Original Article

BRINE SHRIMP LETHALITY TEST AS A BIOLOGICAL MODEL FOR PRELIMINARY SELECTION OF PEDICULICIDAL COMPONENTS FROM NATURAL SOURCE

TESTE DE TOXICIDADE FRENTE ARTEMIA COMO MODELO PRELIMINAR DE BUSCA DE SUBSTÂNCIAS PEDICULICIDAS A PARTIR DE FONTES NATURAIS

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ABSTRACT: Brine shrimp lethality test as a biological model for the preliminary selection of pediculicidal components from a natural source**.** In order to achieve a good correlation between pediculicidal activity and brine shrimp lethality (BSL) test, several pediculicidal substances and active essential oils were tested in BSL test, with the purpose to use the latter as convenient preliminary protocol for pediculicidal activity. Benzyl benzoate, deltametrine and essential oil of *Eucalyptus* were purchased and clove essential oil obtained by hydrodistillation, besides essential oils, chloroform extracts from *Duguetia furfuracea* were also submitted to BSL test. All of them were carried out with same protocol described to pediculicidal assay found in the literature, i.e, flask tests were examined every five minutes in the first half hour and then every ten minutes until all the naupli were dead or no movements were observed (knockdown). During the BSL test, it was possible to observe the effect of a particular lethal dose or only a knockdown in the arthropod, as occurred in the test with lice. The results of the BSL test for essential oils and other active substances are essentially in agreement with those described in literature for pediculicidal activity. Extracts and essential oil obtained from aerial parts of *D. furfuracea* did not present activity, but the essential oil from underground stem bark was active. α-asarone has already been isolated from the underground stem and it has been previously described to possess insecticidal activity.

KEYWORDS: Pediculicidal activity. Bioassay model. Essential oil. *Duguetia furfuracea*.

INTRODUCTION

Infestation with *Pediculus capitis* is a common disease occurring widely throughout the world (KO; ELSTON, 2004). Humans are infested by this parasite for several reasons. In modern society, the main cause of the infestation is not the influence of socio-economic conditions; the predominant factor contributing to its increase is the resistance to insecticides (HODGDON et al., 2010). The problem encountered with the control of this parasitic disease is strictly related to all the stages of the parasite's life along with the mode of transmission; therefore, several products and methodologies have been tried to remove, kill or prevent the infestation of lice (GRATZ et al., 1997; BURKHART et al., 2003).

Insecticidal products, undoubtedly, have been the simplest and most effective method to control the disease. However, side effects and resistance to insecticides, along with the toxicity of currently available treatments, are serious problems to treatment compliance. Thus, plants may provide potential alternatives to the currently used insect-control agents because they constitute a rich source of bioactive chemicals. Therefore, searching for pediculicidal compounds from several sources is necessary; nevertheless, the *in-vitro* pediculicidal protocol used to assess this activity is, in general, complicated (OLADIMEJI et al., 2000; YANG et al., 2004; WILLIAMSON et al., 2007). The bioassays for pediculosis described in literature promote the evolution of more complexity in the parasite itself if used. Several difficulties in the protocols, such as collecting and feeding the lice during the experiment, have been described. In addition to the process of assay development, application of the product to be tested, and the interpretation of the results have been also considered (OLADIMEJI et al., 2000; YANG et al., 2003, 2004).

In folk medicine, pulverised seeds of *Duguetia furfuracea,* a shrub typical of the cerrado of Central Brazil and which is popularly known as "Araticum-seco" are diluted in water and are commonly used to eliminate parasites, particularly louses (CORREA, 1978; SILBERBAUER-GOTTSBERGER, 1981/1982). The purpose of this

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study was to achieve a good correlation between brine shrimp lethality test (BSL test) and the pediculicidal activity of some compounds and extracts from plants, in order to use the BSL test as a convenient preliminary protocol for the screening of the pediculicidal effect. Some compounds and essential oils described as being active on louse were tested; moreover, the essential oil and the extracts of *D. furfuracea* were also tested.

MATERIAL AND METHODS

Preparation of essential oil and extracts from *D. furfuracea*

Benzyl benzoate (BzB), deltametrine (Δ) and the essential oil of *Eucalyptus globulus* Labill. (Eucal) and buds of *Eugenia caryophyllata* Thunb. were purchased commercially. The essential oil of *E. caryophyllata* (clove) was obtained by hydrodistillation.

The leaves, fruit and underground parts (stem bark and wood) of *Duguetia furfuracea* (A. St.-Hil.) Benth. e Hook f. were collected in March 2007, on the UFMS campus in Campo Grande, MS, Brazil, and identified by Prof. R. Mello-Silva. A voucher specimen (No. 023) was deposited in the CGMS Herbarium.

The essential oils and extracts of *D. furfuracea* (Annonaceae), which are described in folk medicine as having pediculicidal properties, were obtained according to the following procedure. The dried natural fruit pulp (211.0 g) was pulverised and subjected to an exhaustive extraction in a Soxhlet apparatus with chloroform for six hours, yielding 277.4 mg (0.13%, D_{fp}) of extract. The seeds (141.8 g) were subjected to the same methodology and 55.9 mg (0.04%, D_{fs}) of the chloroform extract was obtained. Fresh leaves (400 g) and the underground stem bark (300 g) of *D. furfuracea* were pulverised and subjected to an exhaustive extraction by hydrodistillation, and they yielded 2.9 mL (0.73%, w/v, D_{fl}) and 3.6 mL (1.20%, w/v, D_{fusb}) of the essential oil, respectively. All extracts and volatile oils were subjected to a BSL bioassay.

BSL test as a screening test for pediculicidal activity

With the aim of developing a simple methodology for use as a preliminary screening test for pediculicidal activity, various substances and herb extracts previously described as active were subjected to the BSL test, using a protocol similar to that used in the *P. capitis* assay, but with some adaptations. Each flask was examined every five minutes in the first half hour and then every ten minutes until all the naupli were dead or no movements were observed (knockdown). The tested sample was considered ineffective if any modification of *Artemia salina*. behaviour was observed in a span of two hours (120';OLADIMEJI et al., 2000; YANG et al., 2003, 2004).

Assays for the essential oils were also developed, with at least three dilutions and in triplicate, with 1% of dimethyl sulphoxide (DMSO; v/v). Thus, assays with BzB, Δ, and the essential oils clove and Eucal were carried out. In addition, the essential oils and chloroform extracts of *D. furfuracea* were also tested.

Samples with four dilutions, in triplicate, of BzB were prepared in the following concentrations: 5.6, 2.8, 1.4, 0.7 and 0.35 mg/cm^3, as described in literature (OLADIMEJI et al. 2000; YANG et al. 2004). Thus, these samples were solubilised in 20 mL of marine solution with 1% DMSO (v/v), and ten naupli (second instar) of brine shrimp were added in each flask and maintained in direct contact with the solutions. Most of the solutions tested had a milky appearance, but were translucent enough for the development of the bioassay. To avoid the volatilisation of the substances, the flasks were covered with a polyvinyl chloride film. A magnifying glass, a light focus and a dark background were used to conduct the readings.

The same procedure was followed for Δ, with three dilutions and in triplicate, that is, 0.20, 0.10 and 0.05 mg/cm^3 in 20 mL of marine solution with 1% DMSO (v/v). These concentrations are almost the same as those described by Yang (2004) with pyrethroid derivatives in the *P. capitis* assay.

The essential oils from *E. globulus* and *E. caryophyllata* were diluted at concentrations of 0.20, 0.10 and 0.05 mg/cm^3 in marine solutions with 1% DMSO (v/v). Samples from *D. furfuracea* were diluted at concentrations of 1.00, 0.50 and 0.25 mg/cm^3.

Gas chromatography–mass spectrometry Analysis

The samples of volatile oil were subjected to gas chromatography–mass spectrometry (GC–MS) analysis on a Shimadzu QP2010. The apolar extract was purified using a cartridge of silica gel (AccuBond^{II} SPE) with hexane, dichloromethane and methanol. The chloroform fractions were subjected to GC–MS analysis. The GC column was a DB-5MS (30 m in length, 0.25 μm in thickness, 0.25 mm i.d.). The carrier gas was helium, with pressure of 81.9 kPa and flow rate of 1.33 mL/min.

Injector temperature was 250 °C and the split ratio was 1: 50. The temperature was programmed in the range of 60–240 °C increasing at 3 °C/min. The mass spectra were recorded in the electron ionisation mode, with ionisation energy of 70 eV. The temperature of the ion source was 250 °C.

Identification of the constituents

Identification of each individual constituent of the samples (Table 1) was achieved based on the retention indices (calculated using C9 to C22 alkanes), by comparison of the mass spectra data with a computer databank (WILEY 7 and NIST 62) and with reference to published data (ADAMS, 1995).

Statistical methodology

The statistical analysis of the estimated survival time was performed with the application of the Kaplan-Meier nonparametric method. In the present analysis has been not used any assumption about the probability of survival time, and the concept of independence among the events was applied. The survival curves were compared with the log-rank test, and level significance of 5% was considered statistically significant. The values to determine the lethal concentrations were analyzed by using the regression model probit program.

RESULTS AND DISCUSSION

The results of the BSL test using the essential oils and other active substances are described in the Tables 1 and 2, and they are essentially in agreement with those described in literature for pediculicidal activity (YANG et al., 2003, 2004). During the *A. salina* test, it was also possible to observe the effect of a particular lethal dose or only a knockdown in the arthropod, as occurred in the test with lice (OLADIMEJI et al., 2000; YANG et al., 2003, 2004). Substances and extracts were active for a period of up to two hours in the *A. salina* assay. Only the *E. globulus* sample, among those described as active, was relatively less active; however, it still falls within the expected time interval (<2 hours).

Table 1. Relative toxicity of compounds, extracts and essential oils against brine shrimp larvae

Compound, extract or essential oil	Concentration in marine solution (mg/cm3)	Estimate Medians for Survival Time (min)	95% Confidence Interval	
			Lower Bound	Upper Bound
	5.6	10	-	-
	2.8	10	-	-
Bzb	1.40	16	9.62	22.38
	0.70	20	3.93	36.07
	0.35	40	29.36	50.64
Δ	0.20	5	-	
	0.10	15	-	
	0.05	5	-	
clove	0.20	25	22.36	27.64
	0.10	50	39.26	60.74
	0.05	NE	-	-
Eucal	0.20	30	22.49	37.51
	0.10	210	-	
	0.05	210	-	
Dfusb	1.00	30	16.61	43.39
	0.50	90	67.20	112.80
	0.25	210	146.16	273.84
Dfp	(*)	NE	-	-
Dfs	(*)	NE	-	-
Dfl	(*)	NE	-	-

(*) Concentrations of 1.00, 0.50 and 0.25 mg/cm^3. **NE**: no effectiveness. **Dfusb**: essential oil from underground of stem bark of *D. furfuracea*, **Dfl**: essential oil from leaves of *D. furfuracea*, **Dfp**: chloroform extract from pulp fruit of *D. furfuracea*, **Dfs**: chloroform extract from seed of *D. furfuracea*, **Eucal**: essential oil from leaves of *E. globulus*, **clove**: essential oil from bud of *E. caryophyllata*.

Table 2. Lethal dose (DL_{50}) by compound, extract or essential oil in mg/cm^3, against brine shrimp larvae until 50 minutes exposure

Compound, extract or essential oil	DL_{50}	IC 95%	R^2
Bzb	226.1	102.5 – 498.7	0.09
Δ	#	-	-
Clove	102.0	89.9 – 115.8	0.9
Eucal	141.4	127.1 – 157.3	1.0
Dfusb	715.2	608.3 – 840.9	0.85
Dfp*	NE	-	-
Dfs*	NE	-	-
Dfl*	NE	-	-

Total mortality at all concentrations up to 50 minutes exposure; * Concentration of 1.00, 0.50, 0.25 mg/cm^3
NE: no effectiveness. **Dfusb**: essential oil from underground of stem bark of *D. furfuracea*, **Dfp**: chloroform extract from pulp fruit of *D. furfuracea*, **Dfl**: essential oil from leaves of *D. furfuracea*, **Dfs**: chloroform extract from seed of *D. furfuracea*, **Eucal**: essential oil from leaves of *E. globulus*, **clove**: essential oil from bud of *E. caryophyllata*.

Theoretically, the *A. salina* toxicity assay can be used as a pre-evaluation model for pediculicidal activity as long as the bioassay is developed as described in the present work, which follows the protocol proposed for the parasite assay, and the requirements of knockdown and/or death of the naupli stage must be in the range from 50% to 100% during the interval of 120 minutes, as described for lice (YANG et al., 2003, 2004).

Extracts and essential oils of plants are mainly characterised by their complex chemical mixture. In a preliminary assay, the results can be diffuse and not well bio-directed; however, continuing the phytochemical fractionation and testing the sub-fractions using the protocol described previously can identify potential pediculicidal substances.

The chemical profiles of the active substances within the active essential oils, extracts and fractions have been investigated to define the possible potential pediculicidal pattern in terms of monoterpenes, sesquiterpenes and other classes of compounds. Mono-oxygenated nonocyclical monoterpenes have shown more efficiency against lice (OLADIMEJI et al., 2000, PRIESTLEY et al., 2006); moreover, some phenolic compounds, especially benzaldehyde and salicylaldehyde, have shown 30- and 17-fold potency compared to pyrethrin, and eugenol and methyl salicylate, the constituents present in *E. Caryophyllata* have been reported to be active (YANG et al., 2003, 2004). Data relating to pediculicidal or insecticidal activity of sesquiterpene pediculicides are scarce; nevertheless, β-caryophyllene, α-humulene and caryophyllene oxide, for instance, present in the essential oil obtained from the leaves of *D. furfuracea* (Table 3) have been described as inactive against pediculosis (YANG et al., 2003, 2004). For *D. furfuracea*, only the essential oil from the underground stem bark (Dfusb) showed relative activity, whereas the other extracts from the plant were not active at the concentrations tested. α-Asarone was isolated from Dfusb, and this substance has been described as a potential pesticide (LEE et al., 2002, PARK et al., 2003).

Artemia naupli have been suggested for use as a model for several preliminary evaluations of pharmacological and ecotoxicological activities of compounds of greater complexity (MCLAUGHLIN 1993; DVORAK et al., 2010). The BSL test has been used for insecticidal, acaricidal, anaesthetic, and anti-tumour activity evaluations, using different methodologies. (AREEKUL et al., 1960; ROBINSON et al., 1965; HARWING et al., 1971; MCLAUGHLIN 1993) For the correlations cited above, the tests are conducted and the readings taken twenty-four hours after application of the substance. In the present work, readings have been taken every five minutes in the first 30 minutes, followed by further readings every ten minutes, which proved to be a useful search for the substances and extracts with pediculicidal potential.

The extracts and essential oil obtained from the aerial parts of *D. furfuracea* did not show any activity (Table 4); however, the essential oil from the underground stem bark was active; α-asarone has already been isolated from the underground stem and has been previously described to possess insecticidal activity (LEE et al., 2002; PARK et al., 2003)

Table 3. Results of analysis of the survival of the *Artemia* naupli for concentration in marine solution,* Log-rank test compared with *p* value of < 0.05 was considered statistically significant

Compound	Concentration in marine solution (mg/cm^3)	2.80 mg/cm3		1.40 mg/cm3		0.70 mg/cm3		0.50 mg/cm3		0.35 mg/cm3		0.25 mg/cm3		0.10 mg/cm3		0.05 mg/cm3	
		X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.	X^2	Sig.
BzB	5.60	17.9	<0.01*	17.7	<0.01*	52.1	<0.01*			65.3	<0.01*						
	2.80			3.0	0.08	26.1	<0.01*			60.3	<0.01*						
	1.40					15.2	<0.01*			32.2	<0.01*						
	0.70									3.5	0.06						
Δ	0.20													44.1	<0,01*	2.98	0.08
	0.10															11.39	<0.01
	0.05																
clove	0.20													39.3	<0,01*	68.39	<0.01
	0.10															67.70	<0.01
Eucal	0.20													60.61	<0,01*	66.26	<0.01
	0.10															2.03	0.15
Dfusb	1.00							23.12	<0.01*			56.7	<0,01*				
	0.50											20.0	<0,01*				
Dfp	1.00							2.03	0.15			15.3	<0,01*				
	0.50											9.6	<0,01*				
Dfl	1.00							1.00	0.32			2.0	0.15				
	0.50											0.3	0.56				

Dfusb: essential oil from underground of stem bark of *D. furfuracea*, **Dfl**: essential oil from leaves of *D. furfuracea*, **Dfp**: chloroform extract from pulp fruit of *D. furfuracea*, **Eucal**: essential oil from leaves of *E. globulus*, **clove**: essential oil from bud of *E. caryophyllata*.

Table 4. Chemical constituents of extracts, essential oils identified by gas chromatograph-mass spectrometer.

	Relative content (%)						RI
	Dfusb	Dfl	Dfp	Dfs	Eucal	clove	
α-pinene	-	-	-	-	2.3	-	932
β-pinene	-	-	-	-	0.3	-	978
β-myrcene	0.6	-	-	-	0.2	-	988
δ-2-carene	1.6	-	-	-	-	-	992
trans-m-mentha-4,8-diene	6.5	-	-	-	-	-	995
o-cymene	-	-	-	-	6.7	-	1023
Limonene	0.1	-	-	-	9.2	-	1028
β-phellandrene	0.9	-	-	-	-	-	1030
Eucaliptol	-	-	-	-	79.0	-	1032
Linalool	-	-	-	-	0.1	-	1099
cis-limonene oxide	-	-	-	-	0.1	-	1132
trans-limonene oxide	-	-	-	-	-		1137
trans-pinocarveol	-	-	-	-	0.1	-	1139
Terpin-4-ol	0.3	-	-	-	0.3	-	1180
p-cymen-8-ol	-	-	-	-	0.1	-	1185
α-terpineol	-	-	-	-	1.3	-	1189
trans-carveol	-	-	-	-	0.1	-	1218
Carvone	-	-	-	-	0.2	-	1242
(*E,E*)-2,4-decadienal	-	-	-	1.5	-	-	1317
Bicycloelemene	-	0.2	-	-	-	-	1329
δ-elemene	-	3.4	-	-	-	-	1332
Eugenol	-	-	-	-	-	89.4	1354
Cyclosativene	0.1	-	-	-	-	-	1364
α-copaene	0.1	0.5	-	-	-	-	1372
β-elemene	0.9	3.6	-	-	-	-	1385
Cyperene	16.0	-	-	-		-	1397
α-gurjunene	22.2	-	-	-	-	-	1403
NI	1.2	-	-	-	-	-	1408
β-caryophyllene	2.6	11.5	-	-	-	4.1	1415
β-gurjunene	0.2	0.5	-	-	-	-	1428
Aromadendrene	0.1	0.6	-	-	-	-	1434
α-humulene	0.5	1.3	-	-	-	0.5	1451
NI	1.5	-	-	-	-	-	1457
γ-gurjunene	1.9	-	-	-	-	-	1470
α-amorphene	-	1.1	-	-	-	-	1471
γ-muurolene	1.0	-	-	-	-	-	1473
Germacrene D	0.8	13.0	-	-	-	-	1476
β-selinene	0.4	-	-	-	-	-	1484
Viridiflorene	-	1.0	-	-	-	-	1487
Bicyclogermacrene	1.4	16.2	-	-	-	-	1491
α-muurolene	0.6	1.0	-	-	-	-	1495
δ-guaiene	0.2	-	-	-	-	-	1498
γ-cadinene	0.6	0.5	-	-	-	-	1514
Eugenol Acetate	-	-	-	-	-	5.8	1518
δ-Cadinene	-	0.6	-	-	-	-	1519
trans-calamenen	1.3	-	-	-	-	-	1540
Germacrene B	-	2.2	-	-	-	-	1553
2,4,5-trimethoxy-styrene	19.7	-	-	-	-	-	1554
Dodecanoic acid	-	-	-	2.1	-	-	1564
Ledol	1.0	0.1	-	-	-	-	1565
Spathulenol	0.2	17.8	15.0	25.2	-	-	1572
Caryophyllene oxide	0.1	3.6	-	-	-	0.2	1577
Globulol	-	3.5	-	-	-	-	1580
Viridiflorol	-	4.0	-	1.7	-	-	1589

Cedrol	1.1	-	-	-	-	-	1600
Rosifoliol	-	0.4	-	-	-	-	1601
NI	1.5	-	7.5	-	-	-	1605
NI	-	1.6	2.6	-	-	-	1615
NI	-	1.9	-	-	-	-	1624
δ-cadinol	0.4	-	-	-	-	-	1625
Isospathulenol	-	1.1	15.2	3.2	-	-	1628
Hinesol	0.4	-	-	-	-	-	1635
epi-α-cadinol	-	1.3	-	-	-	-	1638
epi-α-muurolol	0.2	1.3	-	-	-	-	1640
α-muurolol	0.9	0.6	3.9	1.7	-	-	1642
trans-isoelemicin	0.2	-	-	-	-	-	1645
NI	-	-	1.0	-	-	-	1649
α-cadinol	0.4	2.6	1.8	-	-	-	1651
Caryophylla-4(12),8(13)-diene-5β-ol	-	0.6	-	-	-	-	1658
NI	-	2.4	-	-	-	-	1664
α-asarone	10.1	-	-	-	-	-	1672
NI	-	-	4.5	-	-	-	1688
NI	-	-	4.6	-	-	-	1692
Asaraldehyde	-	-	14.1	15.5	-	-	1708
Aromadendrene Oxide II	-	-	-	4.1	-	-	1714
NI	-	-	3.4	-	-	-	1727
NI	-	-	-	2.8	-	-	1734
NI	-	-	-	2.7	-	-	1736
(*E,Z*)-farnesol	-	-	1.2	-	-	-	1743
NI	-	-	2.4	-	-	-	1748
Tetradecanoic acid	-	-	-	1.12	-	-	1762
NI	-	-	4.6	-	-	-	1802
NI	-	-	6.5	-	-	-	1855
Hexadecanoic acid	-	-	7.9	20.0	-	-	1966
Ethyl Hexadecanoate	-	-	-	2.5	-	-	1994
n-Octadecanol	-	-	3.5	1.7	-	-	2084
(*Z*)-9-Octadecenoic acid methyl ester	-	-	-	1.4	-	-	2097
(*Z,Z*)-9,12-Octadecadienoic acid methyl ester	-	-	-	1.7	-	-	2125
(*Z*)-9-Octadecenoic acid	-	-	0.4	4.0	-	-	2145
(*Z,Z*)-9,12-Octadecadienoic acid	-	-	-	2.5	-	-	2158

Dfusb: essential oil from underground of stem bark of *D. furfuracea*, **Dfl**: essential oil from leaves of *D. furfuracea*, **Dfp**: chloroform extract from pulp fruit of *D. furfuracea***, Dfs**: chloroform extract from seed of *D. furfuracea*, **Eucal**: essential oil from leaves of *E. globulus*, **clove**: essential oil from bud of *E. caryophyllata*, **NI**: no identification.

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RESUMO: O teste de toxicidade frente a *Artemia salina* (TAS) foi utilizado como modelo biológico preliminar na busca de substâncias potencialmente pediculicidas a partir de fontes naturais. Foi encontrada uma boa correlação entre a atividade pediculicida e o TAS, várias substâncias e óleos essenciais descritos como pediculicidas foram testados sobre o microcrustáceo, com o objetivo de se obter um protocolo preliminar e apropriado para detectar aquela atividade. Benzoato de benzila, deltametrina, e óleos essenciais de eucalipto e cravo foram obtidos comercialmente e/ou por extração. Além desses, extratos e óleos essenciais de *Duguetia furfuracea* também foram testados frente a *Artemia*. Todas as amostras foram conduzidas utilizando o mesmo protocolo proposto para atividade pediculicida, ou seja, os frascos testes foram lidos a cada cinco minutos na primeira meia hora e depois a cada dez minutos até os nauplios estarem mortos ou sem

movimento (*knockdown*). Durante o TAS foi possível observar a dose letal ou somente o *knockdown* na larva, como ocorrido no teste com piolho. Os resultados do TAS para os óleos essencial e os demais compostos ativos estão essencialmente de acordo com o descrito na literatura para a atividade pediculicida. Extratos das sementes e óleos essenciais das folhas e sementes *D. furfuracea* não apresentaram atividade, mas o óleo das cascas do caule subterrâneo foi ativo α-asarona foi isolado das cascas do caule e esta substância apresenta atividade inseticida.

PALAVRAS-CHAVES: Atividade pediculicida. Bioensaio. Óleo essentical. *Duguetia furfuracea.*

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