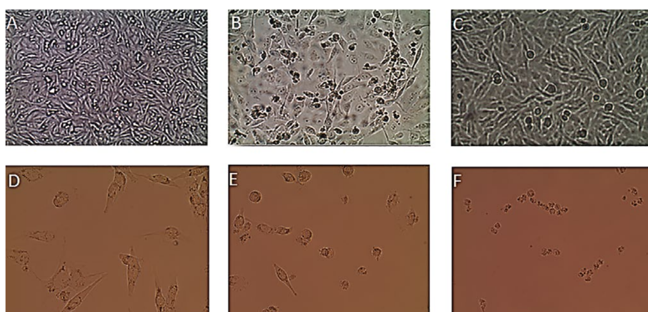

Figure 6. Effects of the copaiba oils. A) Control (untreated cells); B) Doxorubicin (10^{-1}μg.mL^{-1}); C) Copaiba oil 1 (10^{-1} μg·mL^{-1}); D) Copaiba Oil 2 (10^{-1} μg·mL^{-1}); E) Copaiba oil 3 (10^{-1} g·mL^{-1}); F) Copaiba Oil 3 (1 μg·mL^{-1}). (Light microscopy, Olympus brand). 200x.

Discussion

We found a total of 23 compounds through the chromatography methods used, 14 through ESI-MS and 9 through GC-MS. 3 compounds are common to both techniques, Polylactic acid, Copalic acid, and kaur-16-en-18-oic acid. Gas chromatography identifies gases of lower molar mass, the sesquiterpenes, while ESI-MS identifies those of greater molar mass, the diterpenes. The number of compounds found in Oil 1 was 10, also 10 in Oil 2 and 8 in Oil 3.

The MTT assay consists of reducing yellow-colored tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide — Sigma M2128) through the succinic dehydrogenase of the enzyme present in the mitochondria of the tumor cell, which acquires a purplish color, is measured spectrophotometrically at 570 nm [25].

The Neutral Red test is based on the ability of viable cells to incorporate the dye in the lysosomes; thus, the red staining is evaluated by spectrophotometry at 540 nm.

Oil 3 presented the lowest IC_{50} value in the MTT assay, which was lower than the positive control, followed by Oil 1 and Oil 2, however, both presented greater values than doxorubicin.

For the Neutral Red test, all 3 oils presented IC_{50} values lower than the positive control, Oil 2 presented the best value.

Conclusion

This study allowed us to observe some characteristics of copaiba oil and some of its compounds and its effects on tumor cells. Despite having different compositions, all 3 oils affected the cell line U251 positively. The results from the cell viability tests show slight differences regarding the IC_{50} values of Neutral Red versus MTT assays for the 3 types of tested oils, which suggests that the oil interferes at the concentrations studied with cellular metabolic activity and integrity of the liposome membrane.

Acknowledgement

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Conflict of interest

The authors report no conflict of interest associated with this study.

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