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Chemical composition and acaricidal activity of the leaf and fruit essential oils of *Protium heptaphyllum* (Aubl.) Marchand (Burseraceae)[1]

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ABSTRACT

Essential oils from leaves and fruits of *Protium heptaphyllum* collected in Tamandaré beach – Pernambuco/Brazil were analysed by GC/MS and tested for toxicity and repellent effect against the two spotted spider mite (*Tetranychus urticae*). The major constituent identified in the fruits was α-terpinene (47.6 %) whereas oil from leaf contained mainly sesquiterpenes such as 9-epi-caryophyllene (21.4 %), *trans*-isolongifolanone (10.7 %) and 14-hydroxi-9-*epi*-caryophyllene (16.7 %). The fruit oil was found to be more effective against the mite when compared to the leaf oil. Both showed mortality properties and oviposition deterrence in higher concentration (10 μl.l^{-1} air), but only the essential oil from fruits induced repellence on *T. urticae*.

KEY-WORDS

Protium heptaphyllum, essential oil, *Tetranychus urticae*, acaricidal activity

Composição química e atividade acaricida do óleo essencial das folhas e frutos de Protium heptaphyllum *(Aubl.) Marchand (Burseraceae)*[1]

RESUMO

O óleo essencial das folhas e frutos de Protium heptaphyllum *coletada em Tamandaré-Pernambuco foi analisado por CG/EM e testado sua toxicidade e efeito repelente contra ácaro rajado (*Tetranychus urticae*). O constituinte majoritário identificado nos frutos foi α-terpineno (47,6 %) enquanto que nas folhas foram os sesquiterpenos 9-epi-cariofileno (21,4 %),* trans*-isolongifolanona (10,7 %) and 14-hidroxi-9-epi-cariofileno (16,7 %). O óleo dos frutos foi mais eficiente contra o ácaro, comparado com o óleo das folhas. Ambos os óleos revelaram propriedades de mortalidade e deterrência de oviposição na maior concentração (10 μl.l^{-1} air) e apenas o óleo essencial dos frutos induziu repelência no* T. urticae

PALAVRAS-CHAVE

Protium heptaphyllum, óleo essencial, *Tetranychus urticae*, atividade acaricida

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INTRODUCTION

The family Burseraceae is a well-known source of exudates and oleoresins rich in volatile substances which are used for many purposes, e.g. perfumery. In the Neotropical region, this family is largely represented by the genus *Protium*. This is the principal genus in the family, which comprises about 135 species and is one of the most widespread genera in South America (Khalid, 1983). Andrade-Lima (1966), studying the parallel development between the floras of the Brazilian Northeast and that of the Amazon, found that many plant genera and species are common in both regions. This parallel development is supported by the refuge theory, developed by Vanzolini (1970) and Ab'Saber (1970). They justified the origin of these vegetation islands as a consequence of the separation of the Amazonian Hiléa, which during the glacial eras when the South-American climate as well as the global climate was drier and colder than today, retreated to small island forest formations in the middle of an immense savannah, isolating the flora and fauna into different bioma. The posterior drying in glacial periods, and the consequent retreat of the forests, is today's witness of these forest formations in the Brazilian Northeast and Amazonia. So, some genera and species found in forest formations in Pernambuco are directly linked to the rate of how they grow in the Amazonian region. This is the case of *Protium heptaphyllum* (Aubl.) Marchand (Burseraceae) (Loureiro *et al.*, 1978).

P. heptaphyllum is a medicinal plant with the popular names breu, breu branco verdadeiro, which grows widely in the Amazonian region and other parts of Brazil in sandy, wet and dry soils, like the *Restinga* Region of the Brazilian Northeast. In these areas, this species is popularly known as amescla (Ceará, Paraíba, Rio Grande do Norte), almesca in Bahia, and amescla and almécega in Pernambuco (Loureiro *et al.*, 1978). In popular medicine, this species is considered an important therapeutic agent which is used as anti-inflammatory, analgesic, expectorant and healing of wounds. It is also used in the paint industry and covering of boats (Costa, 1975; Corrêa, 1987; Pott & Pott, 1994; Siani *et al.*, 1999a). Other applications include production of oil resins rich in essential oils used as incense or insect repellent (Pernet, 1972; Corrêa, 1987).

Some pharmacological studies using the oil resin verified the therapeutical efficacy, which, by their surprising results, proved their usefulness as anti-inflammatory, antinoceptive, antineoplasic and gastro protective (Oliveira *et al.*, 2004a,b,c; Siani *et al.*, 1999a). The oil composition of the aerial parts and resin of *P. heptaphyllum* have been previously reported from a specimen collected in two regions of Brazil: Manaus in the North (Siani *et al.*, 1999a,b; Zoghbi & Maia, 1995) and Ceará in the Northeast (Bandeira *et al.*, 2001).

Due to its high volatility, the essential oils could be used to control pests found in closed environments, such as greenhouses (Aslan *et al.*, 2004). Recently, studies of essential oils have been made to evaluate its acaricidal (Kim *et al.*, 2004) and insecticidal properties (Choi *et al.*, 2005), especially for stored-product pest control (Bouda *et al.*, 2001; Huang *et al.*, 2000; Kim *et al.*, 2003). This paper reports the chemical composition of the oils from leaves and fruits of *P. heptaphyllum*, a plant collected in the *restinga* region on the Tamandaré beach-Pernambuco, as well as its acaricidal activity against the two-spotted mite *Tetranychus urticae*.

MATERIAL AND METHODS

PLANT MATERIAL AND ESSENTIAL OILS EXTRACTION METHOD

Leaves and fruits of *P. heptaphyllum* were collected in the biological reserve of Guadalupe, Tamandaré beach on the south coast of Pernambuco, Brazil, in December 2004. A sample was deposited in the Herbarium Vasconcelos Sobrinho of the Universidade Federal Rural de Pernambuco (UFRPE) under the number 46329. Fresh leaves and immature fruits were submitted to hydrodistillation for 2 h, and the oils were collected by a modified Clevenger-type apparatus. The oils were separated from water, dried with Na_2SO_4, and stored in sealed vials at low temperature before analysis. Yields were calculated from weight of fresh material. The yield was calculated through the relation of the volatile oil volume from the Clevenger-type equipment to the mass of plant material used in the extraction. All experiments were repeated three times.

GAS CHROMATOGRAPHY AND GAS CHROMATOGRAPHY-MASS SPECTROMETRY

Gas chromatography (GC) analyses were performed on a Hewlett Packard 5890 SERIES II equipped with a flame ionization detector (FID) and a J & W Scientific DB-5 fused silica capillary column (30 m x 0.25 mm x 0.25mm); programmed oven temperature was 50 °C – 250 °C at 4 °C at min^{-1}, integrating purposes. Injector and detector temperatures were 250 °C and 280 °C, respectively. Hydrogen was used as carrier gas, flow rate 1.5 ml min^{-1}, split mode (1:10). A 1.5 µl solution of about 10 mg of oil in ethyl acetate was injected. The retention indices were obtained by co-injecting the oil sample with a C_{11}-C_{24} linear hydrocarbon mixture (retention index from 900 to 1099 range was obtained by extrapolation)

The essential oil analysis was carried out using a Shimadzu QP5050 quadrupole GC/MS fitted with the same column and temperature programme as that for the GC experiments. The carrier gas was helium, flow rate 1.5 ml.min^{-1}, split mode (1:50). 1 µl of 1/100 diluted solution in ethyl acetate was injected. Mass spectra were taken at 70 eV. Scanning speed was 0.5 scan.s^{-1} from m/z 40 to 650.

The essential oils were analysed by GC and GC/MS; identification was made on the basis of retention indices comparison (Van den Doll & Kratz, 1963), as well as by the

computerized matching of the obtained mass spectra with those stored in the NIST mass spectral library of the GC/MS data system and other published mass spectra (Adams, 1995) and percentage compositions were obtained from electronic integration measurements without taking into account relative response factors.

BIOASSAY

The tests were made in the Laboratório de Produtos Naturais Bioativos of Universidade Federal Rural de Pernambuco (LPNB / UFRPE) at a temperature of 25 ± 2 ºC, relative humidity of 70 ± 8 % and 12 h photophase.

BIOLOGICAL MATERIAL

The mite *T. urticae* used for the bioassay was reared in plants of *Canavalia ensiformes* by the Laboratório de Acarologia Agrícola of the Agronomic Department of UFRPE at a temperature of 27 ± 0.5 ºC, relative humidity of 75 ± 5 % and 12 h photophase.

FUMIGANT BIOASSAY

The method to evaluate the activity of essential oils on mites was adapted by Tunç & ªahinkaya (1998), Aslan *et al.* (2004) and Çalmaºur *et al.* (2005).

Glass recipients having a capacity of 2.5 l were used as test chambers. *T. urticae* adult females were collocated in leaf disks of 2.5 cm diameter of *Canavalia ensiformes* leaves, in a Petri dish (9 cm) having 4 filter paper disks saturated with water to maintain the leaf turgor and avoid the exit of mites. The arrangement consisted of a Petri dish having three leaf disks with 10 mites each.

In the experiment, one arrangement per recipient was used. By an automatic pipette, the desired oil quantities were applied on filter paper (5 x 2 cm) fixed on the inner surface of the cover. Each cover received 5, 10, 15, 20 and 25 µl of essential oil which corresponds to 2, 4, 6, 8 and 10 $\mu l.l^{-1}$ of air, respectively. The control contained no oil. The exposure period for the oils was 24, 48 and 72 h. The experiment consisted of six treatments and three repetitions.

Evaluation was made at the end of each exposure period. Mites incapable of moving a distance superior to their body length after a slight touch with a fine brush were considered as dead. Fecundity was evaluated by counting the eggs collocated on the leaf disks. Data obtained in these experiments were submitted to a variance analysis comparing mean values with the Tukey test (P = 0.05) calculated by the Software SANEST 3.0.

REPELLENCE TEST

The repellence tests were made according to the modified method described by Kogan & Goeden (1970). Leaf disks of *Canavalia ensiformes* of 4.5 cm diameter were used to evaluate the repellence of the essential oils. Half of the disk was immersed for 5 seconds in an ethanol solution of the essential oil in three concentrations (0.25, 0.5, 0.75 and 1.0 %), and after drying, the other half of the disk was immersed in pure ethanol, which served as control. Each half circle was immersed in such a way that an area of 0.3 cm between the two halves, where the mites were collocated, remained intact. The leaf was collocated on filter paper on polyethylene foam wetted by water. 10 female adults of mites were put on each disk, each treatment was repeated 10 times.

The evaluation was made after 24 h, where the number of mites present on each half of the leaf disk was counted. Mites found in the neutral area during the evaluation were considered as repellent or attracted, based on their proximity to the blank or to the treatment. The Repellent Index (RI) of the oils was calculated according to the equation: RI = 2G/(G + P) proposed by Kogan & Goeden (1970), where G = number of mites in the treatment and P = number of mites in the control. The security interval used to consider oil as repellent or not was obtained based on the mean value of RI and the respective standard deviations (SD). In other words, if the mean value of the RI was 1 - SD, the oil is repellent, while a mean value higher than 1 + SD, the oil is attractant, and for mean values between 1 – SD and 1 + SD, the oil is indifferent.

RESULTS AND DISCUSSION

CHEMICAL COMPOSITION OF THE ESSENTIAL OILS

The best yield of the essential oils was obtained by fruit extraction (1.3 %, v/w). The essential oil of leaves, obtained with 0.7 % (v/w) yield, was yellow, while that of the fruits was colourless.

The oil analysis by GC and GC/MS permitted the identification of 57 compounds representing 96.4 and 98.0 % of the essential oil constituents from the fruits and leaves, respectively. These analyses also revealed that the major identified components in the leaves were the sesquiterpenes: trans-9-epi-caryophyllene (21.4 %), *trans*-isolongifolanone (10.7 %) and 14-hydroxi-9-*epi*-β-caryophyllene, whereas the monoterpene, α-terpinene (47.6 %), for the fruits, was the principal constituent. The chemical compounds found in these oils are shown in Table 1.

The chemical investigation of the essential oil of *P. heptaphyllum* collected from the Tamandaré beach in Pernambuco revealed a large quantity of sesquiterpenes (84.4 %) in the leaf oil, whereas the fruit oil revealed a predominance of monoterpenes (92.1 %). These data are consistent with the ones reported for the *P. heptaphyllum* species, which grow in different regions of Brazil. The leaf oil composition of *P. heptaphyllum*, from the state of Amazonas - Brazil (Zoghbi & Maia, 1995), revealed more than 45 % of sesquiterpenes, whereas the major components were β-elemene (22.1 %) and β-caryophyllene (11.1 %). On the other hand, the fruit and leaf oil from the specimen collected

Table 1 - Percentage composition of the essential fruit and leaf oils of *Protium heptaphyllum*

Compounds	RI[a] Lit.	RI Cal.	Fruits	Leaves	Compounds	RI[a] Lit.	RI Cal.	Fruits	Leaves
(E)-salvene	865	867	-	0.5	α-longipinene	1351	1352	1.5	-
α-pinene	939	935	1.1	-	neryl acetate	1365	1363	0.8	-
verbenene	967	965	1.1	-	carvacrol acetate	1371	1368	1.5	0.5
myrcene	991	990	2.0	-	isoledene	1373	1367	-	2.7
α-terpinene	1018	1015	47.6	-	linalool isobutyrate	1374	1370	1.6	-
p-cymene	1026	1021	1.5	-	α-copaene	1376	1374	-	7.3
β-phellandrene	1031	1029	-	9.2	β-bourbonene	1384	-	-	1.0
limonene	1031	1027	3.7	0.8	β-cubebene	1390	-	-	0.1
(Z)-β-ocimene	1040	1039	2.5	2.0	β-elemene	1391	-	-	0.1
trans-decahydro naphtalene	1057	1053	0.6	-	β-longipinene	1398	1399	3.5	-
α-pinene oxide	1095	1090	0.8	-	9-*epi*-(E)-caryophyllene	1467	1467	-	21.4
chrysanthenone	1123	1115	1.0	-	γ-muurolene	1477	1474	-	0.6
l-dihydro-linalol	1134	1130	1.0	-	α-zingiberene	1495	1490	-	0.1
trans-verbenol	1144	1136	1.3	-	(Z)-α-bisabolene	1504	1501	-	3.5
karahanaenone	1155	1149	1.1	-	δ-cadinene	1524	1524	-	1.4
cis-pinocarveol	1183	1180	1.8	-	cadina-1,4-diene	1532	1531	-	3.1
verbenone	1204	1200	1.6	-	α-cadinene	1538	1547	-	1.0
p-cymen-9-ol	1206	1202	1.2	-	(E)-nerolidol	1564	1559	-	2.0
trans-carveol	1217	1213	1.1	-	carotol	1594	1590	-	0.7
trans-chrysanthenyl acetate	1235	1230	2.5	-	guaiol	1595	1591	-	3.7
(Z)-ocimenone	1231	1231	0.5	-	β-oplopenone	1606	1608	-	1.1
(E)-ocimenone	1239	1235	1.0	-	*trans*-isolongifolanone	1618	1620	-	10.3
perilla aldehyde	1271	1270	1.1	-	14-hydroxy-9-*epi*-(E)-caryophyllene	1664	1660	-	16.7
3-thujyl acetate	1291	1290	0.5	-	valeranone	1672	1670	-	2.0
trans-ascaridole	1301	1299	1.2	-	8-cedren-13-ol	1688	1690	-	0.7
cis-pinocarvyl acetate	1309	1305	0.9	-	curcuphenol	1715	1710	-	0.7
iso-dihydro carveol acetate	1325	1322	3.7	-	isolongifolol	1726	1721	-	4.1
terpin-4-ol acetate	1340	1335	0.1	-	14-hydroxy-α-muurolene	1775	1770	-	0.7
α-terpinyl acetate	1350	1347	5.0	-	Not identified			3.6	2.0
					Total			100	100

[a]Retention index (RI) values are calculated from retention times relative to that of n-alkanes on the non-polar DB-5 column.

from Ceará – Brazil was found to be entirely monoterpenoid (91.6 %), by the predominance of α-pinene (71.2 %) and sesquiterpenes with 18.6 % of β-caryophyllene, respectively (Bandeira *et al.*, 2001).

A comparison of the chemical profile of the essential oils of the *P. heptaphyllum* species with the ones reported for samples collected in different places in Brazil (Amazonas and Ceará) permits the identification of chemeotypes which belong to the same biosynthetical pathway of caryophyllene. For the essential fruit oil, the major constituent in the sample coming from Ceará is part of the biosynthetic pathway of pinene, while the monoterpene identified as major constituent in the Pernambuco sample is part of the pathway of terpinene. The amount and variation of the oil composition in plants are heavily influenced by climatic factors and geographical parameters as well as genetic factors (Machado *et al.*, 2003; Siani *et al.*, 2004).

FUMIGANT ACTIVITY OF THE ESSENTIAL OILS

MORTALITY

The vapours of the essential oils of leaves and fruits of *P. heptaphyllum* are toxic for *T. urticae* when concentration and exposure times were increased.

The fruit oil is more toxic for mites, provoking a mortality of 63.3 %, when submitted to oil concentrations of 10 $\mu l.l^{-1}$ of air after 72 h of exposure (Table 2). No significant difference was found between the mite mortality for 24 h or 48 h, when applying the same oil concentrations. The minimum oil concentration necessary to promote significant mite mortality is 8 $\mu l.l^{-1}$ of air, when submitted to oil action for 24, 48 and 72 h.

The major mortality provoked by the leaf oils was 41.0 % at the highest concentration of 10 μL L^{-1} of air (Table 2). No significant difference of the toxic action of the oils' vapour was found, when submitted to the same concentrations for 24, 48 and 72 h.

The essential fruit oil of *P. heptaphyllum* is more efficient against mites than the essential leaf oil, as can be seen by the higher mortality. But all oils are active in higher concentrations (8 and 10 μL L^{-1} of air), as can be seen by the significant difference to the control.

FECUNDITY

The essential fruit oil is responsible for the lowest mean value of eggs per leaf disk (16.0) at 72 h exposure time, compared to the other oil (Table 3). The minimum fruit oil concentration necessary for reducing significantly the mite fecundity in 24 h exposure is 4 $\mu l.l^{-1}$ of air. For more than a 24 h exposure, the smallest tested concentration (2 $\mu l.l^{-1}$ of air) is sufficient to reduce oviposition.

Exposure to the leaf oil results in a major reduction of the mean egg value (32.0) at the highest tested concentration (10 $\mu l.l^{-1}$ of air) in 24 h exposure (Table 3). This is the minimum oil concentration necessary to reduce significantly the egg's quantity deposited by mites in 24 h and 72 h. These results indicate that the mites submitted to fruit and leaf essential oils of *P. Heptaphyllum* did not stop oviposition, but drastically reduce fecundity.

REPELLENT ACTION OF THE ESSENTIAL OILS

As shown in table 4, the essential fruit oil of *P. heptaphyllum* is the only one having repellent action of the mite. The smallest used oil concentration in this test (0.25 %) does not show repellent activity. Concentrations equal to and higher than 0.5 % of the essential fruit oil did provoke repellence.

The oils analysis by GC/MS, which resulted in á-terpinene (47.6 %) as the major constituent of the fruit essential oil of *P. heptaphyllum* and other compounds in smaller quantity, like á-pinene (1.1 %), limonene (3.7 %), suggests a probable action of theses volatile components by their acaricide property and repellent action, as well as by their action on oviposition. The literature shows the relating action of these substances in essential oils or isolated as insecticides (Viegas Júnior, 2003; Choi *et al.* 2005) and acaricides (Aslan *et al.*, 2004; Çalmaºur *et al.*, 2005; Iori *et al.*, 2005).

Table 4 - Repellent effect of four different concentrations of essential fruit and leaf oils of *P. heptaphyllum* on mite *T. urticae*.

Essential oil	Concentration (%)	Mean value of Repellence Index[1]	Condition
Fruits	0.25	0.58 ± 0.46	Indifferent
	0.50	0.10 ± 0.01	Repellent
	0.75	0.53 ± 0.33	Repellent
	1.00	0.24 ± 0.13	Repellent
Leaves	0.25	1.20 ± 0.47	Indifferent
	0.50	1.00 ± 0.62	Indifferent
	0.75	0.61 ± 0.38	Indifferent
	1.00	0.96 ± 0.36	Indifferent

[1]Repellence Index calculated according to the equation described by Kogan & Goeden (1970)

Table 2 - Mortality of *T. urticae* exposed to essential fruit and leaf oils of *P. heptaphyllum* in five concentrations and three time periods

Concentration ($\mu l.l^{-1}$ of air)	Mean value of mortality (%)					
	Fruits			Leaves		
	24 h	48 h	72 h	24 h	48 h	72 h
0	0.0±0.0aA	1.0±0.32aA	2.0±1.20aA	3.3±0.57aA	3.3±0.57aA	1.0±0.32aA
2	1.5±0.30aA	8.0±0.87aB	18.0±0.57aC	7.6±0.57aA	13.3±0.87aA	4.3±0.32aA
4	4.0±0.50aA	3.3±1.45aA	29.0±1.20aB	7.6±0.66aA	16.6±0.57aA	10.0±0.57aA
6	21.0±1.70abA	17.6±0.87abA	45.3±1.20abB	17.6±1.20abA	19.0±0.66abA	15.6±2.02abA
8	25.3±1.45bA	28.0±1.76bcA	57.6±0.87bB	21.0±0.66bcA	30.0±1.73bA	18.6±0.87bcA
10	34.6±2.33bA	43.0±1.73cAB	63.3±0.57bB	43.3±2.40cA	28.6±0.87bA	41.0±1.45cA

Mean values followed by the same minor letter in the column and major letter in the line do not differ significantly between themselves based on the Tukey test (*P* d" 0.05)

Table 3 - Fecundity (eggs / leaf disk) of *T. urticae* exposed to essential fruit and leaf oils of *P. heptaphyllum* in five concentrations and three time periods

Concentration ($\mu l.l^{-1}$ of air)	Mean fecundity (eggs / leaf disk)					
	Fruits			Leaves		
	24 h	48 h	72 h	24 h	48 h	72 h
0	127±17.52aA	294.3±4.41aB	343.6±9.84aB	199.6±13.97aA	290.3±9.57aB	303.6±4.26aB
2	89±12.50aA	112.6±2.72bAB	129.0±4.63bB	82.6±2.40bA	102.6±0.87bAB	107.6±2.02bB
4	22±0.30bA	49,0±1.20cB	61.3±3.84cB	69.3±4.26bcA	89.6±4.98bA	89.0±6.80bA
6	18±2,60bA	27.0±2.02cdAB	25.0±0.57dB	50.6±2.51cdA	87.0±6.66bB	79.0±7.81bB
8	23.3±2.18bA	22.0±1.15dA	26.0±3.21dA	40.0±4.98cdA	55.3±3.17cAB	81.6±10.87bB
10	16.0±1.52bA	27.0±1.52dB	32.0±2.31dB	32.0±1.66dA	42.0±1.52cA	43.0±4.93cA

Mean values followed by the same minor letter in the column and major letter in the line do not differ significantly between themselves based on the Tukey test (*P d"* 0.05)

CONCLUSIONS

The chemical investigation of the essential oil of *P. heptaphyllum* collected in Tamandaré beach in Pernambuco was consistent with the ones reported for the *P. heptaphyllum* species which grows in different regions of Brazil. The results obtained suggest that the essential fruit oil of *P. heptaphyllum* shows high toxicity for *T. urticae* as the essential leaf oil at a dose of 10 µl for a minimum exposure of 72 h, or at a dose of 20 µl for 24 h exposure. The fumigant activity of the essential oil of leaves and fruits inhibit the oviposition of mites, reducing the egg number with the smallest tested concentration (2 $\mu l.l^{-1}$ of air) for a minimum exposure period of 24 h.

Acaricidal activity of the essential oils is a promising way to control pests in closed environments. Further studies should be made to evaluate the cost/benefit ratio of the use of these oils in large scale for the protection of species cultivated in commercial greenhouses. These data represent the first reported study of the acaricidal activity of the essential leaf and fruit oils of *P. heptaphyllum*.

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