Journal of Ethnopharmacology 301 (2023) 115744

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Antithrombotic potential of *Lippia alba*: A mechanistic approach

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ARTICLE INFO

Keywords:
Anticoagulants
Herbal medicine
Lippia
Lemon balm
Patient safety
Thrombin generation assay

ABSTRACT

Ethnopharmacological relevance: Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson is traditionally used in Brazil as an adjunct in the relief of mild anxiety, as an antispasmodic, and as an antidyspeptic. This medicinal species was included in the Phytotherapeutic Form of the Brazilian Pharmacopeia 2nd edition (2021) and has already been described as the most used medicinal plant in a study with patients from an Anticoagulation Clinic in Brazil. Meanwhile, no studies were found that support the safety of the use of *L. alba* in patients using anticoagulants, a drug with several safety limitations.

Aim of the study: Provide scientific evidence to ensure the safety of the concomitant use of *L. alba* and warfarin and support the management of these patients by evaluating its *in vitro* anticoagulant effect and chemical composition. And, as a timely complementation, evaluate the potential of this medicinal species in the development of new antithrombotics.

Methods: The chemical profile of *L. alba* derivatives was analyzed by chromatographic methods such as Ultra-Performance Liquid Chromatography (UPLC) coupled with electrospray ionization mass spectrometry (ESI-MS), qualitative UPLC using Diode-Array Detection, and Thin Layer Chromatography. The anticoagulant activity was evaluated by the innovative Thrombin Generation Assay by Calibrated Automated Thrombogram method and using traditional coagulometric tests: prothrombin time, activated partial thromboplastin time, and plasma fibrinogen measurement.

Results: Extracts and fractions prolonged the coagulation time in all the tests and reduced thrombin formation in thrombin generation assay. Coagulation times with the addition of ethanloic extract (2.26 mg/mL) was 17.78s, 46.43s and 14.25s respectively in prothrombin time, activated partial thromboplastin time and fibrinogren plasma measurement. In thrombin generation test, this same extract showed ETP as 323 nM/min compared to control (815 nM/min) with high tissue factor and 582 nM/min compared to control (1147 nM/min) using low tissue factor. Presence of flavonoids, phenylpropanoids, and triterpenes were confirmed by chromatographic methods and 13 compounds were identified by UPLC-ESI-MS. Based on these results and on the scientific literature, it is possible to propose that phenylpropanoids and flavonoids are related to the anticoagulant activity observed.

Conclusion: The results demonstrate the *in vitro* anticoagulant activity of *L. alba*, probably due to the activation of intrinsic and extrinsic pathways. It is concluded, then, that there is a potential for interaction, which needs to be further studied, between *L. alba* and warfarin. Also, this medicinal species shows a great potential for use in the development of new antithrombotics.

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https://doi.org/10.1016/j.jep.2022.115744
Received 16 March 2022; Received in revised form 14 September 2022; Accepted 16 September 2022
Available online 29 September 2022

1. Introduction

The genus *Lippia* is included in the Verbenaceae family and comprises shrubs predominantly distributed in South America, Central America, and tropical Africa. In Brazil, the country that has the largest number of known species of *Lippia*, it is well widespread in the biome cerrado (tropical savannah). The genus is commonly used in food tea preparation, cosmetics, agriculture, and folk medicine, especially because of its nervous system properties (Gomes et al., 2019).

The species *Lippia alba* (Mill.) N.E.Br. ex Britton & P. Wilson is a meaningful example of this genus. It is known in folk medicine as lemon balm ("cidreira") and occurs mainly in Serra do Espinhaço (Minas Gerais). The Brazilian Health Surveillance Agency recommends its use for the treatment of medium anxiety and insomnia. But, *L. alba* is also popularly employed as a digestive and expectorant and to treat cardiovascular diseases (Gomes et al., 2019). Also, given the high relevance of its use in the country, *L. alba* was recently included in the second edition of the Brazilian Pharmacopeia Herbal Medicines Form (ANVISA, 2021).

This plant is known for its phenotypic plasticity and genome variation, and therefore has quite varied chemical composition. It is rich in essential oil, which is even one of the ways to classify the phenotypes of the species (Reis et al., 2015; Hennebelle et al., 2006a, 2006b). In addition, *L. alba* also has iridoids such as geniposidic acid, phenylpropanoids such as verbascoside and its derivatives, and flavonoids such as luteolin, apigenin, and derivatives (Funari et al., 2012; Hennebelle et al., 2006b; Timóteo et al., 2015; Trevisan et al., 2016).

In Brazil, *L. alba* has been pointed out in several ethnopharmacological studies for its high percentage of use, with the main form of use being the tea of its leaves (Hennebelle et al., 2008). In a study conducted in an anticoagulation clinic, this plant was the most cited among patients on warfarin use. The main indication of warfarin is in the prevention of thromboembolic disorders, and it has complex pharmacotherapy with a number of safety limitations. One of the limitations is the potential for interactions with drugs, foods, and herbal medicines (Leite et al., 2016a, 2016b, 2017, 2018, 2021b). Hence, no studies on the safety of concomitant use of *L. alba* and warfarin were found.

Based on this observational study, Brazilian medicinal species widely used by this population group were selected and tested for their anticoagulant potential in order to evaluate the safety of using these species together with warfarin. Most of these medicinal plants showed high anticoagulant activity *in vitro*, suggesting potential interaction with warfarin. These results show how much the plants of traditional Brazilian medicine need to be further studied regarding the scientific validation and safety of their use. Also, in this context, *L. alba* was the medicinal species that showed the highest anticoagulant potential in preliminary *in vitro* tests (Leite, 2015; 2021a, 2022).

Herb-drug interactions started to be considered relevant in recent years and therefore, the way to study them, as well as the scientific basis for this, are still under development. Furthermore, studies of interactions of medicinal plants and their derivatives with anticoagulants, such as warfarin, are complex due to safety issues related to the drug. Therefore, what is most found in the literature about herb-warfarin interactions are *in vitro* studies and case studies. Both have limitations, but they can be excellent for directing scientific research efforts (Brantley et al., 2014; Fasinu et al., 2012; Fugh-Berman, 2000; Ge et al., 2014; Leite et al., 2021a, 2021b; Yeung et al., 2018).

In this sense, there are studies indicating that some compounds present in the genus *Lippia* have anticoagulant and high platelet activities (Oliveira et al., 2014), being a potential source of interaction with anticoagulants such as warfarin. Thus, the study of the anticoagulant activity of *L. alba* could provide scientific evidence that supports the potential interaction of this plant with anticoagulants. And, as a timely complementation, *L. alba*'s potential as an antithrombotic agent is also discussed. In this context, the aim of this study is to evaluate the effect of extracts and fractions of *L. alba* in coagulation, as well as its phytochemical composition, through *in vitro* tests.

2. Methods

2.1. Plant material

Lippia alba (Mill.) N.E.Br. ex Britton & P. Wilson was collected from a medicinal garden in March 2017 in Belo Horizonte - Minas Gerais (MG) – Brazil. The plant name was checked with http://www.theplantlist.org on March 15, 2022. A voucher of the species was identified and deposited in the Herbarium of Universidade Federal de Minas Gerais (UFMG) with the identification number BHCB160513. The registration was carried out in the National System of Genetic Heritage and Associated Traditional Knowledge Management (SisGen Code A20FE36).

2.1.1. Preparation of the extract and fractionation

The dry ethanolic extract of *Lippia alba* was obtained according to the methodology described by Leite et al. (2019a), 2019b (Supplementary material 1). Dried extracts were stored in a freezer until use. The fractions of the ethanolic extract were obtained by liquid-liquid extraction. The ethanolic extract (1 g) was dissolved in water (50 mL) and extracted three times in 50 mL of dichloromethane (DCM) using a separatory funnel. Fractions were dried and also stored in a freezer until use. Ethanolic extracts of *L. alba* collected in the four seasons of the year (summer, autumn, winter, and spring) were also elaborated under these same conditions (Leite et al., 2019a, 2019b) (Supplementary material 1) to achieve a state of seasonal variation in chemical composition and anticoagulant activity *in vitro*.

2.2. Phytochemical analysis

2.2.1. Thin layer chromatography (TLC)

A qualitative evaluation of the presence of certain classes of secondary metabolites was performed by TLC using selective reagents: diphenylboryloxyethylamine/polyethyleneglycol with mobile phase butanol:chloroform:acetone:formic acid (75:16.5:8.5) (flavonoids); ferric chloride with mobile phase chloroform:acetone:formic acid (50:40:10) (tannins); potassium hydroxide with mobile phase ethyl acetate:hexane (1:1) (coumarins), and Libermman-Burchard with mobile phase hexane:ethyl acetate (1:1) (triterpenes/sterols).

2.2.2. Qualitative chemical evaluation with ultra-performance liquid with Diode-Array Detection (UPLC-DAD)

Dried extracts/fractions (5 mg) of *Lippia alba* were solubilized in 1 ml HPLC grade methanol (Tedia Brazil, Rio de Janeiro, Brazil) to prepare the solutions to be used in chromatographic analysis. Chromatographic profiles were obtained by UPLC-DAD according to the method described in Leite et al. (2019a, 2019b) (Table 1). Co-injection experiments were conducted to identify standard substances. The standard solutions were prepared at 1 mg/mL concentration and 100 μL was added to the extract solution.

2.2.3. Ultra-performance liquid chromatography electrospray ionization mass spectrometry (UPLC-ESI-MS)

UPLC-ESI-MS was conducted on a Nexera UPLC (Shimadzu, Kyoto, Japan) coupled to a Compact Electrospray Time-of-Flight Mass Spectrometer (Bruker, Billerica, US). Chromatographic separation of ethanolic extract was conducted using a KINETEX C18 column (100 × 3.0 mm I.D.; 2.6 μm). A volume of 5 μl of the solution was injected into the system at a flow rate of 0.4 ml/min, and the mobile phase and gradient were the same as for analytical UPLC. Mass spectra in negative-ion mode were generated under the following conditions: fragmentor voltage, 100 V; capillary voltage, 4500 V; nebulizer pressure, 72 psi; drying gas temperature, 220° C; and mass range, 100–1000 D.

Table 1
Chromatographic conditions used for the analysis of the ethanolic extract from *L. alba* with UPLC-DAD.

Pre-column	Column	Volume of injection (μl)	Flow (mL/min)	Temperature (°C)	Reading wavelength (λ)	Time (min)	Mobile phase	
							Acetonitrile	H2O 0.1% HCOOH
VanguardTM C18 (2.1 × 5 mm I.D.; 1.7 μm)	Acquity UPLC BEH C18 (100 × 2.1 mm I.D.; 1.7 μm)	2	0.3	40	270	0	5	95
						24	95	5
						26	95	5
						28	5	95

2.3. Participants and plasma sampling

The project was approved by the UFMG ethics committee with registration CAAE 60904316.6.0000.5149. All rules regarding the protection of human subjects were followed and informed consent was obtained from all individual participants included in the study. Members of the Faculty of Pharmacy of UFMG - Brazil were evaluated for eligibility: volunteers of both sexes, aged over 18 years, not users of any medication that could interfere with the hemostatic system (anticoagulant, antiplatelet, contraceptive, non-steroidal anti-inflammatory). Twelve healthy people who met the prerequisites were included in the study. Recruitment and blood collection were conducted in April 2017. Platelet-poor plasma was obtained from the blood to perform coagulometric tests (Leite et al., 2019a,b). Plasma of the 12 participants was used for the elaboration of a pool. This pool of plasmas was aliquoted and stored in a −80°C freezer until use (Supplementary material 1).

2.4. Anticoagulant activity

The samples were prepared for anticoagulant activity assays, *in vitro*, through the dissolution of 5 mg of the respective derivatives of *L. alba* in 1 mL of NaCl 0.9% using an ultrasound bath for 10 min. Aliquots of this mixture were dissolved in 1 mL of plasma pool (obtained concentrations: 1.67 mg/ml, 2.26 mg/mL, and 2.86 mg/mL), to be used in the biological tests. The negative control was done with the addition of the same volume of NaCl 0.9% in the plasma pool.

The anticoagulant activity was evaluated by the following traditional coagulometric tests: prothrombin time, activated partial thromboplastin time, and plasma fibrinogen measurement. In addition, thrombin generation assay was also used to evaluate the whole hemostatic system after addition of the plant derivatives, as it is a more robust technique than standard coagulometric tests. The methodology employed is described in Leite et al. (2019a,b). The three standard coagulometric assays involve, at 37°C, incubating plasma pool (50 μL) with saline (control) or *L. alba* derivatives (extracts or fractions) for 30 min. The specific reagent for each test is then added and the time for clot formation is automatically measured by a DadeBehringBFTII® coagulometer.

Reagents are thromboplastin + calcium (Thromborel®) for prothrombin time; activated cephaloplastin and calcium chloride (Dade Actin®) for the activated partial thromboplastin time; and thrombin (Trinity Biotech®) for plasma fibrinogen measurement. Intra-assay coefficients of variation for prothrombin time, activated partial thromboplastin time, plasma fibrinogen measurement, and thrombin generation assay were respectively 1.29, 1.49, 2.52, and 0.34%, demonstrating the reproducibility of the tests.

Thrombin generation assay was performed with the tissue factor in two concentrations, high tissue factor and low tissue factor; and for the analysis of the results, only the endogenous thrombin potential (ETP) parameter was used. This represents the thrombin formed in the whole process of coagulation and therefore represents better clinical results.

2.5. Statistical analysis

Data were analyzed in the Statistical Package for the Social Sciences (SPSS) version 13.0, and experimental data were expressed as mean ± SD. One-way analysis of variance (ANOVA) was used to investigate whether there were significant differences among the treatments with plant extract. A post-ANOVA Tukey (HSD) multiple mean comparison test was carried out to assess the specificity of the differences. Differences were considered significant with p-value less than 0.05. All the tests were performed in quadruple with similar results and are thus representative of the experiments.

3. Results and discussion

Lippia alba (lemon balm) is a plant widely used for many purposes, but its main use is in the treatment against mild to moderate anxiety and insomnia (Hennebelle et al., 2008). It was reported to be one of the most used plants in patients of an anticoagulant clinic in Brazil, a possible additional risk factor in relation to the potential interaction between herbal medicines and warfarin (Leite et al., 2016a, 2016b; 2018).

Anticoagulation pharmacotherapy has several safety limitations, especially in relation to interactions. In this sense, interactions between drugs and herbs are one of the major clinical and economic concerns for the health system, and the most commonly reported interaction in adults, in general, is the risk of bleeding due to the use of garlic, ginkgo, and ginseng associated with aspirin, warfarin, and other antithrombotic agents (Agbabiaka et al., 2017).

Therefore, the study of the potential interaction between lemon balm and anticoagulants is important to improve the clinical management of these patients. And, in addition, considering the potential of this herb for antioxidant, antiplatelet, and anti-inflammatory activities, it can also be employed in the discovery of new targets and mechanisms of action for the development of new antithrombotics.

3.1. Ethanolic extract of Lippia alba

In all prothrombin time, activated partial thromboplastin time, and plasma fibrinogen measurement, ethanolic extract of *L. alba* increased coagulation time at all the used concentrations (Fig. 1), indicating inhibition of both intrinsic and extrinsic pathways of coagulation. Thus, the analysis of the results allows us to presume that the ethanolic extract of *L. alba* led to a lower formation of fibrin compared to the control.

Although widely used in clinical practice, coagulation times determined by traditional coagulometric tests correspond only to the initiation phase of coagulation. The end point of these tests occurs with 5% of the thrombin formed in the whole coagulation process. However, thrombin generation assay is an alternative, as it directly measures the total amount of thrombin formed and provides more information about the coagulation process (Duarte et al., 2017, 2018, 2019).

The addition of ethanolic extract of the leaves of *L. alba* in the plasma resulted in a decrease of thrombin formation, expressed in ETP, in all the concentrations, using both high and low tissue factors (Figs. 2–4), which indicates inhibition of the extrinsic coagulation pathway and corroborates with results obtained in the standard coagulometric tests. Also tested was the ethanolic extract of *L. alba* flowers, which significantly reduced the ETP. No statistically significant difference was detected in the comparison of ethanolic extracts from leaves versus flowers.

Micromolecular chemistry of *L. alba* was evaluated to investigate the

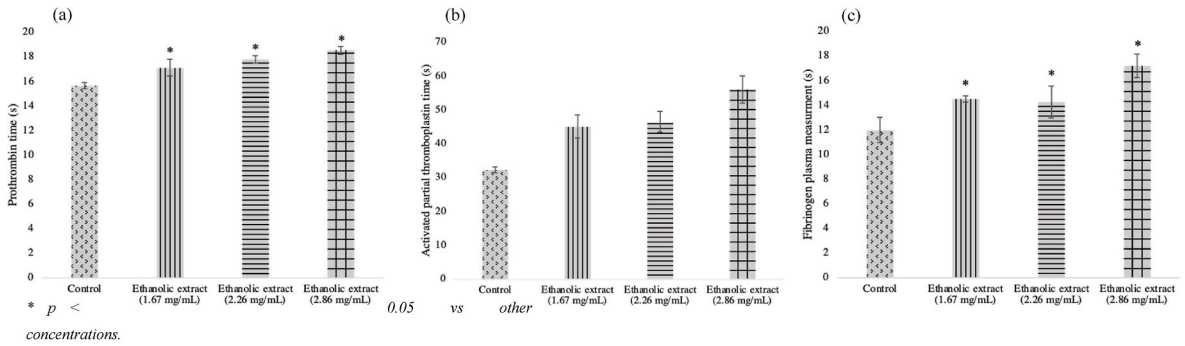


Fig. 1. Anticoagulant activity of ethanol extract of leaves from *Lippia alba* demonstrated by Prothrombin time (a), Activated partial thromboplastin time (b) and Fibrinogen plasma measurement (c).

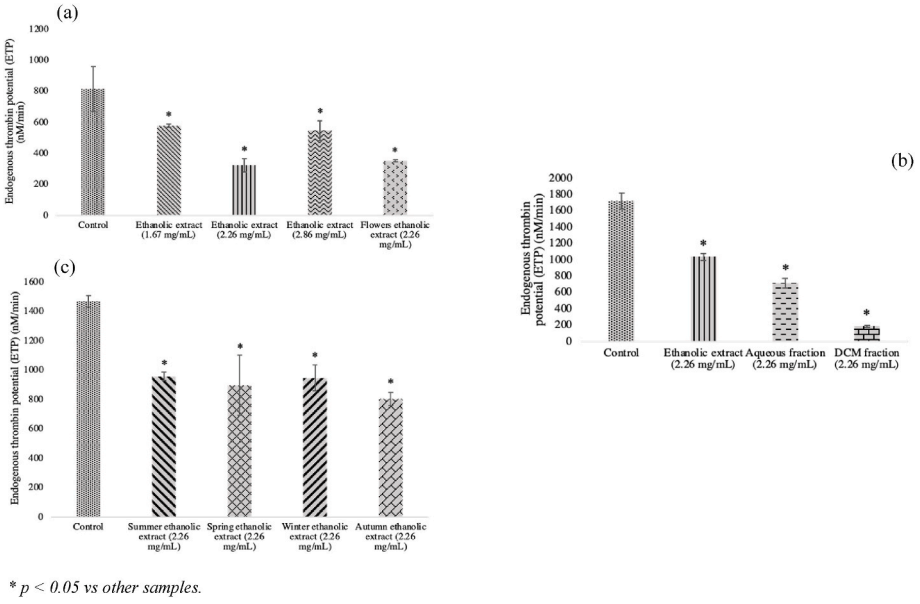


Fig. 2. Anticoagulant activity of extracts and fractions from *Lippia alba* demonstrated by ETP parameter (area under the curve) of thrombin generation using high tissue factor in (a) different concentrations of ethanolic extract of leaves and ethanolic extract of flowers, (b) ethanolic extract and its fractions and (c) ethanolic extract from leaves collected in the 4 seasons of the year.

compounds related to the anticoagulant activity observed. In leaves ethanolic extract, terpenes and flavonoids were detected in the TCL analysis, as well as flavonoids by UPLC chromatogram. The presence of several phenylpropanoids was also observed, due to the characteristic ultraviolet (UV) spectra. These data corroborate with the data of the scientific literature (Hennebelle et al., 2008).

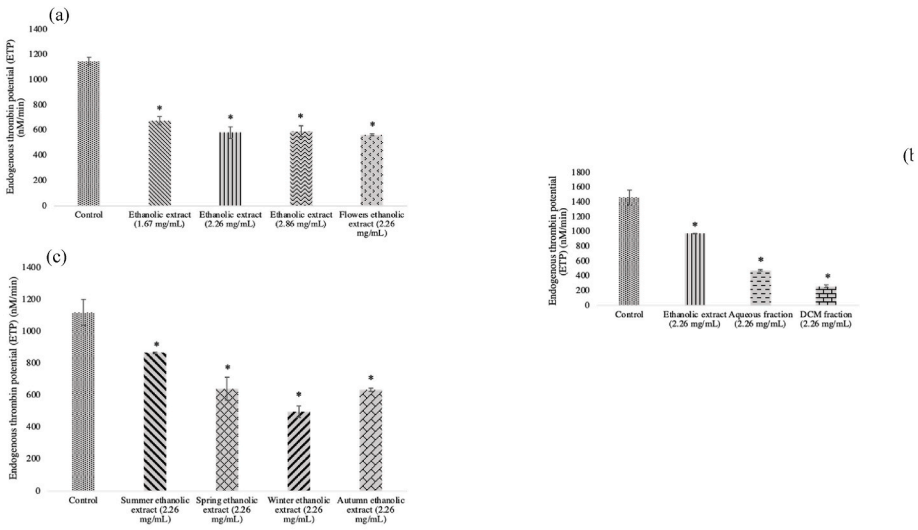
In the ESI-MS analysis, using the fragmentation profile and literature data, it was also possible to identify these classes of compounds, as well as representatives of iridoids, such as geniposidic acid (Table 2). Thirteen substances were identified (Fig. 5): the iridoids shanzhiside - [M-H$^-$] = 391 (1) and geniposidic acid, [M-H$^-$] = 373 (2); the phenylpropanoids decafeoylacteoside - [M-H$^-$] = 461 (3), beta-hydroxyacteoside - [M-H$^-$] = 639 (4), calceolarioside E – [M-H$^-$] = 609 (5), verbascoside - [M-H$^-$] = 623 (6), isoverbascoside - [M-H$^-$] = 623 (7), and martynoside - [M-H$^-$] = 651 (8); the flavonoids spinacetin - [M-H$^-$] = 345 (9), apigenin - [M-H$^-$] = 269 (10), 6-methoxyapigenin - [M-H$^-$] = 299 (11), 5,7,4′-trihydroxy-3,6-dimethoxyflavone - [M-H$^-$] = 329 (12) and 7-dihydroxy-6,4′-dimethoxyflavone - [M-H$^-$] = 313 (13). All of these substances have already been reported in the literature for the species *L. alba* (Funari et al., 2012; Hennebelle et al., 2006b; Trevisan et al., 2016). In addition, apigenin was identified by co-injection experiment (Supplementary material 2).

3.2. Fractionation of ethanolic extract of Lippia alba

Then, the fractionation of the ethanolic extract from *L. alba* was performed to better understand the substances responsible for the observed anticoagulant activity. In TCL, flavonoids were detected in the ethanolic extract and in all fractions, while terpenes were detected only in the ethanol extract and the DCM fraction. In the UPLC analysis, iridoids, phenylpropanoids, and some of the most polar flavonoids were found in the aqueous fraction; and in DCM fraction were some less polar phenylpropanoids and many flavonoids, as expected according to the polarity of these compounds (Supplementary Material 2).

The major peak of this aqueous fraction corresponds to a phenylpropanoid, probably a derivative of verbascoside. The major peak in the DCM fraction as well as in the ethanol extract was compound A, suggesting that it may be one of the compounds related to the observed activity. Compared to the ethanolic extract, it is clear that these fractions were more concentrated in phenylpropanoids and flavonoids, suggesting that these two classes of compounds also contribute a great deal to the activity. It is important to emphasize that this was reproduced in the two concentrations of tissue factor used (Figs. 2 and 3); that is, the extrinsic and instrinsic coagulation pathways seem to have been affected in the same way by the extract and its fractions.

Using high and low tissue factores, both aqueous and



*$p < 0.05$ vs other samples.

Fig. 3. Anticoagulant activity of extracts and fractions from *Lippia alba* demonstrated by ETP parameter (area under the curve) of thrombin generation using low tissue factor in (a) different concentrations of ethanolic extract of leaves and ethanolic extract of flowers, (b) ethanolic extract and its fractions and (c) ethanolic extract from leaves collected in the 4 seasons of the year.

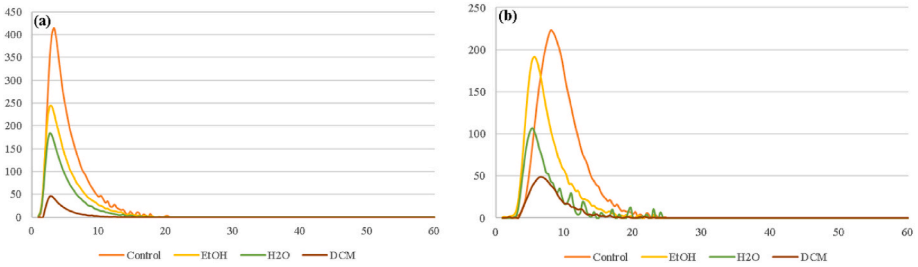


Fig. 4. Thrombin generation curves for control plasma pool and plasma pool added with ethanolic extract (EtOH) of leaves from *L. alba* and the fractions obtained in the fractionation: water (H_20) and dichloromethane (DCM), using (a) High tissue factor, and (b) Low tissue factor.

dichloromethane fractions reduced ETP more than *L. alba* ethanolic extract. As expected, the chemical composition of the ethanolic extract is more complex and phenylpropanoids, flavonoids, and compound A were detected. In the aqueous fraction of this extract, there was a predominance of phenylpropanoids (66%), while in the DCM fraction, flavonoids and compound A represented about 30% each.

Analysis of the micromolecular chemistry and anticoagulant activity of the extract and its fractions also revealed the important role of phenylpropanoids, flavonoids, and compound A in decreasing ETP compared to control, using both high and low tissue factors. It is important to note that the quantity of these substances in the sample is also decisive for the activity, as it was observed that the aqueous fraction containing predominantly phenylpropanoids was more active than the ethanolic extract, as well as the DCM fraction rich in flavonoids and compound A, which was more anticolagulant than ethanol extract and aqueous fraction.

3.3. Seasonal variation of Lippia alba

L. alba is a plant known to have variable chemical composition. Its essential oil, for example, has even been studied for seasonal influence (Gomes et al., 2019). In this context, as biological activity is related to chemical composition, the *L. alba* ethanolic extract collected in the four seasons were evaluated to analyze the chemical composition and its impact on anticoagulant activity.

From the chromatographic profile obtained by UPLC, it is clear that the chemical composition of the four extracts is similar qualitatively, but not quantitatively, and this is reflected in the anticoagulant activity (Supplementary material 3). All extracts showed 2 major peaks, one with retention time of 6 min and characteristic UV of phenylpropanoid and the other for compound A. In addition, the four samples had a large presence of phenylpropanoids and flavonoids in different proportions.

Also, in thrombin generation assay, ethanolic extract of *L. alba* leaves collected in the four seasons of the year (summer, autumn, winter, and spring) was elaborated to evaluate the effect of climatic conditions on the chemical composition of the plant and anticoagulant activity. All extracts reduced ETP, but there was a difference in anticoagulant activity among the extracts of *L. alba* collected in the four seasons (Figs. 2 and 3). This difference was more expressive with the use of low tissue factor.

When using high tissue factor, ethanolic extracts reduced ETP in a similar way, but no statistically significant difference between the summer and autumn collections was observed, which were, respectively, the extracts that least and most reduced ETP. In the use of low tissue factor, there was a greater difference between the four collections: the extract of *L. alba* that presented the highest anticoagulant potential was the winter collection and the one with the lowest anticoagulant potential was the summer collection. No significant difference was observed between plant extracts collected in spring and autumn.

The difference in chemical composition throughout the year was already expected since plant secondary metabolism responds to environmental conditions, such as solar radiation, water availability, altitude, plant maturity, among others (Dewick, 2009). As there is variation throughout the year mainly in relation to temperature and rainfall, it

Table 2
Peaks assignments and UPLC-ESI-MS fragmentation data obtained for the constituents of the leaves of ethanol extract from *L. alba*.

Peak	Name	Rt (min)	Measured mass (negative mode) $[M-H]^-$	Molecular formula	Reference
1	Shanzhiside	1.8	391.1533	$C_{16}H_{24}O_{11}$	(Hennebelle et al., 2006a, 2006b)
2	Geniposidic acid	2.0	373.1417	$C_{16}H_{22}O_{10}$	Timóteo et al. (2015)
3	Decafeoylacteoside	2.2	461.1983	$C_{20}H_{30}O_{12}$	Timóteo et al. (2015)
4	Beta-hydroxyacteoside	4.6	639.2348	$C_{29}H_{36}O_{16}$	Timóteo et al. (2015)
5	Calceolarioside	5.3	609.2237	$C_{28}H_{34}O_{15}$	Timóteo et al. (2015)
6	Verbascoside	5.5	623.2217	$C_{29}H_{36}O_{15}$	Funari et al. (2012)
7	Isoverbascoside	6.0	623.2394	$C_{29}H_{36}O_{15}$	Funari et al. (2012)
8	Martynoside	7.5	651.2556	$C_{31}H_{40}O_{15}$	(Hennebelle et al., 2006a, 2006b)
9	Spinacetin	8.6	345.0768	$C_{17}H_{14}O_8$	Trevisan et al. (2016)
10	Apigenin	9.5	269.0581	$C_{15}H_{10}O_5$	Funari et al. (2012)
11	6-methoxyapigenin	9.7	299.0704	$C_{16}H_{12}O_6$	Trevisan et al. (2016)
12	5,7,4′-trihydroxy-3,6-dimethoxyflavone	9.9	329.0819	$C_{17}H_{14}O_7$	Trevisan et al. (2016)
13	5,7-dihydroxy-6,4′-dimethoxyflavone	11.1	313.0868	$C_{17}H_{14}O_6$	Trevisan et al. (2016)

Note: Chromatographic conditions: Acquity UPLC® BEH C18 column, at 40°C, λ 280 nm, flow rate 0.1 ml/min, scan mode positive and negative within the mass range of m/z 100–1000, cone voltage of 5 kV, capillary voltage of 3.5 kV, nebulizer gas nitrogen, capillary temperature of 320°C, and desolvation temperature of 320°C.

was expected that the chemical composition of the plant would also vary according to its response to these stimuli.

It was also observed that anticoagulant activity varied in response to changes in chemical composition. And once again, the joint importance of phenylpropanoids, flavonoids, and compound A for the anticoagulant activity was perceived. Low tissue factor analysis clearly showed that the *L. alba* that demonstrated the highest activity was collected in winter, when the amounts of phenylpropanoids, flavonoids, and compound A were more balanced. In contrast, the predominance of phenylpropanoids (40%) in the summer collection and compound A (48%) in the autumn collection did not demonstrate superior activity.

3.4. Chemistry of L. alba and possible relation to anticoagulant activity

The main chemical classes of substances present in *L. alba*, flavonoids, phenylpropanoids, and iridoids, which may be responsible for the observed activity, are discussed as to their possible anticoagulant effect, but it is very probable that the observed *in vitro* effect was a result of the phytocomplex, various substances acting together (Leite et al., 2016a, 2016b). In addition, a correlation between the chemical composition and anticoagulant activity of different accessions of this medicinal species was recently published (Leite et al., 2021a, 2021b). The chemical complexity of medicinal plants often enables a synergistic effect between the substances present in the plant and produces a superior therapeutic effect in comparison to the isolated compounds (Malongane et al., 2017).

The flavonoids are compounds of mixed biosynthetic origin formed by three 6-membered rings (Dewick, 2009). In the phytochemical analysis, they are the chemical markers because of the massive presence in *L. alba* extracts and fractions (Supplementary material 2). Throughout all the chromatographic profile, characteristic UV spectra are found, mostly flavones (Mabry, 1970). In addition to apigenin, a flavone that was also identified by co-injection, the flavonoids spinacetin, 6-methoxyapigenin, 5,7,4′-trihydroxy-3,6-dimethoxyflavone, and 5,7-dihydroxy-6,4′-dimethoxyflavone were also identified (Table 2).

As previously discussed, based on the scientific literature and considering that flavonoids are significantly present in *L. alba* derivatives, this class of substances has real potential to be responsible for the presented anticoagulant activity. Flavonoids have recognized anti-inflammatory activity and the inflammation pathway is closely related to platelet aggregation and coagulation through arachidonic acid (Leite et al., 2016a, 2016b; 2017).

Apigenin and luteolin, two examples of flavonoids, are commonly found in *L. alba*, and already had antiplatelet activity described because they inhibit platelet adhesion, aggregation, and secretion (Khan et al., 2018), which activate blood coagulation through the release of mediators that trigger the activation of a coagulation cascade through a mechanical pathway. Luteolin also has shown antithrombotic activity *in vitro* and *in vivo*. Structure-activity studies of flavonoids as inhibitors of thrombin have also been described in the literature (Liu et al., 2010). Therefore, based on *in vitro* results and literature data, flavonoids appear to play a major role in reducing thrombin formation in thrombin generation assay.

Phenylpropanoids are another very characteristic class of compounds of *L. alba* and they are also usually glycosylated, so they have high polarity. In the chromatographic profile they can be identified by characteristic UV spectra and some of them have also been identified in ESI-MS such as decafeoylacteoside, beta-hydroxyacteoside, calceolarioside, verbascoside, isoverbascoside, and martynoside (Supplementary material 2) (Table 2) (Funari et al., 2012; Hennebelle et al., 2006b).

In addition to being one of the major compound classes, the anticoagulant potential of this class of compounds is supported by scientific data (Leite et al., 2019b). One study carried out with 4-hydroxycinnamic acid, an example of a phenylpropanoid, demonstrated that it was able to prolong activated partial thromboplastin time and prothrombin time, as well as inhibit factor Xa and thrombin. In other studies, the inhibition of prostanoid secretion and modulation of arachidonate-phospholipid have been demonstrated, suggesting the antiplatelet potential of phenylpropanoids (Nievergelt et al., 2011). Thus, together with flavonoids, phenylpropanoids also appear to contribute to the demonstrated anticoagulant activity.

Iridoids are an important class of monoterpenes that contain a cyclopentan-pyran system. They are commonly found as glycosides, such as those identified in *L. alba* ethanolic extract: shanzhiside and geniposidic acid (Hennebelle et al., 2006b; Hussain et al., 2019; Timóteo et al., 2015), as evidenced in the TLC through selective reagents for terpenes. As the iridoids are more polar compounds, they must be more present in aqueous fractions, besides the ethanolic extract. These fractions are not the most active but do reduce the amount of thrombin formed. In addition, there are data in the literature that support the participation of this class in anticoagulant activity.

Studies show that iridoids reduced arterial thrombus load in a model of carotid artery thrombosis and inhibited collagen-induced platelet aggregation in rats. Another study tested several iridoids in the aracdonic acid pathway and found that most of them inhibit the formation of thromboxane A2, which in turn leads to platelet activation. Finally, a review article demonstrates the various mechanisms by which iridoids can present anti-inflammatory activity (Viljoen et al., 2012). Thus, this class also plays an important role in the decrease of thrombin formation observed in thrombin generation assay.

3.5. The use of Lippia alba in patients taking anticoagulants

In general, based on the ETP results together, it is possible to

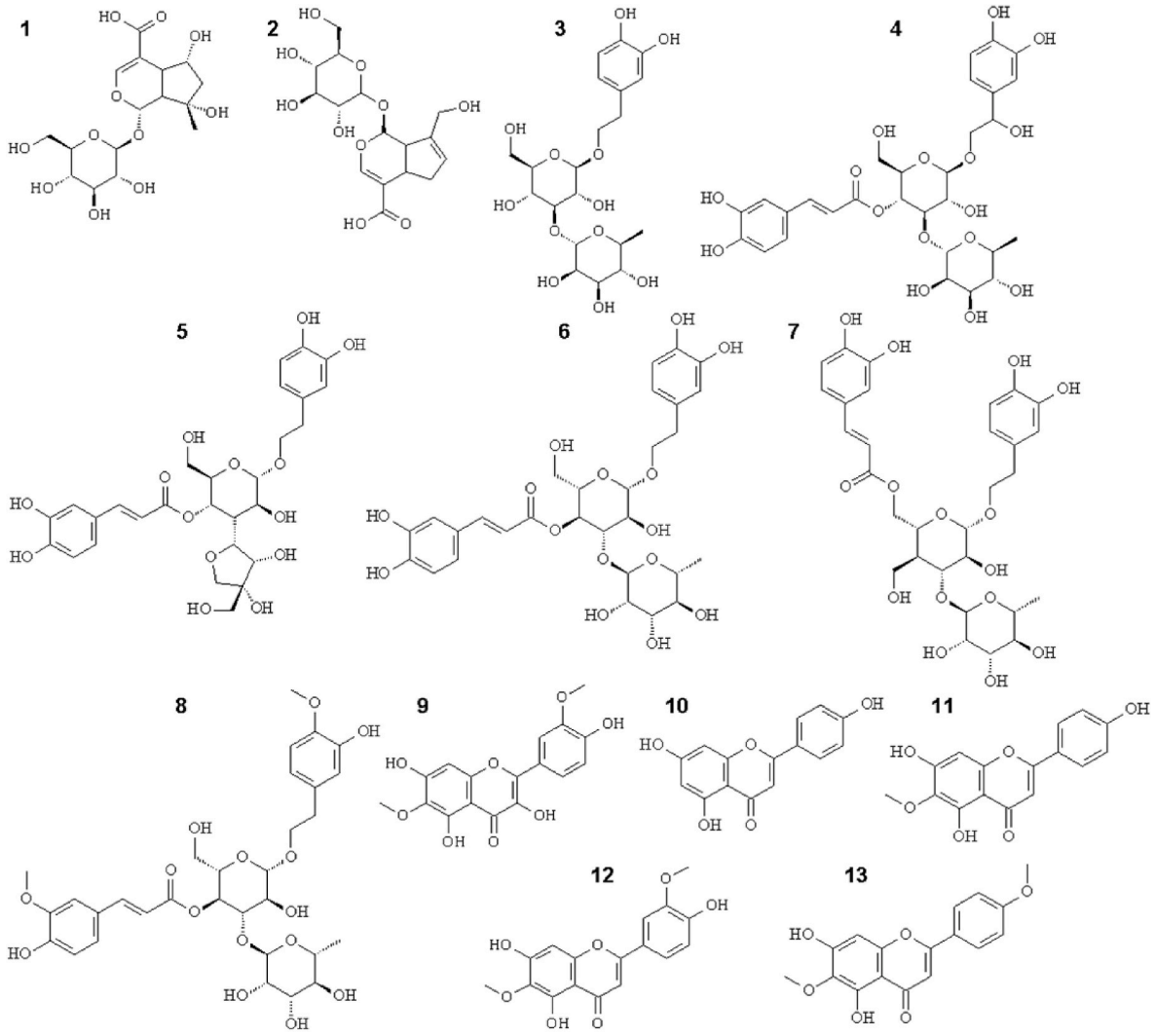


Fig. 5. Chemical structure of the identified substances in ethanolic extract of *Lippia alba*. (1) Shanzhiside**; (2)** Geniposidic acid**; (3)** Decafeoylacteoside**; (4)** Beta-hydroxyacteoside**; (5)** Calceolarioside**; (6)** Verbascoside**; (7)** Isoverbascoside**; (8)** Martynoside**; (9)** Spinacetin**; (10)** Apigenin**; (11)** 6-methoxyapigenin**; (12)** 5,7,4′-trihydroxy-3,6-dimethoxyflavone**; (13)** 5,7-dihydroxy-6,4′-dimethoxyflavone.

conclude that *L. alba* in various ways tested, presented anticoagulant activity through the reduction of thrombin formation in the two coagulation pathways. These *in vitro* results indicate that, if this effect is reproduced *in vivo*, there is a great potential for interaction between lemon balm and anticoagulants, which may result in an increased risk of bleeding in patients who use both in a concurrent manner.

Lippia alba is a plant with widespread use for sedative purposes and was the most cited species in a study that analyzed plant consumption habits by patients using warfarin. In general, it is considered a safe plant due to the traditional consolidated use and there are not many reported contraindications, only use with caution in hypotensive people or patients using hypotension because of the potential to enhance its effect (Leite et al., 2018). Thus, there is no information of the compelling use of this plant species and anticoagulants, such as warfarin. This is probably because this species is still mainly used in folk medicine. Thus, the lack of studies does not mean that it is safe to use anticoagulants and *L. alba*.

Given the widespread use of this plant in patients of the anticoagulation clinic and scientific data demonstrating that some compounds present in the genus *Lippia* have anticoagulant and high platelet activities (Oliveira et al., 2014), further study of the possible interaction between *Lippia alba* and anticoagulants is essential.

Due to the complexity of the warfarin treatment, the patients in use of this drug should be followed up in anticoagulation clinics, where the clinical management and dose adjustment of this drug can be performed (Ageno et al., 2012; Witt et al., 2016). Such clinics also manage interactions with other medicines and with foods such as dark green leafy vegetables because of the high amount of vitamin K. However, interactions with medicinal plants are often neglected, mainly due to lack of professional training and scientific background (Leite et al., 2016a, 2016b; 2018, 2021b; Vazquez, 2018).

Clinical management of interactions, however, can be the best way to improve the quality of treatment offered to the patient in this sense. And, despite the deficiency of robust scientific information on herb-drug interactions, the evaluation of the possible interaction can begin based on data of preliminary studies such as *in vitro*, case reports, and speculation based on chemical composition of the herb (Leite et al., 2016a, 2016b; 2021b; Vazquez, 2018). In clinical practice, this allows the health professional to pay more attention to the use of medicinal plants with real potential interaction with anticoagulants and critically evaluate the available information along with the patient's clinical data (Leite et al., 2021b). That is, the use of *L. alba* among anticoagulated patients, for example, does not need to be discontinued, but such patients should be treated more cautiously.

3.6. The potential of Lippia alba as an antithrombotic agent

Thromboembolic disorders are characterized by the formation of clots in the blood vessel and are one of the major causes of cardiovascular diseases. Cardiovascular diseases, however, is often preventable with changes in lifestyle. And, as reported by Virchow in 1856, a triad of causes lead to thrombosis: stasis, changes in the vessel wall, and blood coagulability. Thus, some biological activities that may aid in the prevention of these causes are antioxidant, anti-inflammatory, antiplatelet, and anticoagulant activities (Esmon, 2003).

The use of medicinal plants such as antithrombotics, in this context, is extremely favorable, as the habit of using tea could be combined with the prevention of cardiovascular diseases, which are the major cause of death worldwide. According to the chemical composition of *L. alba* and the results of the coagulometric tests performed, this plant has the potential to perform these activities to prevent thrombus formation.

Iridoids have demonstrated antiplatelet and anti-inflammatory activities and another study also showed that iridoids can help phenolic compounds in their antioxidant activity (Kucharska et al., 2017). Phenylpropanoids, in turn, have already demonstrated potential anticoagulant and antiplatelet activity (Nievergelt et al., 2011). And at last, flavonoids are highly antioxidant and anti-inflammatory compounds that have been described as antiplatelets and anticoagulants too (Gomes et al., 2008).

Moreover, it can be said that the chemical composition of *L. alba* is quite similar to that of the leaves of the olive tree (*Olea europaea*): a large amount of phenylpropanoids such as verbascoside and derivatives, and a large amount of flavonoids, as well as secoiridoids. *O. europaea* is well known for its ability to prevent cardiovascular disease and is one of the plants with the greatest therapeutic potential (El and Karakaya, 2009). According to these data, then, the plant species *Lippia alba* has real potential to perform antioxidant, anti-inflammatory, antiplatelet, and anticoagulant activities. Thus, the deepening of these studies could lead to the development of an easily accessible antithrombotic for the population.

Ethical approval

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Ethics Committee of UFMG: CAAE number 60904316.6.0000.5149) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Funding

This work was funded by Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES), Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Fundação de Amparo a Pesquisa do Estado de Minas Gerais (FAPEMIG), Pró-Reitoria de Pesquisa da UFMG, and Laboratório de Farmacognosia e Homeopatia daUFMG (GnosiaH) Brazil.

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

ABBREVIATION LIST

ANOVA	One-Way Analysis of Variance
CAT	Calibrated Automated Thrombogram
DAD	Diode Array Detector
DCM	Dichloromethane
ESI-MS	Electrospray Ionization Mass Spectrometry
ETP	Endogenous Thrombin Potential
SPSS	Statistical Package for the Social Sciences
TLC	Thin Layer Chromatography
UFMG	Universidade Federal de Minas Gerais
UPLC	Ultra-Performance Liquid Chromatography
UV	Ultraviolet

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jep.2022.115744.

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