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Essential Oils from *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare*: Inhibition of Phospholipase A_2 and Cytotoxicity to Human Erythrocytes

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Abstract

The essential oils from *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare* were extracted by hydrodistillation and characterized and quantified by GC-MS and GC-DIC. The oils induced hemolysis with all the doses evaluated (0.6 to 1.8 µL), and the diameters of the halos varied between 9 and 15 mm. Pre-incubation of *P. boldus* oil with *Bothrops jararacussu* venom resulted in potentiation of venom-induced hemolysis (30%) (proteases and phospholipases A_2). The essential oil from *M. piperita* (0.6 µL) inhibited venom-induced hemolysis by 45%, whereas 0.6 µL of *R. officinalis* oil increased the hemolysis by 20%. For the essential oil from *F. vulgare*, 100% inhibition of activity (0.6 and 1.2 µL) was observed. The application of *C. citratus* oil induced hemolysis with all the volumes evaluated. Phospholipase activity induced by the venom was only inhibited (10%) with the 0.6 µL volume of *R. officinalis* oil. The oils from *M. piperita* and *F. vulgare* (1.8 µL) and *C. citratus* oil (0.6 µL) potentiated the phospholipase activity. The results highlight the need for a broad characterization and regulation of the use of natural products, because they can have therapeutic or toxic actions.

Keywords

Natural Products, Snake Venoms, Hemolysis, Pharmacological Potential

1. Introduction

Studies have shown that lipophilic substances can often induce hemolysis because they are capable of destabilizing the lipid bilayers present in cell membranes, causing lysis of erythrocytes and increasing plasma hemoglobin levels. This effect can result in several complications: hemolytic anemia, multiple organ failure and even death [1]. However, many natural compounds have properties that cause the reduction in the membrane fluidity of erythrocytes, reducing hemolytic processes that lead to lower blood viscosity. This property has a potential for pharmaceutical use.

Hemolysis is a process of destruction of red blood cells (erythrocytes) in which the rupture of the plasma membrane occurs, resulting in the release of hemoglobin and causing serious damage to vital organs such as liver, kidneys and heart. Hemolysis is caused not only by chemical compounds such as penicillin, methyldopa, some types of antibiotics and anti-inflammatory agents, but also by natural compounds such as animal venoms. Several plant extracts with hemolytic activity have been described, some of which are cytotoxic or genotoxic, making it necessary to perform pharmacological and toxicological analyses of essential oils and plant extracts [2].

The phospholipids constituting the membranes can be degraded by numerous substances, including natural compounds, and they can interact with different compounds, resulting in destabilization of the membranes and alteration in the flow of liquids and ions through the membranes. These substances might also act by inhibiting the action of phospholipases from various sources, both animal and human, thereby exerting anti-inflammatory activity and interfering in processes such as blood coagulation and platelet aggregation. The phospholipids constituting the membranes can be degraded by numerous substances, including natural compounds, and they can interact with different compounds, resulting in destabilization of the membranes and alteration in the flow of liquids and ions through the membranes. These substances might also act by inhibiting the action of phospholipases from various sources, both animal and human, exerting anti-inflammatory activities and interfering in processes such as blood coagulation and platelet aggregation. These processes are closely related to the action of eicosanoids generated from arachidonic acid, one of the products of the breakdown of phospholipids [3].

Ophidic poisoning has been of great concern for public health, especially in tropical and neotropical countries, both because of the incidence and the action of venoms on living organisms [4]. However, snake venoms are mixtures of substances, mainly proteins (for example, phospholipases A_2 and proteases), that have various biological activities such as enzymatic, myotoxic, cardiotoxic and cytotoxic activities [5].

Phospholipases A_2 (PLA_2) and proteases (metalloproteases and serinoproteases), present in venoms of snakes of the *Bothrops* genus, can act directly on erythrocytes, myocytes, blood coagulation cascade factors, epithelial cells and vascular

endothelium, causing severe physiological disorganization and resulting in coagulation or intravascular hemolysis or predisposing the organism to the development of diseases [6]. Venoms have been widely used as laboratory tools for physiological studies and for the characterization of several compounds, mainly of vegetal origin, as exemplified by plant extracts, plant drugs and essential oils. The aim of this study was to evaluate the enzymatic inhibition of phospholipases A_2 and cytotoxicity to human erythrocytes using the essential oils from *M. piperita*, *C. citratus*, *R. officinalis*, *P. boldus* and *F. vulgare*.

2. Material and Methods

2.1. Extraction, Identification and Quantification of Essential Oils

The essential oils from *M. piperita*, *C. citratus*, *R. officinalis*, *P. boldus* and *F. vulgare* were extracted in the Laboratory of Essential Oils of the Department of Chemistry of the Federal University of Lavras by hydrodistillation over a 2-h period using a modified Clevenger apparatus. They were identified by gas chromatography coupled to a mass spectrometer (CG-MS) and quantified by gas chromatography coupled to a flame ionization detector (FID) according to the procedure previously described by Rezende *et al.* (2017) [7].

2.2. Hemolytic Activity: Cytotoxicity to Human Erythrocytes

The analyses involving human blood were approved by the Human Research Ethics Committee (COEP) of the Federal University of Lavras, with registration number 48793115.0.0000.5148. The erythrocyte suspension was prepared using 10 mL of blood collected in tubes containing sodium citrate, which were centrifuged for 10 min at 4˚C and 2500 g. After centrifugation, the plasma was removed, and the erythrocytes were suspended in phosphate-buffered saline (PBS) (pH = 7.2 - 7.4) and centrifuged again under the same conditions. This procedure was repeated three times to obtain a packed red blood cell pellet.

The analysis of the hemolytic activity of the essential oils was accomplished using the methodology of Price, Wilkinson and Gentry (1982) [8], with modifications. The medium was prepared with 1% agar in PBS and 0.01 M calcium chloride, 0.1 g sodium azide, and 1% blood erythrocytes. Cavities 3 mm in diameter were made in the medium after solidification in Petri dishes for the application of 0.6, 1.2 and 1.8 µL aliquots of the essential oils, and the plates remained in a cell culture chamber at 37˚C for 16 hours.

The essential oils (0.6, 1.2 and 1.8 µL aliquots) were incubated with the *Bothrops jararacussu* venom in a water bath at 37˚C for 30 minutes to evaluate a possible inhibitory action of the oils on the hemolysis induced by the venom. The formation of a translucent halo around the cavity in the gel is indicative of activity, and this halo was measured in millimeters for the quantification of hemolytic activity.

2.3. Activity of Phospholipase A_2

The phospolipase activity was determined in a solid medium in accordance with

the method described by Gutiérrez *et al.* (1988) [9]. A gel similar to that described for the determination of hemolytic activity was prepared except that 1% lecithin from egg yolk was substituted for the erythrocytes. After solidification of the medium, cavities 3 mm in diameter were prepared for appliction of the samples.

The essential oils (0.6, 1.2 and 1.8 μL) were incubated with *B. jararacusu* venom in a water bath at 37˚C for 30 minutes and then applied to the plates, which were maintained for 16 hours at 37˚C in a cell culture chamber. The formation of a translucent halo around the hole in the gel was indicative of activity, and this halo was measured in millimeters for the quantification of phospholipase activity.

2.4. Statistical Analysis

For the cytotoxic and phospholipase activities, the test was performed by comparing averages one at a time (each volume was separately compared to the control). The data were submitted to analysis of variance, and the means were compared by the Scott-Knott test at the 5% probability level. The statistical program used was SISVAR [10].

3. Results and Discussion

According to Rezende *et al.* (7), the main constituents of the *M. piperita*, were carvone (84.34%) and limonene (10.97%). The main constituents of the essential oil from *C. citratus* were geraniale (47.74%), neral (35.43%) and myrcene (8.46%). The main constituents in the essential oil from *R. officinalis* were 1,8-cineole (62.26%), camphor (17.34%) and *α*-pinene (9.07%). The main components of the essential oil from *P. boldus* were *α*-terpinyl formate (61.99%), *p*-cymene (15.45%), 1,8-cineole (10.59%), ascaridol (2.73%), and terpinen-4-ol (2.03%), and the main component of the essential oil from *F. vulgare* was methyl chavicol, also known as estragole (89.48%), followed by limonene (6.15%) and fenchone (3.80%).

In **Figure 1**, the classes of constituents present in the essential oil from *M. piperita* can be observed. The oxygenated monoterpene class is predominant (86%). The essential oil from *M. piperita* (mint) contained carvone and limonene as the main components.

The essential oil from *C. citratus* contained approximately 91% monoterpenes, as can be observed in **Figure 2**. The essential oil from *C. citratus* (lemongrass) contained geranial, neral and myrcene as the main constituents.

The classes to which the constituents of the essential oil of *R. officinalis* belong, being 87% oxygenated monoterpenes, are presented in **Figure 3**. The classes of constituents found in the essential oil of *P. boldus* are shown in **Figure 4**. The essential oil of *F. vulgare* was the only one that presented a component belonging to the class of phenylpropanoides in its composition (**Figure 5**). This component represented 90% of the oil.

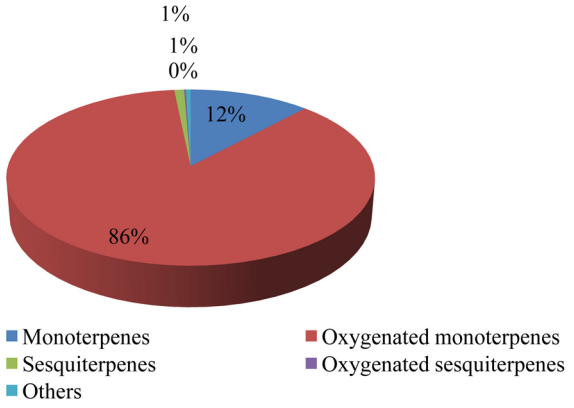

Figure 1. Classification of the constituents of the essential oil from *Mentha piperita*.

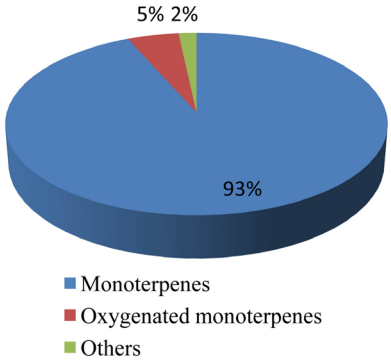

Figure 2. Classification of the constituents of the essential oil from *Cymbopogon citratus*.

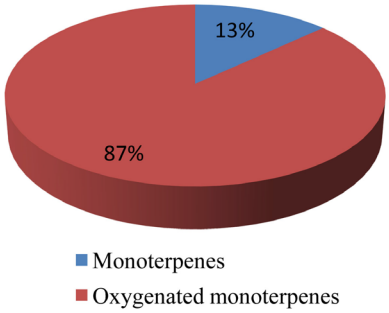

Figure 3. Classification of the constituents of the essential oil from *Rosmarinus officinalis*.

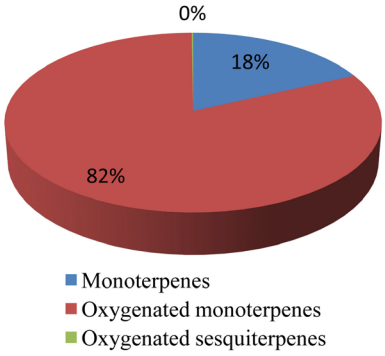

Figure 4. Classification of the constituents of the essential oil from *Peumus boldus*.

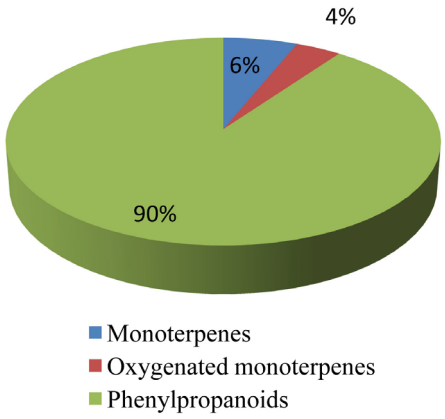

Figure 5. Classification of the constituents of the essential oil from *Foeniculum vulgare*.

3.1. Hemolytic Activity: Cytotoxicity to Human Erythrocytes

With the exception of the essential oil from *R. officinalis*, all the oils evaluated induced hemolysis (**Figure 6**); halos between 9 and 15 mm in diameter were observed for the oils from *C. citratus* and *P. boldus*. The 15 mm halo, referring to the 1.8 µL volume of oil from *P. boldus*, does not differ significantly from the control containing only venom.

Pre-incubation of the essential oil from *P. boldus* with *B. jararacussu* venom resulted in potentiation of hemolytic activity by approximately 30% for all the volumes of oil evaluated (**Figure 7**). An inhibition of approximately 45% occurred with the 0.6-µL aliquot of the essential oil from *M. piperita*, whereas the 1.2- and 1.8-µL volumes potentiated the action of the hemolytic enzymes present in the venom, represented mainly by proteases and phospholipases A_2.

Contrary to the oil from *R. officinalis*, the lowest volume (0.6 µL) of *M. piperita* oil evaluated potentiated the lithotripsy induced by venom (20%) and inhibited this activity (35%) when the highest volumes (1.2- and 1.8-µL) were tested. For the essential oil from *F. vulgare*, 100% inhibition was observed with the 0.6- and 1.2-µL volumes. However, the essential oil from *C. citratus* did not significantly alter the hemolytic activity induced by the venom (**Table 1**). These differentiated activities for each essential oil can be explained by the difference in their compositions.

The different performances of the essential oils evaluated for the hemolytic activity induced by *B. jararacussu* venom are presented in **Table 1**. The data suggest the presence of specific interactions between the constituents of some oils and the hemolytic toxins present in the venom because potentiation was not observed for *C. citratus* oil (hemolytic in volumes of 0.6-, 1.2- and 1.8-µL) and *F. vulgare* (hemolytic with the 1.8 µL volume). Potentiation would be expected if there was a sum of the effect of the oils with that of the venom. In addition, significant potentiation was observed with the 0.6-µL volume of *R. officinalis* oil and significant inhibition with the 1.2- and 1.8-µL aliquots. These results differ from those observed with the oil from *M. piperita*, which caused inhibition at the lowest volume evaluated and potentiation at higher volumes.

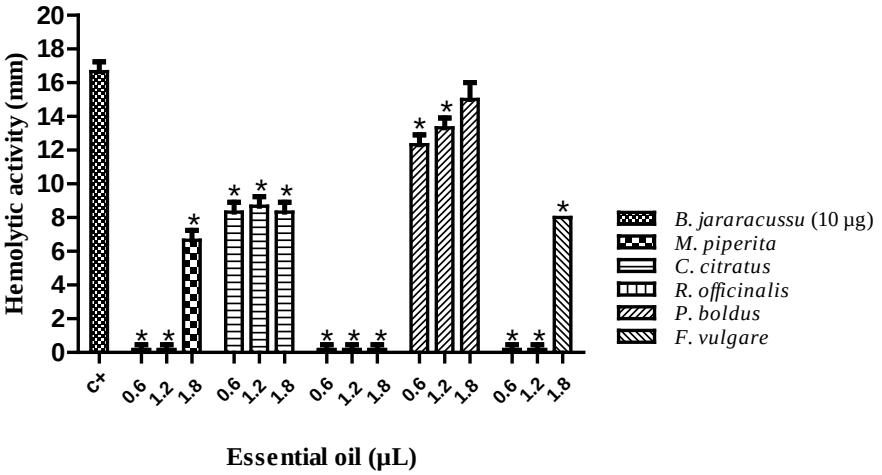

Figure 6. Evaluation of hemolytic activity against human erythrocytes induced by essential oils from *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare* alone and by the *Bothrops jararacussu* venom. *Differ from the control containing only venom by the Scott-Knott test at 5% of significance.

Table 1. Quantitative data of the effect of the essential oils of *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare* on the hemolytic activity induced by *Bothrops jararacussu* venom on human erythrocytes.

Essential oil (µL)		*Bothrops jararacussu* snake venom (10 µg)	
		% inhibition	% potentialization
M. piperita	0.6	45*	-
	1.2	-	10*
	1.8	-	15*
C. citratus	0.6	0*	0*
	1.2	0*	0*
	1.8	0*	0*
R. officinalis	0.6	-	20**
	1.2	35**	-
	1.8	35**	-
P. boldus	0.6	-	30**
	1.2	-	30**
	1.8	-	30**
F. vulgare	0.6	100**	-
	1.2	100**	-
	1.8	0*	0*

*0% value represents the absence of an effect in tests in which the oils did not inhibit or potentiate the action of the venom. **Differ from the positive control (activity of the snake venom considered as 100%) at 5% of significance.

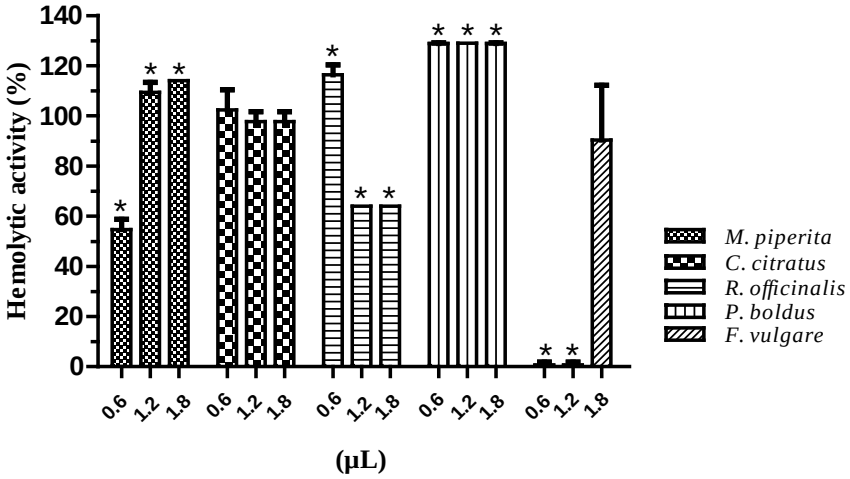

Figure 7. Effect of the essential oils from *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare* on the hemolytic activity induced by *Bothrops jararacussu* venom (10 µg) in human erythrocytes after incubation of the oils with the venom at 37˚C for 30 minutes. The values obtained for the pure venom were considered to represent 100% of activity. *Differs from the positive control by the Scott-Knott test at 5% significance.

The total or partial inhibition of hemolytic activity may be related mainly to the action of phospholipases A_2, which may represent the presence or absence of interactions between the molecules present in the toxins and essential oil constituents, as well as the possibility that the inhibitory action might also result from antioxidant mechanisms [11]. These mechanisms are closely related to the number of molecules (enzymes and active plant compounds) present in the reaction environment and justify the different actions (inhibitory, potentiating or no effect) observed for the various oil volumes analyzed.

3.2. Phospholipase A_2 Activity

According to **Figure 8**, the action of the phospholipases A_2 present in the venom was potentiated only by the 1.8-µL aliquots of the essential oils from *M. piperita* and *F. vulgare* and the 0.6-µL volume of the *C. citratus* oil. The smallest volume evaluated (0.6-µL) of the essential oil from *R. officinalis* caused a 10% inhibition of the phospholipase activity, whereas the essential oil from *P. boldus* did not alter the activity induced by the venom.

Some studies have described the action of plant compounds on the different classes of enzymes present in snake venoms. Silva *et al.* (2017) [12] evaluated the inhibitory potential of essential oils from *Mentha viridis* (L). L. and *Mentha pulegium* L. on phospholipase A_2 present in snake venoms and observed that both essential oils were able to inhibit the degradation of phospholipids induced by *Bothrops* venoms. The essential oils also presented hemolytic activity, the activity of the oil from *Mentha viridis* (L). L. being observed only at the highest concentrations (14.6 and 29 $\mu L \cdot mL^{-1}$).

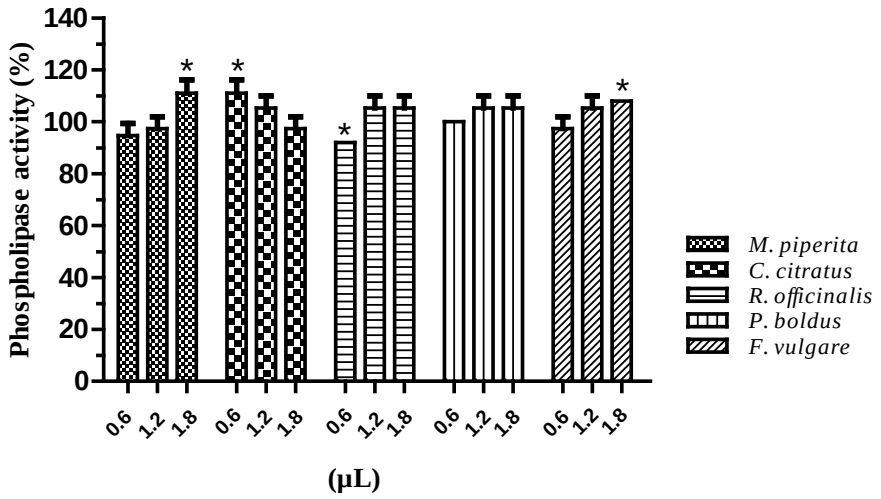

Figure 8. Effect of the essential oils from *Mentha piperita*, *Cymbopogon citratus*, *Rosmarinus officinalis*, *Peumus boldus* and *Foeniculum vulgare* on the phospholipase activity induced by *Bothrops jararacussu* venom (10 µg) after incubation of the venom with the oils at 37˚C for 30 minutes. The values obtained for the pure venom were considered to represent 100% of activity. *Differs from the positive control by the Scott-Knott test at 5% significance.

Miranda *et al.* (2016) [13] investigated the inhibitory properties of the essential oils from *Baccharis dracunculifolia*, *Conyza bonariensis*, *Tithonia diversifolia* and *Ambrosia polystachya* by means of the coagulation and fibrinogenolytic activities induced by *Bothrops* and *Lachesis* snake venoms. They observed that the essential oils exhibited some therapeutic properties because they inhibited the coagulation and fibrinogenolysis induced by the poisons. The authors suggested that the topical use of the oils, in general, does not require specific pharmaceutical preparations and can be applied directly after the extraction. Many oils with antimicrobial, anti-inflammatory and curative properties are described in the literature, and these actions are of great value in the treatment of snake poisonings.

Miranda *et al.* (2014) [14] studied the effect of the essential oil from *Hedychium coronarium* on the fibrinogenolytic and coagulant activities induced by *Bothrops* and *Lachesis* venoms. They observed significant inhibition of the coagulation induced by both venoms, suggesting their possible use as a complementary alternative to serum therapy because the essential oils do not require specific formulations and their topical use can be performed immediately upon extraction.

Yamaguchi and Veiga-Junior (2013) [15] evaluated the hemolytic capacity of essential oils obtained from *Endlicheria citriodora* branches and leaves and observed no damage to the membrane. They also reported that both oils were composed basically of methyl geranate (monoterpenoid ester), which corresponds to 95.15% and 93.75% of the oils from the branches and leaves, respec-

tively.

Phospholipases A_2 in *Bothrops* snake venom induce the hydrolysis of membrane phospholipids and can generate arachidonic acid, which is a precursor of prostaglandins, thromboxanes, leukotrienes and other bioactive lipids that act mainly in inflammatory processes and in the blood coagulation cascade, thereby altering hemostasis [16]. The different venom toxins can be inhibited by several molecules, including chelating agents such as heparin, plasma factors of animal origin and plant extracts [17].

Studies by Silva *et al.* (2017) [12] using *Bothrops* venom showed that both the essential oil from *Mentha pulegium* and that from *Mentha viridis* had an inhibitory effect on phospholipases A_2 on the order of 4.1% at the concentration of 14.6 $\mu L \cdot mL^{-1}$.

In the year 2016, Oliveira *et al.* [11] evaluated the possible interactions between vitamins and enzymes present in *Bothrops atro*x and *Crotalus durissus terrificus* venoms *in vitro*. Inhibition assays for proteolysis, hemolysis, coagulation, and hemagglutination were performed using different proportions of vitamins to inhibit the minimum effective dose of each venom. The authors observed that the vitamins were responsible for the 100% reduction in the cleavage of azocasein by *C.d.t.* poison, induced thrombolysis by the *B. atrox* venom, and also observed the induction of fibrinogenolysis by both poisons.

Oliveira *et al.* (2016) [11] observed possible interactions between the vitamins and the active site of the enzymes. These interactions may occur in the hydrophobic regions present in the enzymes and vitamins, as well as in the inhibitions exerted by the antioxidant mechanism.

According to reports by Borges *et al.* (2000) [18], the aqueous extract of *Casearia sylvestris* inhibited the hemorrhagic activity caused by the venom of several snakes of the *Bothrops* genus. An aqueous extract of *Mandevilla velutina* was an effective inhibitor of phospholipase A_2 and inhibited some of its toxic effects, such as hemorrhage [19].

Carvalho *et al.* (2013) [3] reported the importance of plant species in treating snakebites, especially in places that do not have access to serotherapic treatment. Phospholipases A_2, being among the main constituents of *Bothrops* snake venoms, can be inhibited by components of these plants, such as phenolic compounds, flavonoids, alkaloids, steroids, terpenoids (mono-, di- and triterpenes), and polyphenols (vegetable tannins).

In the present work, approximately 10% inhibition of phospholipases occurred in the presence of the oil from *R. officinalis* (rosemary). This oil is composed of terpenes, alcohols and ethers. This result agrees with the work reported by Mors, Nascimento and Pereira (2000) [20] that demonstrated the inhibitory action of several pentacyclic triterpenes, such as oleanolic acid, lupeol, ursolic acid, taraxerol, taraxasterol, α, β-amirina and friedeline, on snake venoms. Considering that phospholipase activity is only exerted by PLA_2s and that hemolytic activity is exerted by both PLA_2s and proteases, the observed results point to the

presence of specific protease inhibitors in the evaluated oils, especially in the oils from *M. piperita*, *R. officinalis* and *F. vulgare*, which induced significant inhibition of the hemolytic activity exerted by the *B. jararacussu* venom.

4. Conclusion

The essential oil of *R. officinalis* inhibited almost 40% of the proteases and approximately 10% of the phospholipases A_2, followed by the essential oils of *M. piperita* and *F. vulgare* that were able to inhibit protease activity, but did not inhibit of phospholipases A_2. The essential oil of *C. citratus* induces hemolysis and potentiated the activity of phospholipases A_2. The essential oil of *P. boldus* was induced hemolysis and bridged the action of proteases, but showed no effect on phospholipase activity. The results suggest that the essential oils studied can be used as phytotherapics in inflammation processes because they are able to inhibit the action of phospholipases A_2, these enzymes being part of the inflammation cascade.

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