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Article

Thrombolysis, clotting, and genotoxic activities modulated by essential oils extracted from *Lippia alba*

Mariana Araújo Espósito[1], Marcus Vinicius Cardoso Trento[1], Mariana Aparecida Braga[1*], Silvana Marcussi[1]

[1]Biochemistry Laboratory, Department of Chemistry, Federal University of Lavras, Lavras, Minas Gerais, Brazil

The composition and pharmacological properties of *Lippia alba* (Mill.) (*L. alba*) (Verbenaceae) flower and leaf essential oils (EO) were determined in this study. The major constituents in the flower EO were geranial (49.83%) and neral (32.75%), and in the leaf EO were geranial (38.06%), neral (31.02%), and limonene (18.03%). Flower EO inhibited thrombolysis induced by *Bothrops moojeni* (*B. moojeni*) and *Lachesis muta muta* (*L. muta muta*) venoms (0.05-1.2 µL mL^{-1}). When tested against *L. muta muta* venom, the protective effect was smaller in both EO. The EOs prolonged the clotting time induced by *L. muta muta* venom and a procoagulant effect was observed on *B. moojeni*. In the comet assay, the flower EO presented anti-genotoxic action (damage frequency of only 11.6 – 34.9%) against the *L. muta muta* venom. The positive control (Doxorubicin) and the venom alone presented a damage frequency of 80.3% and 70.7%, respectively. The flower EO protected DNA from damage induced by *L. muta muta* venom. *L. alba* leaf and flower EOs presented anti-genotoxic action.

Keywords: Natural products. Hemostasis. Snake venoms as tools. Comet assay. Enzyme inhibitors.

INTRODUCTION

The genus *Lippia* (Verbenaceae family) includes about 200 species that are abundantly present in Central America, South America, and Africa (Viljoen *et al*., 2005). *L. alba* (Mill.) N. E. Brown is a tropical aromatic shrub widely used in traditional medicine (Gomes *et al*., 2019), and its essential oils (EO) have been studied for various applications in industry, agriculture, and human health.

Three chemotypes are found in the Northeast region of Brazil, and the main EO's compounds reported are myrcene-citral (chemotype I), limonene-citral (chemotype II), and carvone-limonene (chemotype III) (Silva *et al*., 2017).

Mishra *et al*. (2010) reported *L. alba* cultivar 'Kavach' as a linalool-enriched oil source. Due to the compound variability of *L. alba* essential oil, different antioxidant, antimicrobial, antifungal, and antitumor activities may outcome (Souza *et al*., 2017; Montero-Villegas *et al*., 2018; Cagol *et al*., 2020; Costa *et al*., 2020).

Natural products are an excellent alternative source of novel antitumor agents. Caspases (cysteine-aspartic proteases) are a family of protease enzymes playing essential roles in programmed cell death (including apoptosis, pyroptosis, and necroptosis) and inflammation. Caspase deficiency has been identified as a cause of tumor development. Due to the results of the cellular inhibitor performance in tumor cell lines and because of their prominent antioxidant activity, the EO may present aggressive activity on DNA from healthy cells (Vale *et al*., 2002). Some plants' EO induces cell death through intrinsic (mitochondrial) and extrinsic apoptotic pathways, revealing that ROS accumulation causes the apoptotic activity. EO-induced apoptosis occurs due to the loss of mitochondrial membrane potential ($\Delta\Psi m$), increase in Bax/Bcl-2 ratio (correlated with age and tumor location), release of cytochrome c, and activation of the proteases

***Correspondence:** M. A. Braga. Laboratório de Bioquímica. Departmento de Química. Universidade Federal de Lavras. Campus Universitário, CP: 3037. Lavras 37200-900, Brasil. Phone: +55 (35)3829-1893. E-mail: marybraga07@yahoo.com.br. Orcid: https://orcid.org/0000-0003-0758-3993. Silvana Marcussi – ORCID: https://orcid.org/0000-0002-4674-6911

caspases (cleaved form of caspase-3, caspase-8, and caspase-9). Some studies have shown isolated components of the EO – such as citral and 1,8-cineole - and their effects on the induction of apoptosis by the colorimetric measurement of caspase-3 (Cha *et al*., 2009; Cha, Kim, Kim, 2010; Lin, Dai, Cui, 2017; Maruoka *et al.,* 2018; Bailly, 2020).

This work's objective was to investigate the inhibitory potential of essential oils extracted from the leaves and flowers of *Lippia alba* against the coagulant and thrombolytic activities induced by *L. muta muta* and *B. moojeni* venoms and evaluate if they exhibit any protective or toxic activity on DNA from human leukocytes.

MATERIAL AND METHODS

Plant material and venoms

L. alba (Mill.) N. E. Brown leaves and inflorescences 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 the morning period during September 2016, in days without precipitation (exsiccata ESAL 23870). This study received the authorization to access the genetic patrimony (CGEN – Brazil) under the process number A00D5C1.

Following the method described by Andrade et al. (2013), the oil extraction and characterization were performed in the Laboratory of Essential Oils of the Department of Chemistry - UFLA, coordinated by Dr Maria das Graças Cardoso. The identification of essential oils' constituents was performed by gas-liquid chromatography coupled to mass spectrometry (GC-MS). The equipment used was an Autosystem XL equipped with a DB-1 fused silica column (30 m × 0.25 mm ID, 0.25 m thick film; J & W Scientific Inc., Rancho Cordova, CA, USA) connected to a Perkin-Elmer Turbomass. The oven temperature was programmed from 45 to 175 °C in increments of 3 °C/min, and subsequently at 15 °C/min up to 300 °C. On reaching 300 °C, the temperature was kept isothermal for 10 min. The temperatures of the transfer line and ionization chamber were 280 °C and 220 °C, respectively. The helium carrier gas was adjusted to a linear velocity of 30 cm/s, a split-flow ratio of 1:40, the ionization energy is 70 eV, ionization current at 60 μA, the mass range is 40–300 u (atomic mass unit), and a scan time of 1 s. The compounds were identified by comparing their retention indices, relative to those of n-alkanes C9-C21, with the data of mass spectra developed in the laboratory of the Centre for Plant Biotechnology, Faculdade de Ciências da Universidade de Lisboa—Portugal (Mendes *et al*., 2011).

The content of each constituent was determined by gas chromatography (GC-FID) in a Perkin Elmer 8700 gas chromatograph equipped with two Flame Ionization Detectors (FID), a data processing system, and an injector, in which two columns of different polarity were installed: DB-1 fused silica, with immobilized methyl silicone phase (30 m × 0.25 mm ID, 0.25 m thick film; J & W Scientific Inc.) and DB-17HT fused silica (30 m × 0.25 mm ID, 0.25 mm thick film; J & W Scientific Inc.). The oven temperature was programmed from 45 °C to 175 °C in increments of 3 °C/min and subsequently at 15 °C/min to 300 °C. On reaching 300 °C, the temperature was kept isothermal for 10 min. The injector and detector temperature were 290 °C and 280 °C, respectively. Hydrogen was used as carrier gas adjusted to a linear velocity of 30 cm/s. The split-flow ratio was 1:50. 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 (Mendes *et al*., 2011). The essential oils were stored at 4 °C. They were diluted in 1:1 (v: v) DMSO: PBS (phosphate-buffered saline) before being used.

Venoms of *Bothrops moojeni* (*B. moojeni*) and *Lachesis muta muta* (*L. muta muta*) were used as the source of enzymes to induce the different activities. They were purchased from the serpentarium Bioagents (Batatais-SP) and previously evaluated in different doses to define the minimum effective dose adequate for each activity. The venoms were weighed and dissolved in PBS, pH 7.4, to perform the assays.

Thrombolytic and clotting activities

Blood samples from healthy volunteers were collected without anticoagulant in BD Vacutainer ®

for the thrombolytic test (Cintra *et al*., 2012). 100 µL blood were distributed in each well of a microplate. After clotting, 30 µL of the samples (*B. moojeni* or *L. muta muta* venom added to different volumes of EOs - 0.05, 0.1, 0.3, 0.6, and 1.2 µL mL^{-1}- incubated for 10 min at 37 ºC) were placed on the thrombus and set for 24 hours at 37 °C in a cell culture chamber. Controls containing only thrombus, thrombus with venoms, or thrombus with oils were tested. The liquid released from the thrombus were aspirated and quantified.

The clotting test was performed with citrated human plasma, according to Miranda *et al*. (2016), with modifications in the venoms used as controls and the volumes of oils evaluated. The difference of 10 seconds between times observed in controls and treatments was considered significant once the prothrombin activation occurs between 10 to 14 seconds.

This study was approved by the UFLA's Committee of Ethics in Research with Humans and filed under registration number 2.376.107.

Comet assay

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

1% low melting point (LMP) agarose, stabilized at 40 ºC, was used to fix the leukocytes previously treated on the slides. The mixtures of 25 µL of each incubation solution plus 75 µL of LMP agarose were homogenized and placed on slides previously covered with 1.5% normal melting point (NMP) agarose. The slides were stored at 4 °C for 10 min and then immersed in lysis solution (0.25 M NaCl; 100mM EDTA; 1% Triton X-100; 5% DMSO; pH 10) for two hours. Then, the slides were kept in the electrophoresis solution (1 mM EDTA; 30 mM NaOH, pH 13) for 20 min at 4 °C, and submitted to an electrophoretic run carried out at 25 V, 300 mA, for 35 min.

The slides remained in a neutralization solution (0.4 M Tris-HCl, pH 7.4) for 30 min. The DNA molecules contained in the slides were then precipitated with absolute ethanol. The staining was performed with 40µL/ slide of propidium iodide solution at 2 µg $µL^{-1}$. Comet patterns were analyzed by visual scores and classified according to fragmentation levels. For additional positive control, Doxorubicin was used in the concentration of 100 µg mL^{-1} (Pereira *et al*., 2012; Marcussi *et al*., 2013).

The procedures described above were carried out in dark conditions since light irradiation causes the death of lymphocytes by apoptosis (Chen *et al*., 2015).

Comet standards were analyzed by visual scores, according to Collins *et al*. (1997). The cells analyzed were classified by DNA injury extent in 5 classes: class 0, without damage (damage < 5%); class 1, low level of damage (5- 20%); class 2, medium level of damage (20- 40 %); class 3, high level of damage (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 damage 1, 2, 3, and 4. To perform comparative analysis, data were presented in arbitrary units (AU) as described by Collins *et al*. (1997). The arbitrary units (0- 400, in which 0 = no damage and 400 = 100% damage) were calculated by 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

Statistical analysis was carried out by one-way analysis of variance (ANOVA) test using the R Core Team (2013) software. The significance of the difference between means was determined by the Scott-Knott test (P<0.05). The analyses were carried out in triplicates and mean±SD (standard deviation) of three parallel measurements.

RESULTS AND DISCUSSION

The constituents identified in the characterization of EOs from *L. alba* leaves and inflorescence are presented in Table I.

TABLE I - Chemical constituents of the essential oils of flowers and leaves of *L. alba*

Essential oil %	Compound	RI
Flower		
9.1	ρ-Cimene	1026
0.06	Limonene	1029
5.16	γ-Terpinene	1058
32.75	Neral	1239
49.83	Geranial	1269
2.07	D-Germacrene	1481
1.03	α-Elemol	1550
Leaf		
0.13	α-Thujeno	924
1	Sabinene	972
*	ρ-Cimene	1.025
18.03	Limonene	1.028
5.02	γ-Terpinene	1.057
31.02	Neral	1.238
38.06	Geranial	1.268
3.17	D- Germacrene	1.480
3.52	α -Elemol	1.549

*Traces: (less than 0.1%). RI: Retention index.

Stashenko, Jaramillo, Martínez (2004) found, using the hydrodistillation technique, more than 40 constituents in *L. alb*a leaves' EO, with carvone (57%) and limonene (37%) being the major components. They also reported that when they used the Headspace technique, they found limonene as the major constituent in the same sample analyzed (77%), but carvone was also present (14%). This suggests that the method employed interferes with the profile of the results obtained. In the present study, the leaves did not present carvone, and limonene only appeared in 18% of the EO.

Four *Lippia* species from different cities throughout Rio Grande do Sul, Brazil were collected and analyzed. The analyses demonstrated the interference of the plant culture site in the component profile from different EOs' (Tomazoni *et al*., 2016). Two of the analyzed samples demonstrated camphor to be the main component, whereas for the other samples, one showed linalool as the primary substance, and the other had citral as the major component. In the present study, camphor and linalool are not present. The profile of constituents modulates the biological properties performed by the EOs and suggests different enzymatic interactions in the metabolism (Bakkali *et al*., 2008). In plant tissue culture, three varieties of *Lippia* produced different profiles of components and responded differently to changes in CO_2 concentrations. In this study, the results support the understanding of the metabolic routes of monoterpene synthesis and the modulation of enzymatic translation/ transcription from environmental effects (Batista *et al*., 2017).

Citral-a (Geranial) and citral-b (Neral) are isomers commonly found in *Lippia* spp. These components' presence have sedative, neuroleptic effects and appears to increase the half-life of barbiturates in tests with mice. This property corroborates with the traditional use already described. Similar to the present work, Soares *et al*. (2016) found carvone as the major constituent (61.7%) and attributed to it the changes in glucose levels and overall leukocyte, neutrophils, lymphocytes, and eosinophils count. The authors reported that this EO causes severe histopathological and hematological changes in the fish *Colossoma macropomum* (tambaqui).

EO from *L. alba* had different effects on the thrombus induced by the venoms of *B. moojeni* and *L. muta muta*. Figure 1 presents the results obtained in the controls (*L. alba* EOs, PBS, and venoms) and incubated samples.

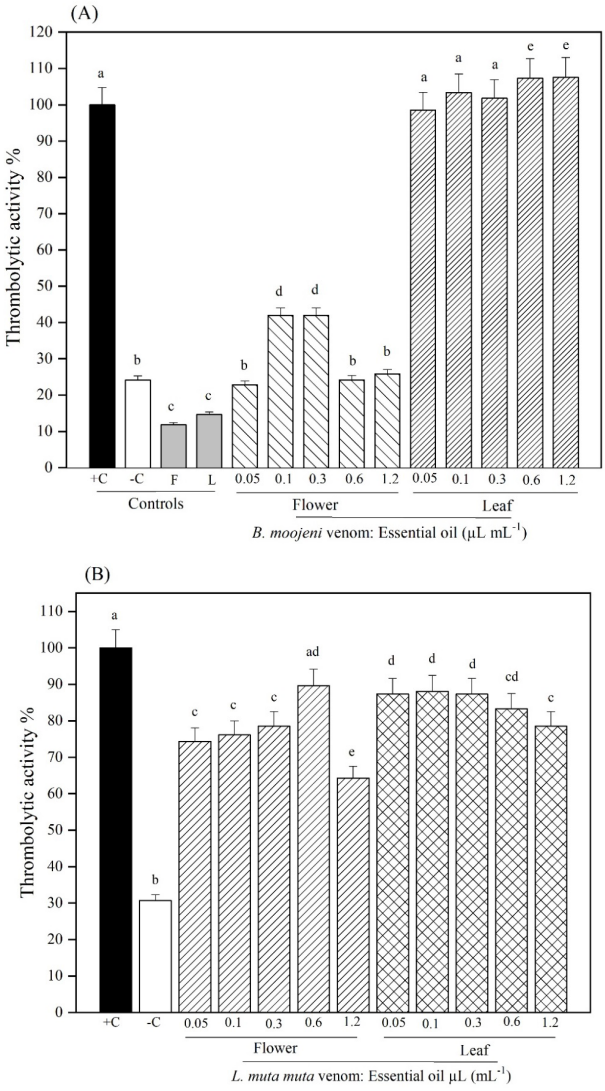

FIGURE 1 - Activity on human blood thrombi. (A) Thrombolytic activity of essential oils from *L. alba* flower and leaf and activity induced by *B. moojeni* venom. (B) Effect of essential oils on the thrombolytic activity induced by *L. muta muta* venom. (+)C: Positive Control: 40 µg mL^{-1} of *B. moojeni* or *L. muta muta* venom; (-)C: Negative Control: PBS; *L. alba* flower and leaf essential oil (F and L; 1.2 µL mL^{-1}). Incubated: Venoms + essential oils at different ratios. Each letter statistically differs ($p <$ 0, 05) using Skott-Knott test.

Previous incubation of *L. alba* leaves EO with *B. moojeni* venom presented a procoagulant activity at the doses of 0.6 and 1.2 µL mL^{-1}. However, *L. alba* flower EO presented thrombolytic activity at 0.1 and 0.3 µL mL^{-1} (Figure 1A). This results may be due to the presence of several components, such as ρ-cymene in the flowers and limonene in the leaves essential oils. *L. alba* EOs presented activity in all the doses tested. The dose 1.2 µL mL^{-1} from flowers EO was more efficient in inhibiting the enzymes present in *L. muta muta* venom (Figure 1B).

The clotting activities induced by *B. moojeni* and *L. muta muta* venoms were inhibited by *L. alba* flower and leaf EOs (Table II). The partial inhibitory effect may be related to the volumes of oils used (chosen based on the limitations of the method to the use of compositions of low polarity), incubation time with the venoms, and varied composition of the venoms and oils.

TABLE II - Effect of essential oils from *L. alba* on clotting time of human citrated plasma induced by *B. moojeni* and *L. muta muta* venoms

Samples *L. alba* oil (µL mL^{-1})		Clotting time (s)	
		***B. moojeni* venom**	***L. muta muta* venom**
Control (without oil)		66.66 ± 2.1	61.33 ± 2.1
Leaf	0.05	78.6 ± 1.5[a]	74.6 ± 2.3[a]
	0.1	47.6 ± 1.2[b]	90.3 ± 2.1[a]
	0.3	44.0 ± 2.0[b]	92.6 ± 0.6[a]
	0.6	45.6 ± 1.5[b]	110.0 ± 2.0[a]
	1.2	44.3 ± 0.6[b]	113.0 ± 1.0[a]
Flower	0.05	57.0 ± 1.2 *	80.0 ± 0.6 [a]
	0.1	65.6 ± 1.2 *	65.9 ±1.5*
	0.3	57.0 ± 1.7 *	65.9 ±0.6*
	0.6	52.3 ± 2.5[b]	73.6 ±2.9[a]
	1.2	46.3 ± 2.5[b]	83.6 ±2.3[a]

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

The data of *L. alba* EOs in contact with *B. moojeni* venom presented in Table II show a procoagulant action, which was previously reported in the literature by Miranda *et al*. (2016) when evaluating the *Hedychium coronarium* essential oil. This can occur due to the structural variability of molecules present in essential oils, mainly of hydrophobic character. The results suggest that the oils may interact with plasma proteins involved in the coagulation cascade, making them more susceptible to the proteolytic action of the venoms, and consequently, accelerating the plasma coagulation (procoagulants). This interaction probably occurs between the individual constituents of the essential oils and proteins, such as thrombin, fibrinogen, or fibrin, which are the main targets of the coagulant toxins. The clot formation time was prolonged in the *L. muta muta* venom, and the oils could be considered as potential anticoagulants or inhibitors of coagulant proteases. *L. muta muta* and *B. moojeni* venoms have diverse constitutions and, consequently, their toxins have different mechanisms of action.

However, both venoms evaluated act on the blood-clotting cascade, inducing coagulation in the absence of calcium, mainly due to the action of serine proteases. EOs were able to modulate the enzymatic activity and alter their *in vitro* performance. These findings suggest possible interactions between the terpene compounds present in the oils and proteases present in the venoms. Considering the coagulation times obtained with the

different oils and venoms, it was not possible to detect an inhibition pattern. Several terpenes may have been responsible for specific interactions with different toxins present in the evaluated venoms. The enzymatic inhibition can be attributed to the sesquiterpenoid (D-Germacrene and α-Elemol) content present in *L. alba* EO since there was an elongation of the coagulation time in the test with the *L. muta muta* venom. This sesquiterpenoid content was only 3.10% for flower and 6.69% for leaf essential oils.

The genotoxicity analysis (Comet assay) is presented in Table III and Figures 2 and 3.

TABLE III - Effect of *L. alba* EO on DNA from human blood leukocytes

Amount (µL mL^{-1})	Samples	Damage frequency (%)	Arbitrary units; A ± SD
	(-) C	**7.1**	**28.3 ± 8.02^{a}**
100 µg mL^{-1}	(+) CD	80.3	321.3 ± 45.4^{b}
50 µg mL^{-1}	(+) CL	70.7	282.7 ± 33.8^{b}
1.5	Flower EO	14.4	57.8 ± 6.15^{c}
3.0		13.3	53.3 ± 2.31^{c}
6.0		15.4	61.7 ± 3.51^{c}
1.5	Leaf EO	7.7	30.7 ± 4.16^{a}
3.0		7.6	30.0 ± 3.60^{a}
6.0		8.4	33.0 ± 5.6^{a}
1.5	*L. muta muta* + Flower EO	58.8	235.0 ± 11.31^{b}
3.0		26.8	107.0 ± 4.24^{d}
1.5	*L. muta muta* + Leaf EO	14.5	58.0 ± 1.41^{c}
3.0		11.6	46.5 ± 6.36^{c}

(+) CD: Positive Control Doxorubicin; (+) CL: Positive Control *L. muta muta* venom, (-) Negative control (PBS); EO: Essential oil. A± SD: Average of arbitrary units corresponding to triplicates obtained in three independent assay ± Standard Deviation. Each letter differs significantly ($p < 0.05$) by Scott-Knott Test. * *L. alba* flower oil tested at 1.5 and 3.0 µL mL^{-1} only because of the small volume of oil available.

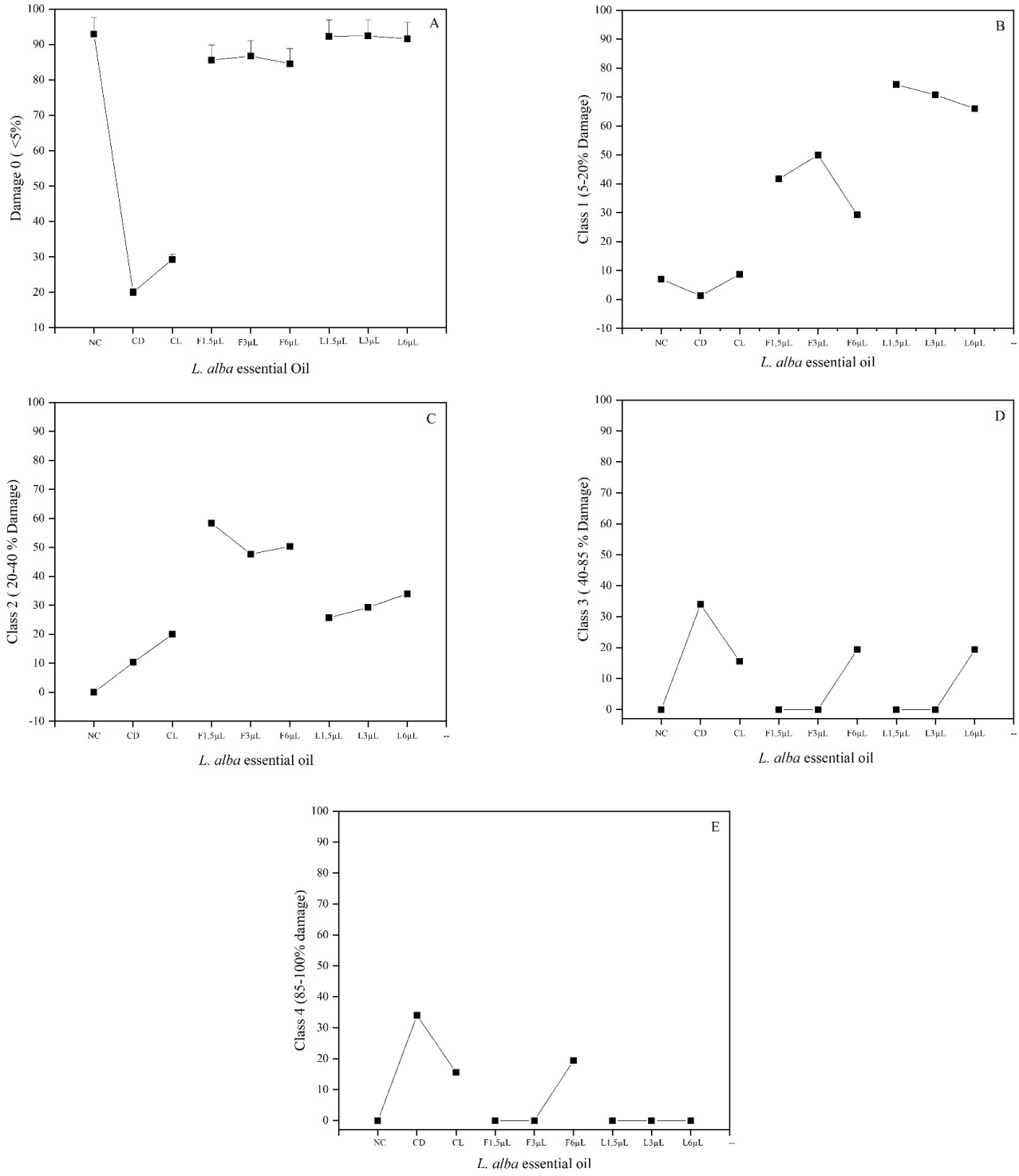

FIGURE 2 - Effect of *L. alba* flower and Leaf EO on DNA from human blood leukocytes. Percentage of nucleoids in the different comet classes corresponding to the varying degradation levels of the DNA molecules. Data represent average of values obtained from triplicate assays where were evaluated 300 nucleoids/treatment/assay. Comets were classified according to Collins *et al*. (1997). NC: negative control (PBS); +CD: positive control (Doxorrubicin, 100 µg mL^{-1}); +CL: positive control (*L. muta muta* venom, 50 µg mL^{-1}). Oil + Venom (*L. muta muta* venom). F: flower oil. L: leave oil. Volume of oil in µL.

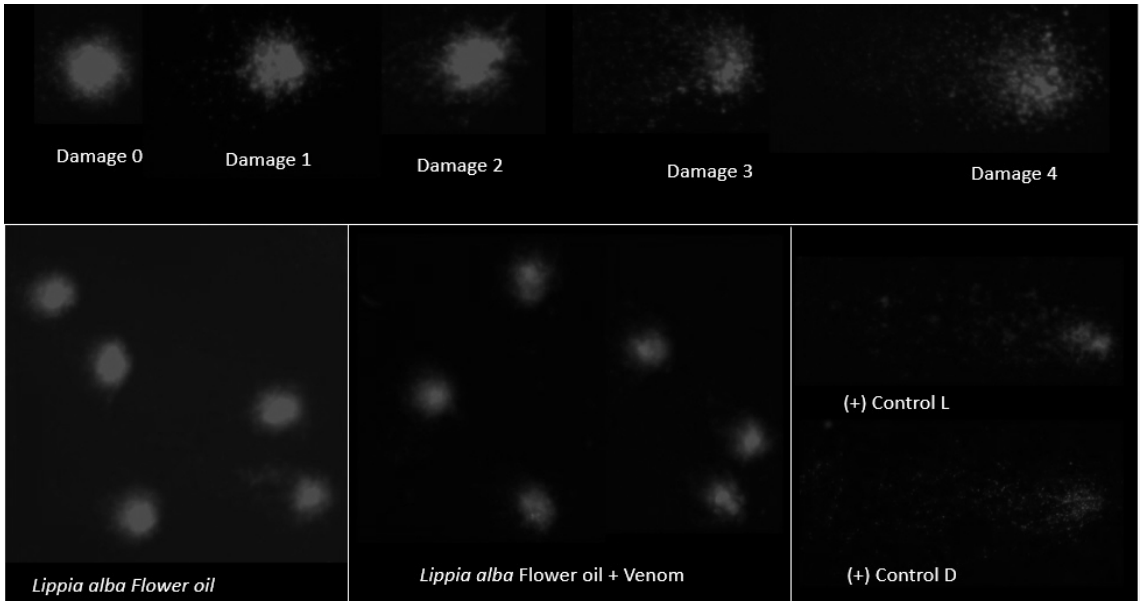

FIGURE 3 - Comet assay (single cell gel electrophoresis) in human blood leukocytes. Demonstrative images of scores observed in different Damage Classes (form 0 to 4). (+)CL: Positive Control with *L. muta muta,* 50 µg mL^{-1}; (+) CD: Positive Control with Doxorubicin, 100 µg mL^{-1}.

The alkaline comet assay is widely used in genotoxicity tests to evaluate DNA damage and repair mechanisms. This assay enables alkaline pH treatment and electrophoresis to detect DNA single- or double-strand breaks, which are alkaline-labile sites, through the alkylation of electronegative DNA groups and cross-links (Collins *et al*., 1997). The EOs from *Lippia* spp. were chemically characterized through GC-MS, and their anesthetic activity and genotoxicity were evaluated in fish and mammals (Kampke *et al*., 2018). In the anesthetic activity, the authors reported no difference between the control and treatment neither at 300 mg kg^{-1} when evaluated by the methods of inhalation or gavage. Higher percentages of damage were found in fish, possibly due to the immune system's action since they detected the inhibition of nitric oxide (NO) release (Kampke *et al*., 2018).

In the present study, leaf EO presented damage similar to the negative control (PBS). When the EOs were tested in combination with venom, they presented a protective activity in the DNA. The enzymatic inhibition could be observed (Figure 3) when 1.5 µL and 3 µL mL^{-1} of leaf EO were used.

In the work of Kampke *et al*. (2018), the doses tested considering the mice weight (32.5 ± 3.43g A± SD) corresponds to 2.9 to 10.7g of oil. In our study using *in vitro* assays, the doses of 1.5 and 3 µL mL^{-1} caused different effects. It is suggested that *in vitro* assays do not have all the immunological signaling and chemotaxis issues that a xenobiotic agent produces in a living organism.

Our study is the first to present experimental genotoxicity data using the alkaline comet assay in *L. alba* leaf and flower EOs. The alkaline comet provides considerable advantages over conventional cytogenetic methods (e.g., chromosomal aberrations, sister chromatid exchange, and micronucleus test) used to detect damage in DNA since the cells in this test do not need to be mitotically active (Ali, Kumar, 2008).

The natural inhibitors act on PLA_2s and proteases through different mechanisms. Most of them are still not completely understood, including binding to specific domains in the structures of the enzymes and forming complexes with divalent ions (Miranda *et al*., 2016). Several substances have been evaluated regarding their effects against snake venoms and isolated toxins, including plant extracts, compounds from mammals and snakes serum plasma, poly or monoclonal antibodies, and synthetic molecules. Research involving these inhibitors

may be useful to understand the mechanism of action of different classes of enzymes and their role in snakebite envenomations. Furthermore, inhibitors' biotechnological potential may provide therapeutic molecular models with antiophidian activity to supplement the conventional serum therapy (Oliveira *et al*., 2016).

CONCLUSION

The essential oils of *L. alba* showed modulatory effects (through inhibition or potentiation) on the biological activities induced by *L. muta muta* and *B. moojeni* venoms, highlighting their anti-genotoxic potential, and modulator of hemostasis-related processes. These results suggest the benefits of using both oils as a natural source of antioxidants and as protection against DNA damage.

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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