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RESEARCH ARTICLE

Chemical profile of *Schinus molle* L. essential oil and its antihemostatic properties

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Abstract

About 17.7 million people die each year from cardiovascular disorders and the essential oils have great importance in the development of research. The aim was to evaluate the chemical composition of the essential oil from *Schinus molle* (EOSM) and its antihemostatic activity. EOSM was obtained from leaves by hydrodistillation and characterized by GC-FID-MS. Anticoagulant assay was performed by activated Partial Thromboplastin Time (aPTT) and Prothrombin Time (PT) assays, and the platelet aggregation assays were performed using adenosine diphosphate (ADP) and collagen as inducers. Forty-one compounds were identified mainly sesquiterpenes (88.1%) of which *epi*-α-cadinol (22.8%) and γ-cadinene (9.7%) are the major compounds. EOSM did not show anticoagulant activity (40-400 mg/mL). EOSM oil was active against ADP inducer at 5 mg/mL (80%) and 40 mg/mL (48%), but against collagen the EOSM showed no activity. This is the first report on the antiplatelet action of *Schinus molle*.

Keywords: *Schinus molle,* adenosine diphosphate, antihemostatic, antiplatelet, thrombosis.

Introduction

According to the World Health Organization, Cardiovascular diseases are responsible for 17.7 million deaths each year, with a projection of 25 million deaths worldwide in 2020 (WHO, 2017). Despite technological advances and the progress of medicine, 80% of the population of developing countries need traditional medicine for basic health care (Tomazzoni et al., 2006). Essential oils (EO) have several properties and their actions have been reported for the treatment of infections and inflammation (Ji et al., 2019; Ogunwande et al., 2019; Saldanha et al., 2019) and thrombosis (Ballabeni et al., 2007; Tognolini et al., 2007). However, few studies have been published about the effects of essential oils on the cardiovascular system (Asiful et al., 2016). *Schinus molle* (Annacardiaceae) is native in South America (Gomes et al., 2013) and several compounds are known in its leaves and fruits, such as phenolic acids, flavonoids and terpenes (Masateru et al., 2008). Dried leaves of *S. molle* contain about 1% EO rich in monoterpenes and sesquiterpenes and possess some biological properties (Batista et al., 2016). A brief literature search did not return any scientific papers that evaluated the *in vitro* antihemostatic effect of *S. molle* essential oil. Therefore, considering the importance of natural products and phytotherapy as an alternative to synthetic drugs, the present study evaluated the *in vitro* antihemostatic effect of EOSM.

Materials and Methods

Leaves of *Schinus molle* were collected in Seropédica, Rio de Janeiro, Brazil (22º42'56''S 43º43'01''W) in August 2017. A voucher specimen has been deposited in the herbarium of Biology Institute (UFRRJ) with the following ID: RBR 35791.

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Essential oil extraction

From leaves of *Schinus molle* dried in a forced-air oven at 37°C for 72 h essential oil was obtained by hydrodistillation in a Clevenger apparatus for 3 h. Essential oil was collected and dried with anhydrous Na_2SO_4. Oil yield on moisture-free basis was 2.44%.

Chromatographic analysis and identification

To separate, detect and quantify the constituents, 1 μL of the essential oil (10 μL/mL) was injected into the gas chromatograph (GC). A Hewlett-Packard 5890 Series II (Palo Alto, USA), equipped with flame ionization detection and a split/splitless injector, in a split ratio of 1:20 was used to separate and detect the constituents in the essential oil. The compounds were separated on a non-polar fused silica capillary column, similar to DB5 with 30 m × 0.25 mm (i.d.) × 0.25 μm (film thickness). Helium was used as the carrier gas at a flow rate of 1.0 mL/min. The column temperature was programmed as follows: 60°C for 2 min followed by heating at 5◦C min^{-1} to 110°C, followed by heating at 3◦C min^{-1} to 150◦C and finally by heating at 15°C min^{-1} until 290◦C and holding constant for 15 min. The injector temperature was 220◦C and the detector temperature was 290◦C.

For GC/MS analysis, 1 μL of essential oil was injected in the gas chromatograph coupled to mass spectrometer (GC-MS) QP-2010 Plus(Shimadzu, Japan). The flow of the helium gas carrier, the capillary column and the temperature conditions for the GC-MS analysis were the same as described for the GC. The temperature of the injector was 220°C and the temperature of the interface was 250°C. Mass spectra were obtained with a quadrupole detector operating at 70 eV, with 40–400 *m/z* mass range and scanning rate equal to 0.5 scan s^{-1}. The identification of volatile compounds in the essential oil has been based on Linear Retention Indices (LRI) and mass spectra of the samples, compared with authentic standards injected under the same conditions, with the NIST database (2008) and the index Kovats, IK (Adams, 2007). The LRI was calculated based on co-injection of alkanes series (Van Den Dool & Kratz, 1963).

Biological assays

All biological assays were submitted to the Research Ethics Committee of the University Hospital Clementino Fraga Filho/HUCFF/UFRJ and approved by the number CAAE: 60160716.3.0000.5257.

In vitro determination of activated partial thromboplastin time (aPTT) and prothrombin time (PT)

The ability of *Schinus molle* essential oil to interfere with plasma coagulation was assessed by measuring the aPTT and PT of human plasma on an Amelung KC4A coagulometer. For aPTT tests, cephalin (aPTT reagent, Diamed, RJ, Brazil) was incubated for 1 min with 50 μL of pre-warmed plasma (Lyphochek Coagulation Control, Bio Rad, USA) and *S. molle* essential oil at 40, 200 and 400 mg/mL The reaction started by the addition of 100 μL of pre-warmed $CaCl_2$ (25 mM). For PT tests, 50 μL of pre-warmed plasma was incubated with *Schinus molle* L essential oil at 50, 250 and 500 mg/mL for 1 min (37°C) and reaction started by the addition of 100 μL of pre-warmed thromboplastin with calcium (PT reagent, Labtest Diagnostica, RJ, Brazil).

Platelet aggregation assays

Human blood was collected using BD®Vacutainer tubes containing 3.8% sodium citrate (1/10 volume). Platelet-rich plasma (PRP) was prepared by centrifugation (150 x g, 10 min) at room temperature. The platelet-poor plasma (PPP) was prepared by centrifugation of the PRP (1500 x g, 10 min) at room temperature. Platelet aggregation was monitored by the turbidimetric method on a Chrono-Log aggregometer (Soslau et al., 2001). PRP (350 μL) was incubated (37°C, 1 min) with continuous stirring at 900

rpm. Platelet aggregation was induced by ADP (5 µM) or collagen (5.7 µg/mL). *S. molle* essential oil at 5, 10, 20, 30 e 40 mg/mL (pure) or vehicle (PBS buffer) was added to PRP samples 1 min before addition of the agonists. Aspirin was used with positive control.

Statistical analysis

All the data presented represent mean ± S.D. Differences in mean values were analyzed using Dunnet test. When more than one group was compared with the same control one, significance was evaluated using one-way analysis of variance (ANOVA). *P*-values <0.05 were considered to be statistically significant.

Results and Discussion

The essential oil yield of the leaves of *Schinus molle* (EOSM) was 2.44% (w/w). Forty-one compounds were identified (Table 1, Figure 1) and showed an EO rich in sesquiterpenes oxygenated (50.03%) and non-oxygenated (38.07%). γ-cadinene (9.66%), and *epi*-α-cadinol (22.85%) were the major compounds.

Table 1. *Schinus molle* essential oil chemical composition.

N	Compound	LRI_C	LRI_L	(%)
1	α-Pinene	934	932	0.78
2	β-Pinene	978	974	1.62
3	α-Campholenal	1128	1122	0.34
4	Nopinone	1139	1135	0.10
5	*trans*-Pinocarveol	1141	1135	1.74
6	*cis*-Verbenol	1143	1137	0.09
7	*trans*-Verbenol	1147	1140	1.16
8	n.i.	1151	-	0.07
9	Pinocarvone	1165	1160	0.85
10	*p*-Mentha-1,5-dien-8-ol	1169	1166	0.36
11	Terpinen-4-ol	1179	1174	0.10
12	α-terpineol	1193	1186	0.08
13	Myrtenal	1199	1195	2.80
14	Verbenone	1212	1204	0.53
15	*trans*-Carveol	1222	1215	0.17
16	α-Copaene	1380	1374	0.26
17	β-Elemene	1396	1389	0.34
18	α-Gurjunene	1414	1409	0.33
19	β-Caryophyllene	1425	1417	4.44
20	Aromadendrene	1444	1439	0.13
21	*cis*-Muurola-3,5-diene	1451	1448	0.19
22	α-Humulene	1459	1452	0.73
23	9-*epi*-*β*-Caryophyllene	1467	1464	5.50
24	Germacrene D	1481	1484	0.25
25	γ-Amorphene	1487	1495	2.86
26	Bicyclogermacrene	1502	1500	5.67
27	α-Muurolene	1505	1500	1.64

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Table 1. *Schinus molle* essential oil chemical composition (cont.)

28	γ-Cadinene	1520	1513	9.66
29	δ-Cadinene	1529	1522	5.23
30	α-Cadinene	1543	1537	0.84
31	n.i.	1575	-	0.67
32	Spathulenol	1581	1577	7.62
33	Caryophyllene oxide	1586	1582	5.45
34	Globulol	1594	1590	2.80
35	Ledol	1611	1602	1.80
36	Humulene epoxide II	1616	1608	0.66
37	1,10-di-*epi*-Cubenol	1622	1618	5.19
38	1-epi-Cubenol	1635	1627	0.67
39	*epi*-α-Cadinol	1650	1638	2.,85
40	α-Cadinol	1662	1652	2.20
41	Cadalene	1682	1675	0.27
42	n.i.	1695	-	0.10
43	Shyobunol	1700	1688	0.44
44	Caryophyllene acetate	1717	1701	0.08
45	n.i.	1752	-	0.30
	Monoterpene hydrocarbons	2.40		
	Oxygenated monoterpenes	8.32		
	Total monoterpenes	10.72		
	Sesquiterpene hydrocarbons	38.07		
	Oxygenated sesquiterpenes	50.03		
	Total sesquiterpenes	88.10		
	Total identified	**98.82**		
	Essential oil yield (% w/w)	2.44		

Composition was analyzed by GC-MS. Relative concentration (%) was calculated from the total area under the peaks obtained by GC-FID. Compounds are listed in elution order in the chromatographic column and identified by crescent number (N). Linear retention index calculated (LRI_C) and Linear Retention index from literature (LRI_L). Not identified compound (n.i.).

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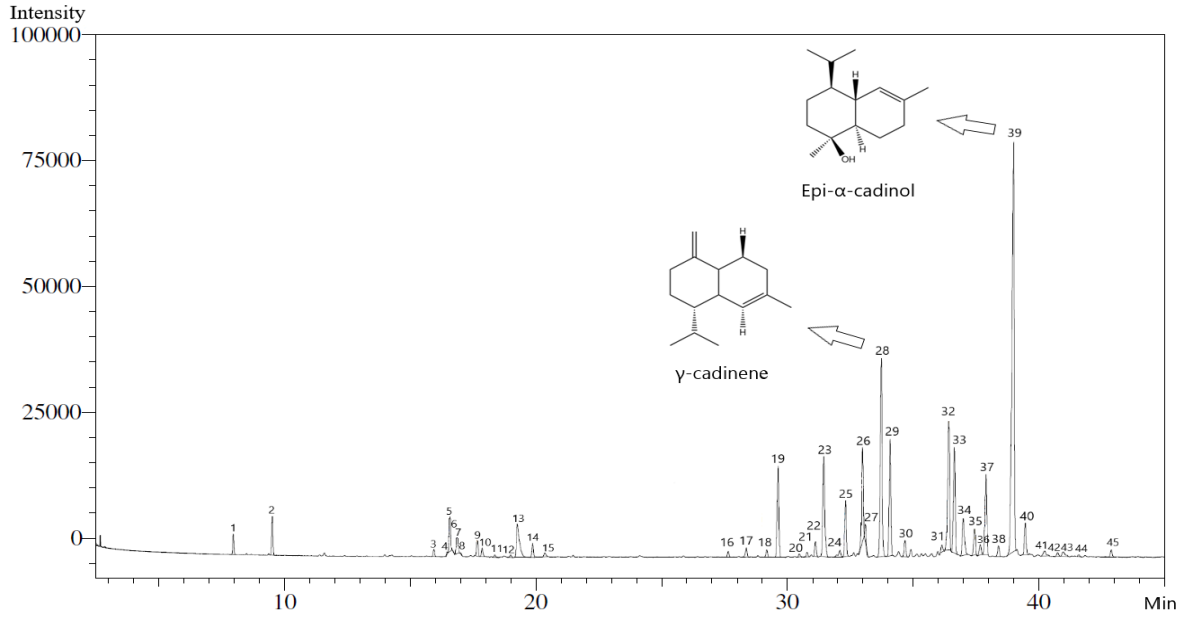
Figure 1. Chromatogram obtained by GC-FID analysis from *Schinus molle* essential oil. The numbers are listed in Table 1 based on elution order in the chromatographic column and identified by crescent number. The major compounds are shown in the chromatogram.



EOSM obtained in this work showed a chemical profile different from that reported by Santos et al. (2009) and Alnavari et al. (2018), rich in sabinene and limonene, and Gomes et al. (2013) rich in α-pinene and β-pinene. However, we verified a similarity in the chemical profile of EOSM as reported by Cavalcanti et al. (2015). Our results showed that the EOSM did not prolong the coagulation time in any of the assays used. In aPTT test, at the highest concentration (400 mg/mL) 44 seconds of coagulation time was observed in comparison with control (30 s) (Figure 2). In PT test the EOSM (500 mg/mL) again showed no significant change in coagulation time (Figure 3).

Figure 2. *Schinus molle* essential oil (EOSM) *in vitro* effect on coagulation after the intrinsic pathway activation induced by the aPTT reagent. * represent significant result based on Dunet test (5%, n=3). Heparin was used as positive control.

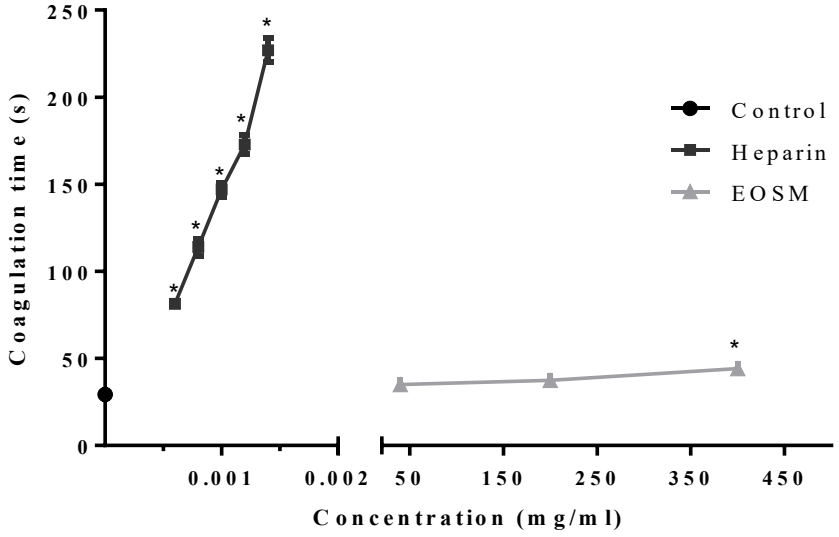


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Figure 3. *Schinus molle* essential oil (EOSM) *in vitro* effect on coagulation after the extrinsic pathway activation induced by PT reagent. *represent significant result based on Dunet test (5%, n=3). Heparin was used as negative control.

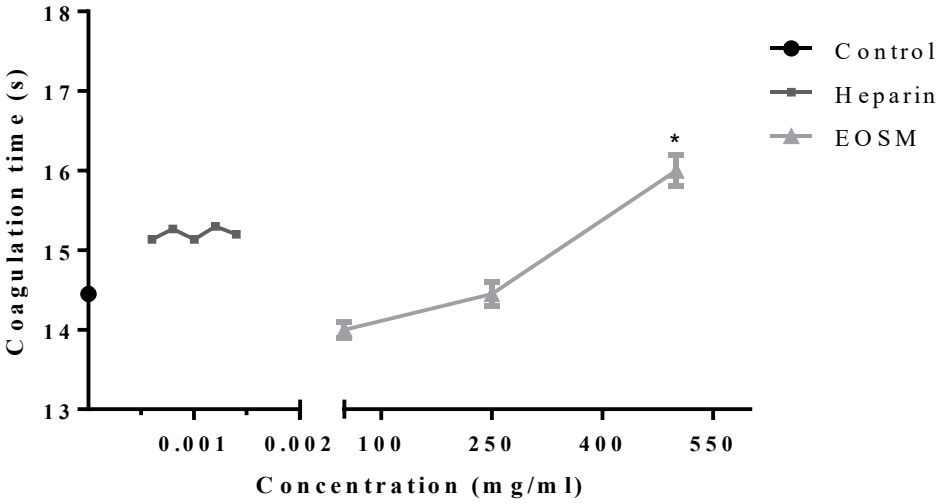


Figure 4 shows the antiplatelet activity of the EOSM against the ADP inducer. The lowest concentration (5 mg/mL) showed 80% of inhibition while the highest concentration (40 mg/mL) inhibited 48% of platelet activation, which means that the lower the oil concentration, the greater the antiplatelet activity. The antiplatelet activity of OESM was different using collagen as inducer. So, in the Figure 5 we can observe that the EOSM inhibited only 25.2% of the platelet aggregation at 80 mg/mL, and at the lowest concentrations (5 mg/mL) showed 4.82% denoting dose-response behavior. When the platelet aggregation inducer was replaced by collagen, the inhibitory potential of the essential oil was different.

Figure 4. Inhibitory effect of *Schinus molle* essential oil (EOSM) (5.02 mg/mL) on the platelet aggregation in vitro induced by ADP (5 μM) using human platelet-rich plasma. * represent significant result based on Dunet test (5%, n=5). Aspirin was used as positive control.

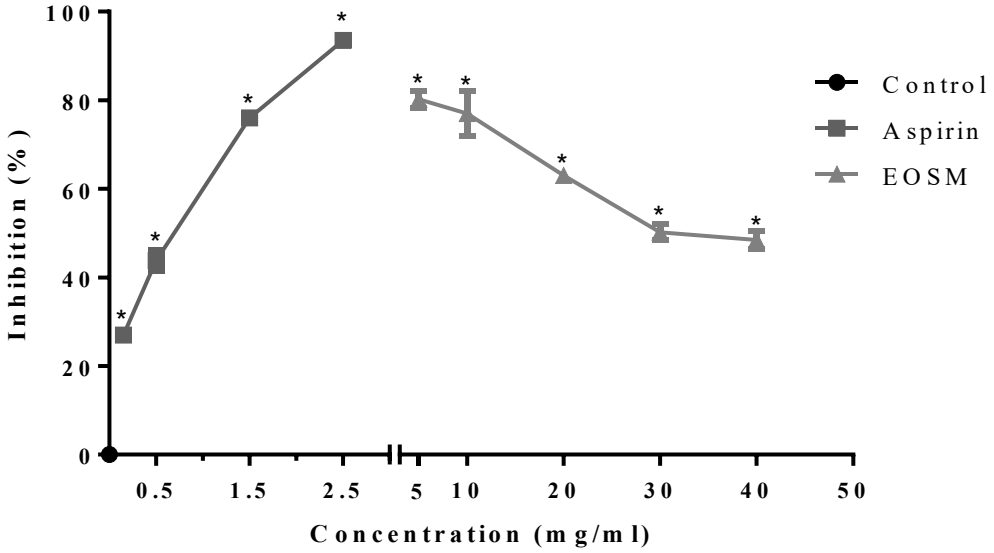
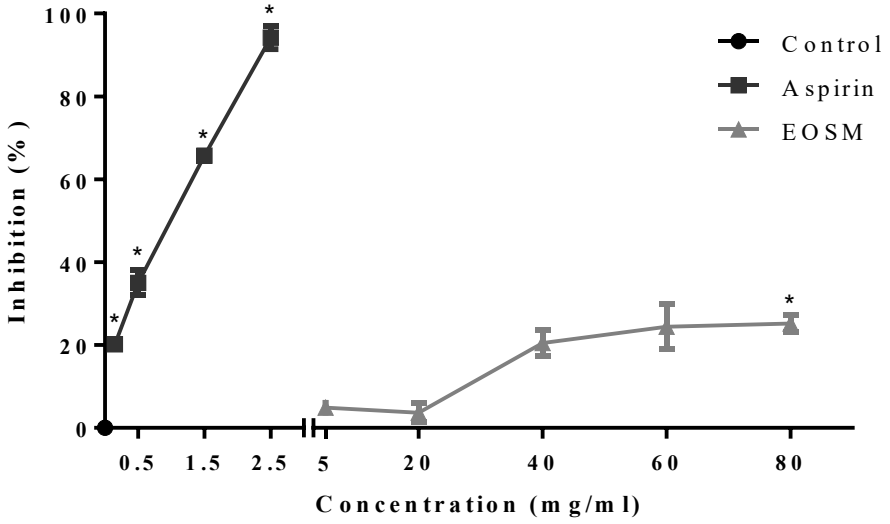


Figure 5. Inhibitory effect of *Schinus molle* essential oil (EOSM) (5,7x10^{-3} mg/mL) on the platelet aggregation in vitro induced by collagen (5 μM) using human platelet-rich plasma. * represent significant result based on Dunet test (5%, n=5). Aspirin was used as positive control.



This result can also be considered positive, since it indicates that the activity of the essential oil occurs due to the activation pathways induced by ADP. However, to confirm this hypothesis further experiments are needed with other inducers of platelet aggregation, in addition to future *in vitro* and *in vivo* tests. The antithrombotic activity of curdione from *Rhizoma curcumae* showed *in vitro* and in *vitro* significant platelet antiaggregant activity, preferably by platelet-activating factor (PAF) and thrombin pathway, exhibiting IC_{50} of 60 μM (Xia et al., 2012). Tao and Wang 2010 showed two sesquiterpenes isolated from *Dalbergia odorifera* inhibited the platelet aggregation *in vitro* in 50% (10 μmol/mL). These studies suggest a connection between the antiplatelet activity of the EOSM and the dominant class of its chemical compounds (sesquiterpenes). Ballabeni et al (2004) reported that the major compounds of *Lavandula hybrida* essential oil did not exhibit antiplatelet effect compared to the crude essential oil, and the complete inactivation was observed for the main compound (linalyl acetate) at 1 mg/mL (Ballabeni et al., 2004). EOSM showed a promising antihemostatic activity *in vitro*, which occurred by the inhibition of platelet aggregation by ADP, once the essential oil did not interfere in the coagulation pathways. However, studies using other inducers of platelet aggregation are required so that the route of action can be proposed. These results suggest that the essential oil of *Schinus molle* is a promising therapeutic source for cardiovascular diseases. This is the first report of the antiplatelet action of the essential oil from *Schinus molle*.

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