Vol.64: e21210109, 2021
https://doi.org/10.1590/1678-4324-2021210109
ISSN 1678-4324 Online Edition

Article - Human and Animal Health

Eucalyptus grandis and *Eucalyptus microcorys* Essential Oils: *in vitro* Modulation of Hemostasis and Genotoxic Activity

Mariana Araújo Esposito[1]
https://orcid.org/ 0000-0002-6724-8321

Pedro Henrique Souza Cesar[1]
https://orcid.org/0000-0003-3271-7797

Rafaela Magalhães Brandão[1]
https://orcid.org/ 0000-0002-2925-6961

Tatiane Silva de Abreu[1]
https://orcid.org/ 0000-0002-3809-5442

Silvana Marcussi[1]*
https://orcid.org/0000-0002-4674-6911

[1]Federal University of Lavras, Department of Chemistry, Lavras, Brazil.

Editor-in-Chief: Alexandre Rasi Aoki
Associate Editor: Daniel Fernandes

Received: 2021.02.26; Accepted: 2021.06.18.

*Correspondence: marcussi@ufla.br; Tel: +55-35-38291622. (S.M.)

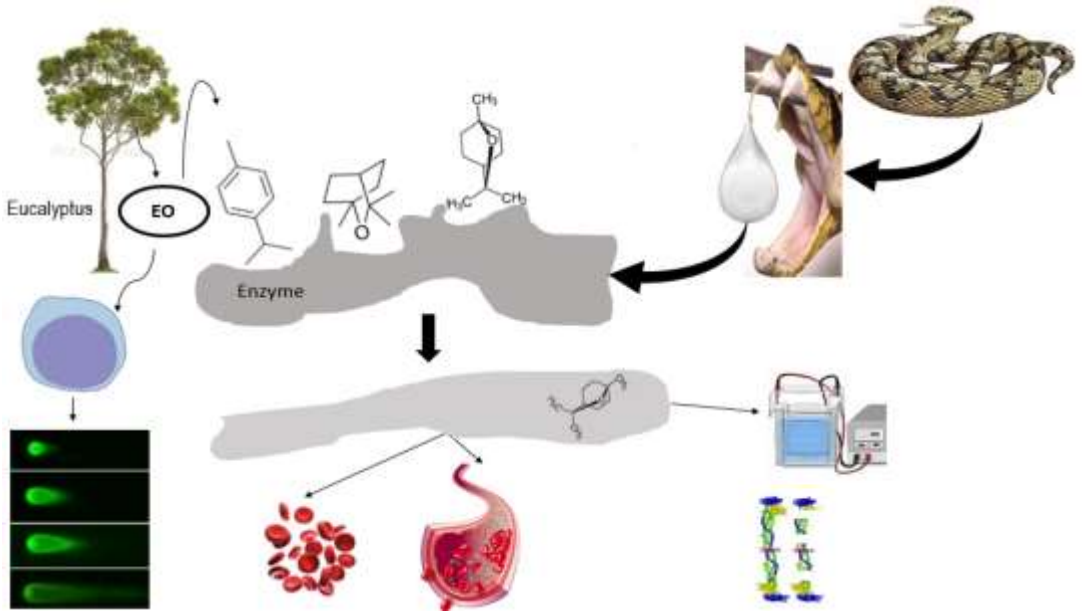
HIGHLIGHTS

- Secondary metabolites modulating the action of fibrinogenolytic proteases.
- Protecting DNA molecules from enzymatic degradation.
- Inhibiting hemorrhagic proteases.

Abstract: The genus *Eucalyptus* present high content of essential oil (EO). This study evaluated the pharmacological properties of *Eucalyptus grandis* (EG) and *Eucalyptus microcorys* (EM) EOs. The major component in both EOs was 1,8-Cineole. Both essential oils prevented thrombus dissolution and reduced clotting, hemolysis, and genotoxicity induced by snake venoms. 50% (EM) and 73% (EG) were the greatest inhibitions obtained in the thrombolytic assay - thrombolysis induced by *B. moojeni* venom. Increases in clotting time were also observed, with values considered significant between 10-27 seconds. Lysis values 50% lower than the negative control were observed in both EOs. The EOs also protected fibrinogenolysis induced by snake venom. EM EO was more effective in reducing venom-induced DNA fragmentation in the comet assay, with arbitrary unit values 66.15% lower than the positive control. These oils present wide application potential considering the pharmacological properties observed in this study.

Keywords: 1,8-cineole; snake venom proteases; Comet assay; chemical characterization; enzyme inhibitors; hemostasis.

GRAPHICAL ABSTRACT



INTRODUCTION

Eucalyptus (genus) are the world's most widely planted hardwood trees. Their outstanding diversity, adaptability, and growth have made them a global renewable resource of fiber and energy. *Eucalyptus* also shows the highest gene diversity for specialized metabolites such as terpenes. *Eucalyptus* leaves present high essential oil (EO) content (up to 33%), which can have up to 48 different compounds. Almost all EO present the terpene 1,8-Cineole, known as eucalyptol, as their major constituent. Myrtaceae EOs are rich in p-Cymene, carvacrol, cuminaldehyde, and linalool, which are often cited as responsible for the great antioxidant property observed. Besides that, these EOs are related to antimicrobial, anti-inflammatory, antitumor, and enzymatic modulating properties [1].

The several classes of enzymes present in snake venoms (proteases and phospholipases) possess high homology with mammal's equivalent enzymes. Therefore, they can be used as adequate tools to simulate hemostatic, inflammation, genotoxic, and cytotoxic effects. *Eucalyptus microcorys* (EM) is one of the least exploited *Eucalyptus* species in terms of its phytochemical content and biological activity [2,3]. On the other hand, *Eucalyptus grandis* (EG) has extensive research on its biological effects. In the present work, the chemical composition of EG and EM were characterized. In addition, their essential oils were evaluated against the biological effects induced by snake venoms (hemostasis and genotoxicity on human leukocytes).

MATERIAL AND METHODS

Plant material and venoms

EG and EM leaves were collected at the campus of the Federal University of Lavras - UFLA (Latitude 21° 13' S, Longitude 44° 58' W, and an average altitude of 915 m) in September 2016 (in days without precipitation and during the morning). The species were identified by the botanist Manuel Losada Gavilanes, and the exsiccates were deposited at UFLA's Herbarium. The extraction of the essential oils was performed at the Organic Chemistry Laboratory—Essential Oils, Department of Chemistry – UFLA (coordinated by Dr Maria das Graças Cardoso), with a modified Clevenger apparatus using the hydrodistillation technique (±98˚C for 2 hours), according to Camargo and collaborators [4]. The essential oils were stored at 4°C and diluted in DMSO: PBS (1:1).

The essential oils chemical characterization was carried out at UFLA's Chemical Analysis and Prospecting Center (CAPQ) using gas chromatography-mass spectrometry (GC/MS) and gas chromatography with flame ionization detection (GC/FID). The essential oils compounds were identified using a Shimadzu GC-17 A device with a mass-selective detection (model QP 5050 A). The following were the

experimental conditions used: DB-5 fused-silica capillary column (5% phenyl-95% dimethylpolysiloxane; 30 m × 0.25 mm id × 0.25 µm film); helium as carrier gas at a flow rate of 1.18 mL min^{-1}; initial temperature at 60 °C and then increases of 3 °C/min up to 240 °C and 10 °C/min up to 300 °C, which remains for 7 minutes; the injector and detector temperatures were 220 °C and 240 °C, respectively; 0.1 µL of sample diluted in hexane (1:100) and a mixture of hydrocarbons (C9-C26) were injected; the impact energy was 70 eV.

The quantification of the compounds was performed using gas chromatography (Shimadzu GC – 17A) with flame ionization detection (GC/FID). The experimental parameters were the same used in the characterization with GC/MS analysis, with the detector temperature at 300 °C, and the concentration of each component was obtained by normalization of areas (%). The compounds were identified comparing the retention rates and respecting the homologous series of alkanes [nC8- nC18] [5], extrapolating to C19 and C20) to the indexes of two data available on the equipment - NIST107 and NIST2. Therefore, it was possible to compare the spectra obtained from the samples with those already existing in the literature. The integration of peak areas defined the percentage of oil constituents without using correction factors. The values shown correspond to the average value of two injections. The used venoms were purchased from the serpentarium Bioagents (Batatais-SP). They were weighed (10 mg), dissolved in 1 mL of phosphate-buffered saline (PBS), and adjusted to pH 7.4. The venoms were previously evaluated to define the minimum doses that induce the activity required in each test.

This study received the authorization to access the genetic patrimony (CGEN – Brazil) under the process numbers AAA3244 (*Eucalyptus*) and ABA4AB3 (*Bothrops moojeni* and *Lachesis muta* venoms).

Thrombolytic, Fibrinogenolytic, Clotting, and Hemolysis tests

Blood samples from healthy volunteers were collected without anticoagulant in BD Vacutainer® for the thrombolytic test [6]. 100 µL blood were distributed in each well of the microplate. After clotting, different volumes of essential oils were added to 30 µL of snake venom (*Bothrops moojeni* or *Lachesis muta*), which were incubated for 10 minutes at 37ºC. Afterwards, these samples were placed on the thrombi and left inside a cell culture chamber for 24 hours at 37°C. Controls containing only venoms or essential oils were also tested. The liquid released from the thrombi were aspirated and quantified.

For the hemolysis test, a 10 mL blood sample was collected in a heparin tube, homogenized, and centrifuged at 700 g for 5 minutes. Afterwards, PBS (2 mM NaH_2PO_4, 3mM Na_2PO_4, 154 mM NaCl, pH 7.4) was added to the same volume of erythrocytes and centrifuged under the same conditions.

Erythrocytes were washed 3 times with PBS and then diluted to a hematocrit of 2%. The negative control was performed with PBS (quantify mechanical hemolysis), and the positive control with distilled water (quantify total hemolysis). After incubating erythrocytes at 2% with different volumes of oils (37 °C for 1 hour), centrifugation was carried out at 1500 g for 5 minutes. Absorbance was recorded at 412 nm in a spectrophotometer to calculate the hemolysis activity [7].

Fibrinogenolytic activities were evaluated according to Miranda and collaborators [8]. The first is the fragmentation profile analysis of fibrinogen molecules in polyacrylamide gel electrophoresis under reducing conditions (SDS-PAGE). The other is the clotting test, which is performed with citrated human plasma. In this test, the difference of 10 seconds between the controls and each treatment was considered significant (prothrombin activation occurs between 10 to 14 seconds).

This study was approved by the UFLA Committee of Ethics in Research with Humans (registered under the number 2.376.107).

Comet assay

This assay was performed to assess DNA damage in human leukocytes. 3 or 6 µL of EOs were added to blood aliquots and remained at 37 °C for 3 hours in a cell culture chamber. A cell suspension of approximately 106 cells mL^{-1} was used to obtain up to 10,000 leukocyte nucleoids per slide. Blood from three volunteers was used in independent experiments in which 300 nucleoids per treatment were evaluated and classified, totalizing 900 nucleoids per treatment.

One percent (1%) low melting point (LMP) agarose (75 µL aliquots), stabilized at 40ºC, was used to include the leukocytes previously treated (25 µL aliquots) and fix these cells into slides previously covered with 1.5% normal melting point (NMP) agarose. Slides were coverslipped and stored at 4°C for 10 minutes, and then immersed in freshly prepared lysis solution (0.25 M NaCl, 100 mM EDTA, 1% Triton X-100, 5% DMSO, pH 10) for two hours. Afterwards, they were kept inside the electrophoresis solution (1 mM EDTA;

30 mM NaOH, pH 13) for 20 minutes at 4°C and submitted to the electrophoresis run (25V, 300mA, 35 minutes).

After the run, the slides remained in a neutralization solution (0.4M Tris-HCl, pH 7.4) for 30 minutes. DNA molecules contained in the slides were then precipitated with absolute ethanol. Staining was performed with a propidium iodide solution at 2 $\mu g.\mu L^{-1}$ (40µL/slide). 100 $\mu g.\mu L^{-1}$ of doxorubicin was used as positive control. The procedures described above were carried out in dark conditions since light irradiation causes lymphocyte death by apoptosis.

Comet patterns were analyzed by visual scores and classified by fragmentation levels [9]. The analyzed cells were classified by DNA injury extent in 5 classes: class 0= without damage (damage < 5%); class 1= low damage level (5-20 %); class 2= medium damage level (20-40 %); class 3= high damage level (40-85 %); and class 4= totally damaged (damage > 85%). The average frequency of damage was calculated from the sum of the percentages of nucleoids with damages 1, 2, 3, and 4. In order to perform comparative analysis, data were calculated in arbitrary units (AU). The arbitrary units (0-400, which 0 = no damage and 400 = 100% damage) were calculated using the equation (1 x number of nucleoids grouped in class 1) + (2 x number of nucleoids in class 2) + (3 x number of nucleoids in class 3) + (4 x number of nucleoids in class 4).

Statistics

One-way analysis of variance (ANOVA) was carried out using the R Core Team (2013) software, and the significance of the means was determined by the Scott-Knott test ($P<0.05$). Analyses were carried out in triplicates and in three parallel measurements, in which were obtained the means ± SD.

RESULTS AND DISCUSSION

EOs characterization

The constituents identified in the characterization of *Eucalyptus* EOs are presented in Table 1. In both *Eucalyptus* species evaluated, the compound in higher quantity in the EOs was 1,8-cineole, known as eucalyptol. This compound represented 55.24% of EG essential oil and 57.14% of EM oil. p-Cymene was identified in centesimal amounts in the essential oil of EM (0.56%).

Thrombolytic, Fibrinogenolytic, Clotting, and Hemolysis tests

The action of *Eucalyptus* EOs on thrombi and their effects on thrombolysis induced by snake venoms are presented in Figure 1.

The essential oils did not cause thrombi dissolution under the evaluated conditions (data not shown). Thrombolytic activity induced by *Bothrops moojeni* venom was intensified at the highest volumes of EOs, with a more pronounced thrombolytic effect at concentrations 0.6 and 1.2 µL for EG and 1.2 µL of EM EOs. However, at 0.1 µL of *E. grandis* and 0.05 µL of *E. microcorys* essential oils, the action of enzymes (mainly proteases) upon thrombi decreased (Figure 1A). This effect was more heterogeneous for *Lachesis muta muta* venom, with no relation between the EOs volumes evaluated and the percentage of inhibition observed. All doses tested decreased the venom-induced thrombolytic activity, but 0.1 µL of EG EO and 1.2 µL of EM EO were the most efficient doses for enzymatic inhibition (Figure 1B).

Clotting activity induced by *B. moojeni* and *L. muta* venoms was inhibited by EG and EM EOs. A longer coagulation time was observed after the incubation of 1.2 µL of EG with *L. muta* (clotting time increased from 63s to 90s). The partial inhibitory effect may be related to the volumes of essential oils used (chosen based on the limitations of the method to the use of compositions of low polarity), their incubation time with the venom, and variations in the composition of venoms and oils (Table 2).

The essential oils in the evaluated conditions exerted partial protection on the fibrinogenolysis induced by *B. moojeni* and *L. muta* venoms. A reduction in the α and β chain fragmentation, and consequent reduction on the intensity of bands corresponding to fibrinopeptides, was observed when comparing the electrophoresis profiles obtained for the controls containing snake venoms added to fibrinogen and only fibrinogen (data not shown). Both EOs were tested for hemolysis. During the assay, a small hemolysis may arise from manipulation of the samples (mechanical hemolysis). In this study, both EOs were considered as non-hemolytic. In fact, they were able to reduce even the mechanical hemolysis when compared to PBS. This protection may be related to EO compounds that interact with the erythrocyte's membrane, increasing its stability and resistance.

Comet assay

The genotoxicity analysis is presented in Table 3 and Figure 2. *E. grandis* EO showed similar effects to the positive controls in all tested doses. Conversely, this does not occur with any EM doses since this EO caused DNA damage (Table 3). The difference in the behavior of these two EOs can be explained by a much larger quantity of p-Cymene in EG and α-Pinene in EM.

The composition of plant essential oils is subject to environmental variations. For example, Soyingbe and collaborators [10] have described an EG EO composed of α-Pinene (29.6%), p-Cymene (19.8%), and 1,8-Cineole (12.8%) as major constituents. In another work, Santadino and collaborators [11] identified α-Pinene (48.8%), 1.8-Cineole (18.8%), and limonene (2.2%) as major components of EG EO. In the present work, the samples analyzed had 4.32 times and 2.9 times more eucalyptol (1,8-Cineole) than the ones in Soyingbe and collaborators and Santadino and collaborators studies, respectively. Besides, no limonene was found in our assessment. The only aromatic compound was p-cymene, and all of them are monoterpenes, also known as C10 compounds. Regarding the clotting time, similar results were observed by Miranda and collaborators [8] when evaluating the performance of the essential oils from the Asteraceae family plants. In their study, all EOs evaluated increased the time to form clots when compared to the same venoms tested in our study. Both studies tested the same concentrations of venom (10 µg) and essential oils.

In the present study, an EO dosage 24 times smaller than the one evaluated by Miranda and collaborators [8]. was tested to obtain partial or gradual enzymatic inhibition. The inhibitory effect was observed for all EO dosages evaluated except for 1.2 µL of EG. The partial inhibitory effect may be related to the volumes of essential oils used (chosen based on the solubility limitation of low polarity compounds), incubation time with the venoms, and variable venom composition.

B. moojeni and *L. muta* venoms have different constitutions and, consequently, have different mechanisms of action. However, all of them act on the coagulation cascade inducing coagulation in the absence of calcium, mainly due to proteases. *Eucalyptus* EOs were able to modulate the enzymatic activity and change the snake venom performance when evaluated *in vitro*. These findings suggest possible modulatory interactions between the terpenes present in the essential oils and the proteases present in the venoms.

Considering the variable coagulation times obtained with the different EOs and venoms, it was not possible to detect an inhibition pattern. Several terpenes may have been responsible for specific interactions with each toxin present in the snake venoms evaluated. Possible mechanisms of inhibition may include chelation of ionic cofactors by oil components, binding of terpenes to catalytic sites present in enzymes, and the interaction of oil components with hydrophobic amino acids in toxin structures. These interactions may alter the three-dimensional structure of proteins, which affect their solubility and, therefore, interfere with their catalytic activity [12,13]. Miranda and collaborators attribute the enzymatic inhibition observed in their work to the sesquiterpenoid content (19.0%) in *Hedychium coronarium* EO. However, this mechanism does not occur with the *Eucalyptus* EOs of our study since they do not have sesquiterpene components.

Transient receptor potential channels (TRP channels) are a group of ion channels located mostly on the plasma membrane of numerous animal cell types. These channels mediate a variety of sensations, such as pain. Caceres and collaborators clarify that 1,8-Cineole modifies inflammatory enzymes through TRP channels that lead cells to produce less inflammatory cytokines (IL-1β, TNF-α, and IL-6). Eucalyptol also exhibited strong analgesic effects through interactions with membrane receptors [3]. Sharma and collaborators [14] suggest that geraniol disrupts cell membrane integrity by interfering with ergosterol biosynthesis and significantly inhibits PM-ATPase, a type of Proton-ATPase. The authors tested this monoterpene on yeasts, which caused membrane lysis and, therefore, fungicidal action. They also reported that lysis occurs only in doses equal or 5 times higher than the Minimum Inhibitory Concentration (MIC: 30 to 130 $\mu g.mL^{-1}$).

Silva and collaborators [15] tested *Casearia sylvestris* EOs on seven different types of red blood cells and observed hemolysis in all of them at doses between 0.6 and 600 $\mu g.mL^{-1}$. However, in their work, the EOs were dissolved in Ethanol: DMSO, which could also have influenced hemolysis results.

The results of the studies previously mentioned demonstrate that doses below 150 $\mu g.mL^{-1}$, depending on the EO components, can induce pharmacological activities without the undesirable effect called hemolysis. Dörsam and collaborators [16] reported that isolated eucalyptol is a weakly genotoxic compound, inducing oxidative damage in DNA from proficient cells in repair mechanisms without causing cell cycle arrest and death. In our study, however, it is shown that the EO, mostly composed by eucalyptol, can cause severe damage to lymphocyte DNA and its behavior is quite similar to doxorubicin (100 µg) ($p < 0.05$).

Some plant species have a well-described and characterized EO antioxidant activity. This activity is one of the mechanisms that contributes to antiproliferative properties [17]. Saleh and collaborators suggest that the growth control mechanisms of tumor cells in the presence of EO occur by apoptosis triggered by mitochondrial death. This mechanism was described for leukemic HL-60 cells after the addition of *Artemia vulgaris* L. EO [17]. In their study, the authors suggested that the major components of the isolated EOs would be responsible for apoptosis induction. These components are caryophyllene, α-zingiberene, borneol, and α-curcumene. Except for borneol, all the others are sesquiterpenes.

Similarly, aerial parts of *Salvia bracteata* Banks and *Salvia rubifolia* Boiss had their essential oils extracted and subjected to the comet assay on human melanoma cells [5]. This research described that *Salvia rubifolia* essential oil presented a higher concentration of sesquiterpene hydrocarbons (41.4%). This EO was also more active than the essential oil of *Salvia bracteata* in reducing cell vitality, altering cell membrane integrity, and inducing genomic DNA fragmentation. Therefore, the results on melanoma cells suggest that the essential oils' anticancer activity may be related to active sesquiterpenes acting in synergism. The results from these authors had a solid scientific background since α-humulene is active against A-549, DLD-1, and LNCaP cell lines [18], and caryophyllene and α-caryophyllene exhibited antiproliferative activity against K562 cell [19].

Figures and Tables

Table 1. Chemical components of the essential oils extracted from *E.grandis* and *E.microcorys* leaves.

Essential oil	Compound	%	RI
E. grandis	α-Pinene	6.1	933
	p-Cymene	28.1	1024
	1,8-Cineole (Eucalyptol)	55.2	1032
	γ-Terpinene	5.8	1058
	α-terpinylacetate	4.5	1346
E. microcorys	α-Pinene	25.4	933
	Camphene	1.3	943
	p-Cymene	0.5	1033
	1,8-Cineole (Eucalyptol)	57.1	1032
	α-Fenchol	1.2	1121
	Trans-Pinocarveol	6.7	1142
	Pinocarvone	2.4	1163
	Isoborneol	2.7	1174
	α-Terpineol	2.3	1197

RI: retention index.

Table 2. Effect of *E. grandis* and *E. microcorys* essential oils on clotting time of human citrated plasma induced by *Bothrops moojeni* and *Lachesis muta* venoms.

Samples		**Clotting time (s)**	
		***B. moojeni* venom**	***L. muta* venom**
Control		62.33 ± 2.51	63.00 ± 3.0
Essential oil	**(μL)**		
E.grandis	0.05	72.6 ± 3.2 [a]	77.6 ± 0.6 [a]
	0.1	71.6 ± 3.0 [a]	70.3 ± 1.5 [a]
	0.3	56.3 ± 1.5[b]	69.3 ± 2.5 [a]
	0.6	74.0 ± 1.0[a]	86.6 ± 1.5 [a]
	1.2	62.6 ± 3.2 *	90.0 ± 2.0 [a]
E.microcorys	0.05	76.6 ± 2.1[a]	70.3 ± 0.6 [a]
	0.1	76.0 ± 2.0 [a]	78.0 ± 2.6 [a]
	0.3	74.33 ± 1.2[a]	73.6 ± 1.5 [a]
	0.6	65.6 ± 1.2 *	76.3 ± 1.5 [a]
	1.2	78.6 ± 1.5 [a]	74.6 ± 2.3 [a]

Data represent the means and standard derivation of triplicates. For each treatment of essential oil plus each venom analyzed, the means followed by letters "a" (in anti-clotting action) or "b" (pro-clotting action) differ significantly from the control at 5% probability by Scott-Knott Test. * Do not differ significantly from the control at 5% probability by Scott-Knott Test.

Table 3. Effect of EO from *E. grandis* and *E. microcorys* on DNA from human blood leukocytes.

Samples	Amount	Damage frequency (%)	Arbitrary units (± SD)
C(-)	-	7.0	28.3 ± 8.02[a]
(+)CD: Doxorubicin	100µg	80.8	323.3 ± 2.4[b]
(+) CL: *L. muta* venom	50µg	70.5	282.7 ± 33.8 [b]
E.grandis (EO)	3 µL	59.8	239.0 ± 1 [b]
	6µL	60.0	240.7 ± 10.4 [b]
	9µL	66.0	264.0 ± 9.0 [b]
E.microcorys (EO)	3µL	23.9	95.67 ± 21.4 [c]
	6µL	31.6	126.3 ± 14.2 [c]
	9µL	33.0	132.0 ± 1.0 [c]

(+)CD: Positive Control = Doxorubicin; (+) CL: Positive Control = *Lachesis muta* venom, (-) Negative Control (PBS); EO: Essential oil. A± SD: Average of arbitrary units corresponding to triplicates obtained in three independent assays± Standard Deviation. Each letter = differs significantly ($p < 0.05$) by the Scott-Knott Test.

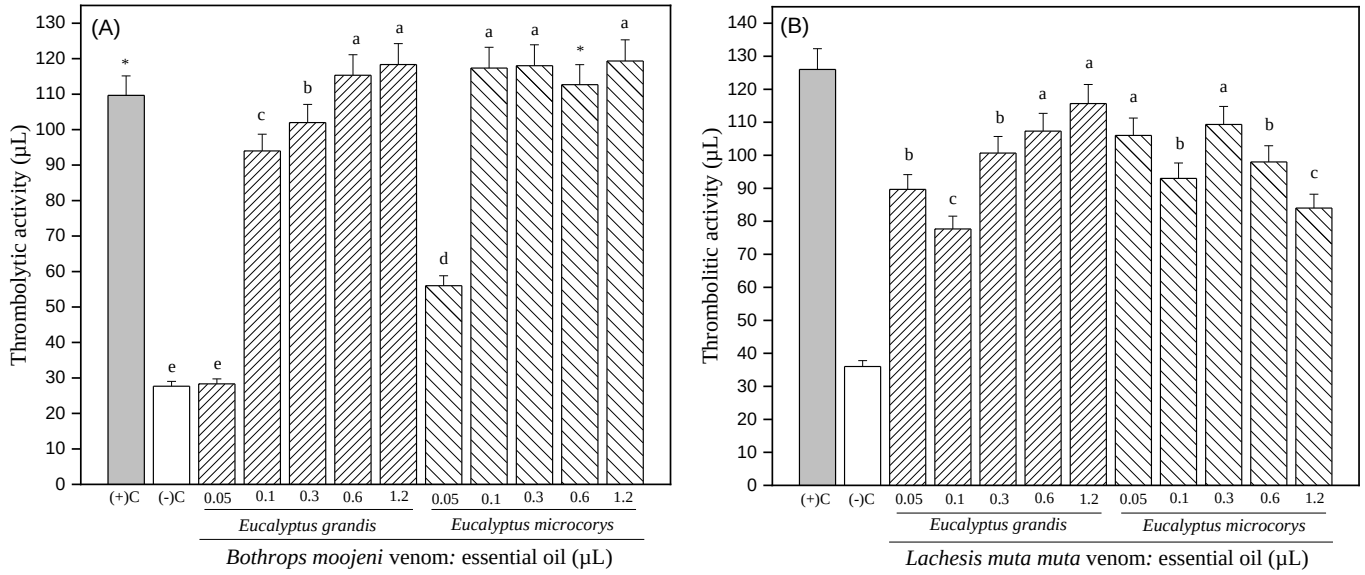


Figure 1. Activity on human blood thrombi. (A) Effect of essential oils on the thrombolytic activity induced by *Bothrops moojeni* venom (30µg). (B) Effect of essential oils on the thrombolytic activity induced by *Lachesis muta* venom (30µg). (-)Negative control: PBS. (+) Positive control: venom. Data represent the average of triplicates evaluated for *Eucalyptus grandis* and *E. microcorys* EOs at different volumes in three independent experiments. Each letter = differs significantly ($p < 0.05$) by the Scott-Knott Test.

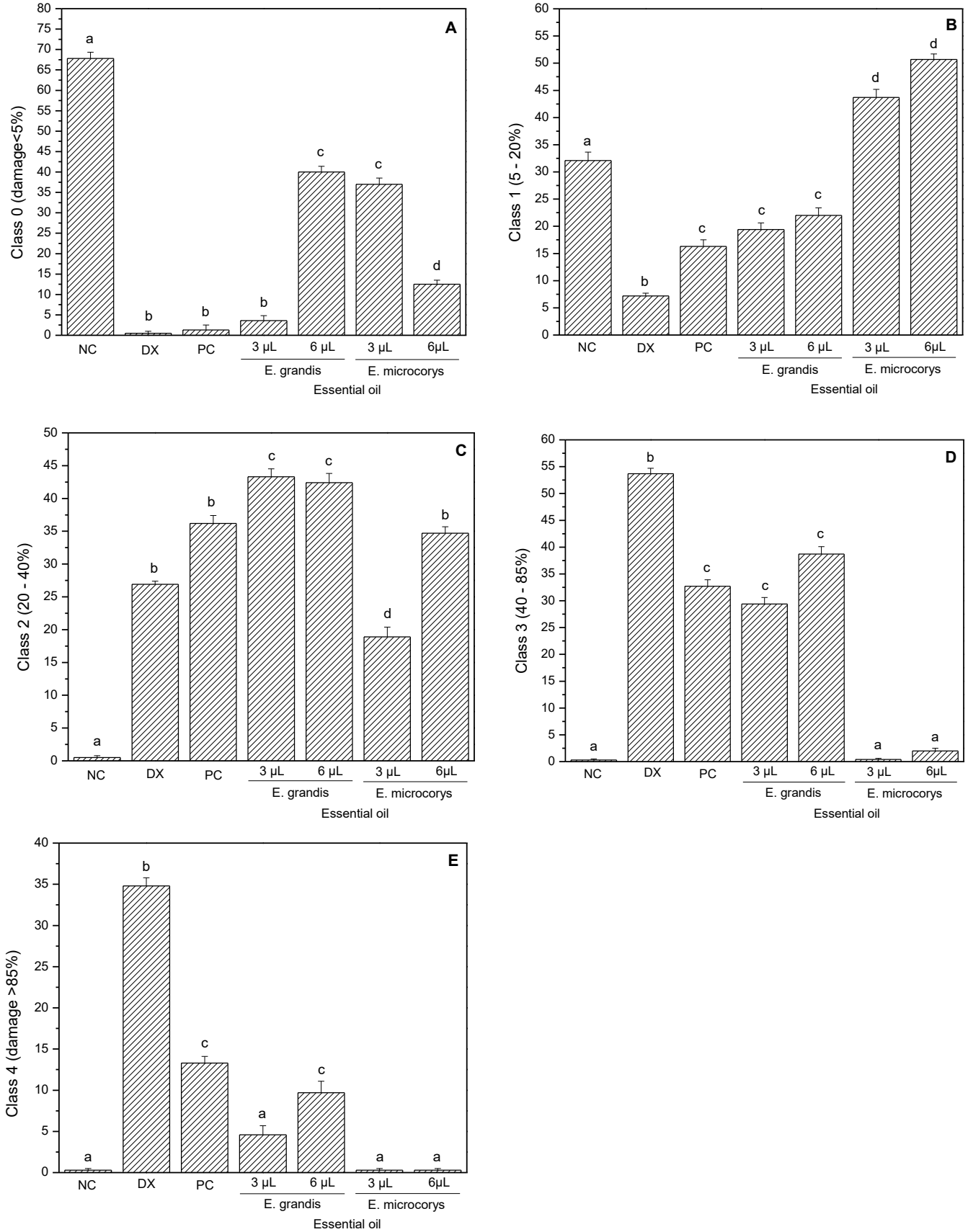


Figure 2. Effect of *Eucalyptus grandis* and *Eucalyptus microcorys* EOs on DNA from human blood leukocytes. Percentage of nucleoids in the different comet classes corresponding to the varying degradation levels of DNA molecules. Data represent the average of values obtained from triplicate assays where 300 nucleoids/treatment/assay were evaluated. Comets were classified according to Collins and collaborators [7]. NC: negative control (PBS); DX: positive control (Doxorubicin, 100µg); PC:positive control (*Lachesis muta* venom, 50µg). Each letter= differs significantly ($p < 0.05$) by the Scott-Knott test.

CONCLUSION

The findings in the present study suggest that the inhibitory activity is due to the monoterpene compounds present in both essential oils analyzed. The doses used may cause inhibition of the coagulation cascade enzymes, which increases the coagulation time and formation of the fibrin net, hinders the thrombolytic effect, and protects against hemolysis. In the tested doses of 0.05 and 1.2 µL, EG essential oil exerts a genotoxic action similar to doxorubicin. Meanwhile, EM EO is less genotoxic than the positive controls evaluated.

Acknowledgments: National Council for the Improvement of Higher Education (CAPES) and National Council for Scientific and Technological Development (CNPq).
Conflicts of Interest: The authors declare no conflict of interest.

REFERENCES

1. Bakkali F, Averbeck S, Averbeck D, Idaomar M. Biological effects of essential oils–a review. Food Chem Toxicol. 2008;46(2):446-5.
2. Bhuyan DJ, Vuong QV, Chalmers AC, van Altena IA, Bowyer MC, Scarlett CJ. Phytochemical, antibacterial and antifungal properties of an aqueous extract of *Eucalyptus microcorys* leaves. S Afr J Bot. 2017;112:180-5.
3. Caceres AI, Liu B, Jabba SV, Achanta S, Morris JB, Jordt SE. Transient Receptor Potential Cation Channel Subfamily M Member 8 channels mediate the anti-inflammatory effects of eucalyptol. Br J Pharmacol. 2017;174(9):867-9.
4. Camargo KC, Batista LR, de Souza PE, Teixeira ML, Sales TA, Ferreira VRF, et al. Antimicrobial Activity of the Essential Oil from *Hyptis carpinifolia* Benth. Am J Plant Sci. 2017;8(11):2871.
5. Cardile V, Russo A, Formisano C, Rigano D, Senatore F, Arnold NA, et al. Essential oils of *Salvia bracteata* and *Salvia rubifolia* from Lebanon: Chemical composition, antimicrobial activity and inhibitory effect on human melanoma cells. J Ethnopharmacol. 2009;126(2):265-72.
6. Cintra ACO, De Toni LGB, Sartim MA, Franco JJ, Caetano RC, Murakami MT. et al. Batroxase, a new metalloproteinase from *B. atrox* snake venom with strong fibrinolytic activity. Toxicon. 2012;60(1):70-82.
7. Rangel M, Malpezzi EL, Susini SM, De Freitas J. Hemolytic activity in extracts of the diatom Nitzschia. Toxicon. 1997;35(2):305-9.
8. Miranda CASFD, Cardoso MDG, Marcussi S, Teixeira ML. Clotting and fibrinogenolysis inhibition by essential oils from species of the Asteraceae family. Braz Arch Biol Technol. 2016;59. doi: 10.1590/1678-4324-2016150775.
9. Collins AR. The comet assay for DNA damage and repair. Mol Biotechnol. 2004;26(3):249.
10. Soyingbe OS, Oyedeji A, Basson AK, Opoku AR. The essential oil of *Eucalyptus grandis* W. Hill ex Maiden inhibits microbial growth by inducing membrane damage. Chin Med. 2013;4(1):7-14.
11. Santadino M, Lucia A, Duhour A, Riquelme M, Naspi C, Masuh H. et al. Feeding preference of *Thaumastocoris peregrinus* on several *Eucalyptus* species and the relationship with the profile of terpenes in their essential oils. Phytoparasitica. 2017;45(3):395-406.
12. Oliveira CH, Simão AA, Trento MV, César PH, Marcussi S. Inhibition of proteases and phospholipases A2 from *Bothrops atrox* and *Crotalus durissus terrificus* snake venoms by ascorbic acid, vitamin E, and B-complex vitamins. An Acad Bras Ciênc. 2016;88(3):2005-16.
13. Samy RP, Gopalakrishnakone P, Chow VT. Therapeutic application of natural inhibitors against snake venom phospholipase A2. Bioinformation. 2012;8(1):48-57.
14. Sharma Y, Khan LA, Manzoor N. Anti-Candida activity of geraniol involves disruption of cell membrane integrity and function. J Mycol Med. 2016;26(3):244-54.
15. Silva SLD, Chaar JDS, Figueiredo PDMS, Yano T. Cytotoxic evaluation of essential oil from *Casearia sylvestris* Sw on human cancer cells and erythrocytes. Acta Amaz. 2008;38(1):107-12.
16. Dörsam B, Wu CF, Efferth T, Kaina B, Fahrer J. The *Eucalyptus* oil ingredient 1,8-cineol induces oxidative DNA damage. Arch Toxicol. 2015;89(5):797-805.
17. Saleh AM, Aljada A, Rizvi SA, Nasr A, Alaskar AS, Williams JD. In vitro cytotoxicity of *Artemisia vulgaris* L. essential oil is mediated by a mitochondria-dependent apoptosis in HL-60 leukemic cell line. BMC Complement Alter Med. 2014;14(1):226. doi: 10.1186/1472-6882-14-226.

18. Loizzo MR, Tundis R, Menichini F, Saab AM, Statti GA, Menichini F. Cytotoxic activity of essential oils from Labiatae and Lauraceae families against in vitro human tumor models. Anticancer Res. 2007;27(5A):3293-99.
19. Lampronti I, Saab AM, Gambari, R. Antiproliferative activity of essential oils derived from plants belonging to the Magnoliophyta division. Int J Oncol. 2006:29(4):989-95.