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Spermostatic activity of *Eugenia brejoensis* and *Myroxylon peruiferum* essential oils toward human spermatozoa

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In this study, the *in vitro* spermostatic action, hemolytic action in combination of parabens of two essential oils (EOs) from *Eugenia brejoensis* Mazin (Myrtaceae, EbEO) and *Myroxylon peruiferum* L. (Fabaceae, MpEO) is reported, in addition to the first chemical characterization of MpEO. The EOs were obtained by hydrodistillation and characterized by gas chromatography-mass spectrometry (GC-MS). Different concentrations of both EOs (25 to 400 µg/mL) were mixed with human ejaculate and sperm motility was assessed after 5, 15 and 30 min. A total of 44 compounds were identified in MpEO; the major components were spathulenol, α-pinene, caryophyllene oxide, limonene and β-elemene. Both EOs exhibited spermostatic action in a dose-dependent manner. The best results were obtained for EbEO at all treatment times, showing IC_{50} values of 454.75 µg/mL (5 min), 417.68 µg/mL (15 min) and 403.93 µg/mL (30 min). *M. peruiferum* also showed significant immobilizing potential with IC_{50} values of 620.39 µg/mL (5 min), 652.70 µg/mL (15 min) and 579.37 µg/mL (30 min). Both oils showed low cytotoxicity against human erythrocytes. This is the first report on human spermostatic action for EOs from *E. brejoensis* and *M. peruiferum* which has antimicrobial activity and low cytotoxicity. These results suggest the need for further research on toxicological aspects of both EOs and their components in order to develop new pharmaceutical preparations using them as leader compounds.

Key words: *Eugenia brejoensis* and *Myroxylon peruiferum* essential oils, spermostatic activity, human spermatozoa.

INTRODUCTION

Several contraceptive methods are described in the medical literature as alternatives to avoid unplanned pregnancy. These approaches include different compounds from various chemical classes, of various action mechanisms and with different levels of effectiveness. Barrier methods, for example, consist of the use of substances that prevent fertilization of the ovum by sperm by establishing physical or chemical barriers (Kumar et al., 2012). Vaginal spermicides, including creams, gels and foaming aerosols are examples of chemical barriers (Batár, 2010). Spermicidal substances are designed to prevent pregnancy through death or immobilization of spermatozoa so that they are unable to reach the oocyte and fertilize it (Schreiber et al., 2006). Despite their advantages, they are based mostly on nonoxynol-9, a compound that is capable of causing side-effects such as cervical and vaginal irritability and potentially favoring the emergence of infectious microorganisms and/or altering the growth of lactobacilli (Ojha et al., 2003). There is urgent need for replacement of such agents with safer and more effective alternatives such as natural contraceptives.

In this context, it is important to search for new spermicidal compounds from plants, especially those endemic to different and under-exploited ecosystems, such as the Caatinga (a biome exclusive to Brazil). This area is marked by extreme environmental conditions typical of a semi-arid tropical climate, such as high temperatures (annual average temperature above 25°C) and scarce and erratic rainfall with long periods of drought. It is believed that these climatic features play an essential role in the production of bioactive metabolites by plants of the Caatinga (de Albuquerque et al., 2012). Such metabolites provide a range of proven biomedical applications (de Oliveira et al., 2012; da Silva et al., 2013), thereby encouraging the search for new compounds from plants of the Caatinga.

This study aimed to evaluate the spermostatic activity of essential oils from two plants collected in the Caatinga: *Eugenia brejoensis* Mazin and *Myroxylon peruiferum* L. The first plant, *E. brejoensis* (Myrtaceae), is a recently described species first recorded from humid, highland forests, called *brejos*, in the Caatinga of Pernambuco State, Brazil. Later collections documented an expanded distribution now known to include other states of the Brazilian Northeast (Paraiba, Alagoas and Sergipe) and Southeast (Espírito Santo) and showed that the species occurs in both the Caatinga and Atlantic forest domains (Mazine and Souza, 2008; Giaretta and Peixoto, 2014). On the other hand, *M. peruiferum* (Fabaceae) is a well-known species with a wide distribution in Brazil and

MATERIALS AND METHODS

Collection of plant

Samples of leaves from *E. brejoensis* and *M. peruiferum* were collected from the "Parque Nacional do Catimbau" (Catimbau National Park, Buíque, Pernambuco, Brazil) in September, 2012. Botanical identification was done at the Herbarium of the Instituto Agronômico de Pernambuco (Agronomic Institute of Pernambuco; IPA-PE, Brazil) and specimen vouchers were deposited in the same herbarium (IPA 84.033 and IPA 84.113, respectively).

Extraction of the essential oils

Approximately, 200 g of powdered leaves of each plant (pre-dried at room temperature and ground in a mill) were subjected to hydrodistillation by heating for 3 h in a Clevenger-type apparatus. Each essential oil layer was separated, dried over anhydrous sodium sulfate and stored in a hermetically sealed glass vial. The essential oils were named from *E. brejoensis* as EbEO and from *M. Peruiferum* as MpEO. They were stored at 4°C prior to each assay. This protocol was repeated 3 times at least .

GC and GCMS analyses

Gas chromatographic-mass spectrometric (MS) analyses were carried out using an Agilent 5975C Series quadrupole GC/MSD (Agilent Technologies, Palo Alto, USA) instrument equipped with an Agilent J&W non-polar DB-5 fused silica capillary column (60 m × 0.25 mm i.d.; film thickness 0.25 µm). For each sample, 1 µL was injected in split mode (50:1) with the injector temperature set to 250°C.

The GC oven temperature was set at 40°C, held for 2 min, increased to 230°C at 4°C/min, then held for 5 min. Helium (He) carrier gas flow (1 mL/min) was maintained at a constant pressure of 7.0 psi. MS Source and quadruple temperatures were set at 230 and 150°C, respectively. Mass spectra were taken at 70 eV (in EI mode) with a scanning speed of 1.0 scan from 35 to 350 m/z. The same conditions were used in the GC analysis performed in a Thermo Fisher Scientific (Waltham, MA, USA) Trace GC Ultra gas chromatograph equipped with a flame ionization detector (FID), a split/splitless injector and a Hamilton Bonaduz (Bonaduz

Switzerland) HB-5 fused silica capillary column (30 m × 0.25 mm; film thickness 0.25 µm). The composition of essential oil was expressed in the form of percentages of total peak area as recorded by the GC-FID. The GC analyses were performed in triplicate. The medium percentage of peaks were processed.

Initial identification of the individual components of the essential oils was carried out by comparison with previously reported values of retention indexes (RI), obtained by co-injection of oil samples and C_9-C_{30} linear hydrocarbons and calculated according to the Van den Dool and Kratz equation (Van Den Dool and Dec Kratz, 1963; Adams, 2009). Subsequently, the MS acquired for each component was matched with those stored in the mass spectral library of the GC-MS system (MassFinder 4, NIST08 and Wiley Registry™ 9th Edition) and with other published mass spectral data.

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Analysis of immobilizing activity

Semen samples were collected by masturbation from 12 fertile volunteers who had been pre-tested, were non-smoking, healthy, aged 18 to 45 years, and whose sperm showed normal morphology (> 60%), motility and viability more than 40 and 70%, respectively. Semen samples were collected after sexual abstinence of 48 to 120 h, as recommended by the WHO standard protocol (World Health Organization, 2010). A routine semen analysis was performed immediately after coagulation, followed by reliquefaction at room temperature. A free and informed consent form was signed by each volunteer before participation in the study. All experiments were performed according to ethical standards and were approved by the Ethics Committee of ASCES College (Caruaru, Brazil), under license number 02604312.6.0000.5203.

Different concentrations (25, 50, 100, 200 and 400 µg/mL) of each oil (prewarmed to 37°C) were mixed with human ejaculate at a 1:1 volume ratio. A solution of warmed DMSO (5%), diluted in phosphate-buffered saline (PBS; pH 7.4), was used as a control. Motility assessment was performed by putting 10 µL of each experimental mixture (Semen + EO or Semen + Control) on prewarmed slides, and at least 10 fields were viewed for a total count of 200 spermatozoa for each slide (Paul and Kang, 2011). The tests were carried out in triplicate for each sample after incubation for 5, 15 and 30 min. Sperm motility was assessed following the WHO protocol for seminal processing (World Health Organization, 2010). Tests were performed independently and six patients were tested for each essential oil.

Hemolytic assay

Blood (5 to 10 ml) was obtained from healthy volunteers by venipuncture and placed in heparinized tubes, after written informed consent was obtained. Human erythrocytes were isolated by centrifugation (1,500 rpm, 10 min at 4°C). The erythrocytes were washed three times with phosphate-buffered saline (PBS; pH 7.4). Each tube received 1.1 mL of erythrocyte suspension (1%) and 0.4 mL of various extract concentrations (0.15625 to 2.5 mg/mL). The controls were only solvent (negative) and *Quillaja* saponin (0.0025%, positive). After 60 min incubation, cells were centrifuged and the absorbance of supernatant was recorded at 540 nm. The hemolytic activity was expressed by the following formula (de Oliveira et al., 2012):

Hemolytic activity (%) = (As - Ab) × 100 / (Ac - Ab)

where Ab = solvent absorbance, As = sample absorbance, and Ac = saponin absorbance.

Statistical analysis

The results are expressed as mean ± standard deviation (SD). Statistical significance was determined by Student t and analysis of variance (ANOVA) tests comparing treated groups with untreated control. A p-value of <0.05 was considered to be statistically significant. In all graphs, the bars represent the mean value ± SD. Determinations of IC_{50} (concentration of 50% immobilized spermatozoa) and HC_{50} (the concentration needed for 50% of hemolysis) were performed by linear regression.

RESULTS AND DISCUSSION

The chemical composition of the oils used in this paper was performed by GC-MS. As shown in Table 1, 47 and 41 components were detected in the oils of *M. peruiferum* (MpEO) and *E. brejoensis* (EbEO), respectively. Of these, 44 compounds were identified in MpEO (which corresponds to 96.38% of the total). This oil was found to be rich in sesquiterpenes (63.37%) and monoterpenes (21.31%). Its major components (58.84%) were spathulenol (13.29%), α-pinene (9.64%), caryophyllene oxide (7.27%), limonene (7.17%), β-elemene (6.79%), bicyclogermacrene (5.49%), β-bourbonene (4.96%) and α-muurolene (4.23%) (Table 1). On the other hand, EbEO composed almost entirely of sesquiterpenes (both sesquiterpene hydrocarbons and oxygenated sesquiterpenes; 62.66 and 26.64%, respectively). Its major compounds were δ-cadinene (22.6%), β-(E)-caryophyllene (14.4%), α-muurolol (9.34%), α-cadinol (8.49%) and bicyclogermacrene (7.93%) (da Silva et al., 2015).

The effects of EbEO and MpEO on human spermatozoa motility is as shown in Figure 1. The essential oil from *E. brejoensis* showed the best spermicide activity. When treated with EbEO, significant immobilizing action was observed with all tested concentrations and at all times as compared to controls ($p<0.05$), except at a concentration of 25 µg/mL. The spermostatic activity of EbEO was time- and dose-dependent and ranged from 8.53 to 42.70% at 5 min, from 9.38 to 44.10% at 15 min, and from 9.60 to 47.56% at 30 min.

The IC_{50} values were 454.75, 417.68 and 403.93 µg/mL at 5, 15 and 30 min, respectively. Previous studies has showed the biomedical potentials of oils from *Eugenia* plants (Ogunwande et al., 2005; Siebert et al., 2015), including antifertility activities for *Eugenia jambolana* (Rajasekaran et al., 1988). Similarly, the presence of β-caryophyllene, a chemical component identified in *Eugenia brejoensis*, has been reported in the oil of *Trachyspermum ammi* which also has spermicidal action (Paul and Kang, 2011). Other constituents identified in EbEO such as bicyclogermacrene, δ-cadinene, α-muurolol and α-cadinol are reported in other bioactive essential oils (Martins et al., 2015; Karapandzova et al., 2014; Su et al., 2015).

Regarding the action of MpEO, significant inhibition was found at 50 µg/mL after 15 and 30 min of incubation, and at higher concentrations (100, 200 and 400 µg/mL) in all treatment periods ($p< 0.05$). The immobilizing values were also dose-dependent and ranged from 6.2 to 33.33% at 5 min, 7.6 to 32.73% at 15 min, and from 9.66 to 36.66% at 30 min. The IC_{50} values at 5, 15 and 30 min were 620.39, 652.70 and 579.37 µg/mL, respectively. It is worth emphasizing that there are no reports in the literature regarding contraceptive potential for the genre *Myroxylon*. As part of its phytochemical composition, *M. peruiferum* possesses, volatile oils, esters, alcohols, triterpene alkaloids, phenols, proteins and glycosides (Schwarcz et al., 2014).

Chemical components identified in *M. peruiferum*

Table 1. Identification of constituents of the essential oil obtained from *Eugenia brejoensis* and *Myroxylon peruiferum*.

Number	Identify compound[a]	Retention times	Retention indices		Content (as % of total oil) + SD	
			Determined[b]	Literature[c]	*E. brejoensis*	*M. peruiferum*
1	α-Pinene	9.396	932	932	-	9.64 ± 0.20
2	Thuja-2.4(10)-diene	10.132	952	953	-	0.23 ± 0.01
3	β-Pinene	10.945	974	974	-	0.44 ± 0.01
4	Myrcene	11.550	991	988	-	0.15 ± 0.03
5	ρ-Cymene	12.753	1024	1020	-	0.10 ± 0.04
6	Limonene	12.903	1028	1024	-	7.17 ± 0.38
7	γ-Terpinene	15.238	1058	1054	-	0.12 ± 0.07
8	Terpinolene	16.541	1088	1086	-	0.05 ± 0.02
9	α-Campholenal	16.978	1126	1122	-	0.95 ± 0.04
10	*trans*-Pinocarveol	16.978	1138	1135	-	0.38 ± 0.06
11	*trans*-Verbenol	17.208	1144	1140	-	1.17 ± 0.15
12	Pinocarvone	17.869	1163	1160	-	0.08 ± 0.05
13	α-Phellandre-8-ol	18.024	1167	1166	-	0.20 ± 0.00
14	Terpinen-4-ol	19.089	1177	1174	-	0.41 ± 0.01
15	*trans*-Carveol	19.878	1219	1215	-	0.22 ± 0.01
16	Isovaleric acid	20.489	1234	1232	-	0.22 ± 0.01
17	α-Cubebene	24.395	1351	1348	1.28 ± 0.02	1.19 ± 0.01
18	α-Copaene	25.221	1378	1374	1.23 ± 0.01	2.63 ± 0.11
19	β-Bourbonene	25.517	1387	1387	-	4.96 ± 0.21
20	US	25.687	1392	-	-	0.66 ± 0.02
21	β-Elemene	25.742	1394	1389	0.84 ± 0.01	6.79 ± 0.20
22	US	26.177	1407	-	-	0.55 ± 0.03
23	α-Gurjunene	26.344	1413	1409	1.56 ± 0.02	-
24	*trans*-Caryophyllene	26.619	1422	1417	8.43 ± 0.09	3.51 ± 0.11
25	β- Copaene	26.955	1432	1430	0.53 ± 0.01	-
26	Aromadendrene	27.261	1442	1439	0.14 ± 0.01	-
27	α-Himachalene	27.395	1447	1449	1.78 ± 0.02	-
28	α-Humulene	27.685	1456	1452	0.28 ± 0.01	1.19 ± 0.06
29	Caryophyllene <9-epi-(E)->	27.913	1464	1464	1.67 ± 0.02	0.22 ± 0.00
30	Cadina-1(6),4-diene <*trans*->	28.354	1477	1475	1.97 ± 0.03	-
31	γ-Muurolene	28.392	1479	1478	2.50 ± 0.03	0.42 ± 0.02
32	Germacrene D	28.540	1486	1484	-	1.06 ± 0.04
33	β-Selinene	28.695	1489	1489	0.88 ± 0.01	1.63 ± 0.04
34	α-Selinene	28.961	1498	1498	-	1.22 ± 0.03
35	Bicyclogermacrene	29.007	1499	1500	4.27 ± 0.10	5.49 ± 0.20
36	α-Muurolene	29.114	1503	1500	2.61 ± 0.06	4.23 ± 0.50
37	Germacrene A	29.273	1508	1508	-	2.67 ± 0.08
38	γ-Cadinene	29.554	1517	1513	5.37 ± 0.05	0.83 ± 0.02
39	δ-Cadinene	29.807	1526	1522	15.57 ± 0.07	2.84 ± 0.02
40	Cadina-1,4-diene <*trans*->	30.062	1535	-	0.35 ± 0.01	0.18 ± 0.02
41	α-Cadinene	30.240	1541	1537	1.03 ± 0.01	0.13 ± 0.08
42	α-Calacorene	30.386	1546	1544	0.72 ± 0.03	0.37 ± 0.01
43	US	30.693	1556	-	-	0.79 ± 0.01
44	Palustrol	31.142	1572	1567	1.04 ± 0.01	-
45	Spathulenol	31.411	1581	1577	10.04 ± 0.20	13.29 ± 0.48
46	Caryophyllene oxide	31.575	1586	1582	4.81 ± 0.14	7.27 ± 0.27
47	Viridiflorol	31.861	1596	1592	1.09 ± 0.01	-
48	Salvial-4(14)-em-1-one	31.885	1597	1594	-	0.96 ± 0.05
49	Guaiol	32.017	1602	1600	1.39 ± 0.01	-
50	Ledol	32.175	1607	1602	2.44 ± 0.02	-

Table 1. Cont`d.

51	β-Oplopenone	32.358	1613	1607	0.97 ± 0.01	-
52	Cubenol <1,10-di-epi->	32.505	1619	1618	0.75 ± 0.01	-
53	Cubenol <1-epi->	32.851	1631	1627	-	0.15 ± 0.01
54	UC	32.881	1633	-	0.27 ± 0.07	-
55	UC	32.981	1635	-	2.13 ± 0.01	-
56	Aromadendrene eposxide <allo->	33.148	1642	1639	-	-
57	α-Muurolol	33.231	1645	1640	5.25 ± 0.04	0.12 ± 0.04
58	α-Cadinol	33.612	1659	1652	9.24 ± 0.17	-
59	Eudesma-4(15).7-dien-1β-ol	34.447	1689	1687	-	0.33 ± 0.27
60	US	34.582	1694	-	-	0.16 ± 0.08
61	US	35.050	1711	-	-	0.66 ± 0.13
62	US	35.307	1721	-	-	0.49 ± 0.13
	Monoterpenes	-	-	-	-	21.31
	Sesquiterpenes	-	-	-	90.03	63.37
	Unidentified sesquiterpenes (US)	-	-	-	-	3.62
	Fatty acids	-	-	-	-	0.22
	Unidentified compounds	-	-	-	2.40	9.84
	Total	-	-	-	92.43	98.36

[a]Constituents listed in order of elution on a non-polar DB-5 column; [b]Retention indices (RI) calculated from retention times in relation to those of a series of C_9-C_{30}. *n*-alkanes on a 30 m DB-5 capillary column; [c]Values taken from Adams (2009).

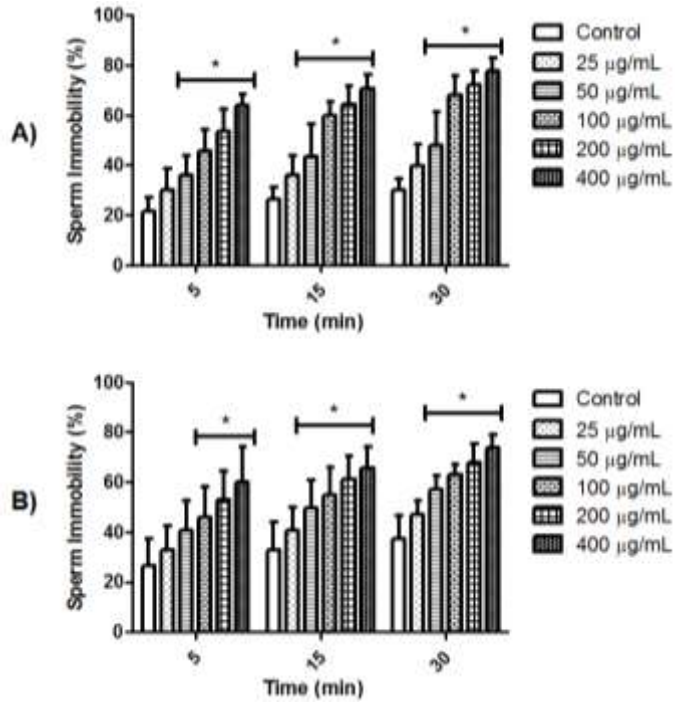

Figure 1. Spermostatic effect of essential oils from *Eugenia brejoensis* and *Myroxylon peruiferum,* respectively.

essential oil such as α-pinene, γ-muuroleno, spathulenol and caryophyllene oxide were not reported as contraceptive actives in previous studies. On the other hand, some major components (such as α-pinene, γ-muurolene, caryophyllene oxide) have been detected in essential oils antimicrobial, antioxidant, and gastro-protective activities (Almeida et al., 2015; Saraswathy et al., 2010; Policegoudra et al., 2012).

The cellular toxicity were evaluated using human erythrocytes as test system, both essential oils showed low toxicity with HC_{50} of 8.66 and 6.85 mg/mL, for MPEO and EBEO, respectivelly. These concentrations are higher than IC_{50} values found for both oils.

Conclusion

This study demonstrated that essential oils from *E. brejoensis* and *M. peruiferum* show a potential spermostatic effect on human sperm with low cytotoxicity. Future studies, including the identification of active compounds in each essential oil, their action mechanisms and *in vivo* activity, are necessary to support the use of these essential oils to develop new pharmaceutical preparations.

CONFLICT OF INTERESTS

The authors have declared any conflict of interests.

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