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Insecticidal activity of *Gallesia integrifolia* (Phytolaccaceae) essential oil

[Actividad insecticida del aceite esencial de *Gallesia integrifolia* (Phytolaccaceae)]

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Abstract: This study evaluated the insecticidal activity of *Gallesia integrifolia* essential oil from fruits, leaves, and flowers against *Aedes aegypti* larvae and pupae. The essential oil was obtained by hydrodistillation and characterized by gas chromatography-mass spectrometry. Sulfur compounds represented 95 to 99% of the essential oil from fruits, leaves, and flowers. Essential oil major compounds were 2,8-dithianonane (52.6%) in fruits, 3,5-dithiahexanol-5,5-dioxide (38.9%) in leaves, and methionine ethyl ester (45.3%) in flowers. The essential oils showed high activity against larvae, and low for pupae with $LC_{99.9}$ of 5.87 and 1476.67 µg/mL from fruits; 0.0096 and 348.33 µg/mL from leaves and 0.021 and 342.84 µg/mL from flowers, respectively. The main compound with insecticide activity is probably n-ethyl-1,3-dithioisoindole, from isoindole organothiophosphate class, found in greater amount in flower and leaf essential oil. The great insecticide activity of *G. integrifolia* essential oil suggests that this product is a natural insecticide.

Keywords: Anticholinesterase; Dimethyl trisulfide; Insecticide; Lenthionine; 1,3,5-trithiane; Methyl p-tolyl sulfide.

Resumen: Este estudio evaluó la actividad insecticida del aceite esencial de frutos, hojas y flores de *Gallesia integrifolia* contra larvas y pupas de *Aedes aegypti*. El aceite esencial se obtuvo por hidrodestilación y se caracterizó por cromatografía de gases acoplada a espectrometría de masas. Los compuestos de azufre representaron del 95 al 99% del aceite esencial de frutas, hojas y flores. Los compuestos principales del aceite esencial fueron 2,8-ditianonano (52,6%) en frutas, 3,5-ditiahexanol-5,5-dióxido (38,9%) en hojas y éster etílico de metionina (45,3%) en flores. Los aceites esenciales mostraron alta actividad contra larvas y baja para pupas con $LC_{99.9}$ de 5.87 y 1476.67 µg/mL de frutos; 0,0096 y 348,33 µg/mL de hojas y 0,021 y 342,84 µg/mL de flores, respectivamente. El principal compuesto con actividad insecticida es probablemente el n-etil-1,3-ditioisoindol, de la clase de los organotiofosfatos de isoindol, que se encuentra en mayor cantidad en el aceite esencial de flores y hojas. La gran actividad insecticida del aceite esencial de *G. integrifolia* sugiere que este producto es un insecticida natural

Palabras clave: Anticolinesterasa; Trisulfuro de dimetilo; Insecticida; Lentionina; 1,3,5-tritiano; p-tolil sulfuro de metilo.

INTRODUCTION

Mosquitoes are the main transmitter of diseases such as dengue and malaria and have been affecting hundreds of thousands people worldwide (Suesdek, 2019). The incidence of diseases caused by *Aedes aegypti* (Linnaeus) has notably increased in the last years, and besides dengue, other arboviruses are also transmitted by this vector such as dengue hemorrhagic fever, chikungunya fever, Zika virus and yellow fever (Pavela, 2015). In 2019 (until 31st of august), 1,439,471 dengue-related cases were reported in Brazil with an incidence of 690.4 cases per 100 thousand inhabitants with 591 deaths. For chikungunya fever, 110,627 cases were recorded with an incidence of 53.1 cases per 100 thousand inhabitants and with 57 deaths. Zika virus was responsible for 9,813 cases with an incidence of 4.7 cases per 100 thousand inhabitants with two deaths (Brasil, 2019). Moreover, in the Americas, outbreaks of Zika, a virus transmitted by *A. aegypti* and *Aedes albopictus* (Skuse), for instance, caused microcephaly to thousands of newborns with devastating long-term side-effects (de França *et al*., 2018).

Several strategies have been used to prevent and control dengue outbreaks such as the eradication of the mosquito breeding sites and the population's motivation and involvement through education and mobilization campaigns. Organophosphates are one of the most used chemical insecticides to control mosquitoes in Brazil and worldwide, even though they pollute the environment and increase insect resistance (Tiwary *et al*., 2007). Their action mechanism consist of binding to the esterase center of acetyl cholinesterase (AChE), making it impossible the hydrolysis of acetylchline neurotransmitter (ACh) into choline and acetic acid, increasing the level of acetylcholine in synapses (Cavaliere *et al*., 1996). Moreover, organophosphates have non-prolonged effects and, therefore, numerous applications are necessary. This increases the vector's resistance to the chemical insecticide; in addition, the broad spectrum of the pesticide alters natural enemy communities and may facilitate secondary pest outbreaks. An alternative utilized to substitute organophosphates is the use of pyrethroids that is not cumulative; however, they are extremely toxic for fish, bees, and aquatic arthropods (Braga & Valle, 2007). An alternative to those synthetic products could be the use of natural products with insecticide action such as essential oil from plants. Some plant extracts have insect repellent properties with very low impact on the environment and less risks for human and animal health (Kim & Ahn, 2017).

Gallesia integrifolia (Spreng.) Harms belongs to the Phytolaccaceae family and is a native plant from South America found in Peru and Brazil, from Ceará to Paraná states (Sambuichi *et al*., 2009), with has several synonymous names such as *Gallesia gorazema* (Vell. Conc.) Moquin, *Gallesia integrifolia* var. ovata (O. C. Schmidt) Nowicke, *Gallesia ovata* O. C. Schmidt, *Gallesia scorododendrum* Casar., and *Thouinia integrifolia* Spreng (Hassler, 2018).It is a large tree with strong alliaceous and characteristic odor that is associated with sulfur compounds and found in essential oil of this plant bark (Barbosa *et al*., 1999).

Most of *G. integrifolia* biological activity is related to chemical extracts such as root dichloromethanic extract and leaf ethanolic extract with antinociceptive, anti-inflammatory, and antiviral activities (Silva Júnior *et al*., 2013); bark dichloromethanic and methanolic extracts against dermatophytes *Microsporum gypseum* (E. Bodin) Guiart & Grigoraki and *Trichophyton mentagrophytes* (C. P. Robin) Sabour. (Freixa *et al*., 1998); leaf aqueous extract against fungus *Botrytis cinerea* Pers. (Silva *et al*., 2017); leaf hydroalcoholic extract against cattle tick *Rhipichephalus microplus* Canestrini (Dias et al., 2018); leaf hydroalcoholic extract against bacteria *Pseudomonas aeruginosa* (Schroeter) Migula, *Shigella flexneri* Castellani and Chalmers, *Streptococcus pyogenes* Rosenbach, and *Staphylococcus aureus* Rosenbach (Arunachalam *et al*., 2016) but with moderate activity when leaf aqueous extract was used against ectoparasite insect *Monalonion dissimulatum* Dist. that attacks cacao (Theobroma cacao L.) (Callisaya *et al*., 2017).

Moreover, there are few studies about *G. integrifolia* essential oil with biological activity such as fruit essential oil against genera *Aspergillus* and *Penicillium* (Raimundo *et al*., 2018); fruit, leaf, and flower essential oil against cattle tick (*R. microplus*) (Raimundo *et al*., 2017) and minor allelopathic effect of the essential oil against *Bidens pilosa* L. seeds (Moura *et al*., 2013). In addition, it has been claimed that fumes of burned leaves of this plant might have repellency action against *Anopheles gambiae* Giles, a malaria-carrying insect, probably because of its essential oil (Pérez, 2002). However, few studies have been found on *G. integrifolia* essential oil to control insects. Thus, this study aimed to obtain essential oil from *G. integrifolia* fruits, leaves, and flowers, determine its chemical composition, and evaluate its insecticidal activity against third-stage larvae and pupae of *A. aegypti*.

MATERIAL AND METHODS

Plant material

Fruits, leaves, and flowers of G. integrifolia were harvested in Umuarama at latitude 23º46'16''S and longitude 53º19'38''W, and altitude of 442 m. Fruits were collected in the morning on May 2015, and leaves and flowers in the morning on December 2015. The species was identified and a sample was deposited in the Herbarium of Western Paraná State University, Campus of Cascavel, Center of Biological Sciences and Health under the number 1716. This species is registered in the National System for the Management of Genetic Heritage and Associated Traditional Knowledge (SisGen) under the registration number AB67595.

Essential oil extraction

The essential oil of *G. integrifolia* fruits, leaves, and flowers (fresh) was obtained by hydrodistillation with a modified Clevenger for 3 h (Raimundo *et al*., 2017; Raimundo *et al*., 2018). The essential oil was withdrawn with n-hexane, dried with anhydrous sodium sulfate (Na_2SO_4), and stored in an amber flask at 4ºC (Schindler & Heinzmann, 2017). The essential oil yield was calculated by mass of the essential oil divided by the mass of the plant expressed in percentage, using 250 g of fresh plant material. The extractions were performed in quintuplicate

Essential oil chemical characterization

The essential oil from fruits, leaves, and flowers of *G. integrifolia* (100 mg) was analyzed by gas chromatography-mass spectrometry (GC-MS; Agilent 19091S-433) coupled to a mass spectrometer (Agilent 19091J-433). An analytical column of 5% HP-5MS (30 m x 0.25 mm x 0.25 µm) was used with initial temperature of 60°C and kept for 3 min; next, with a ramp of 5°C/min, the temperature was increased to 300°C, and was kept for 10 min and, finally, reached 310°C with a ramp of 10°C/min for 10 min. The utilized carrier gas was helium with linear speed of 1 mL/min until 300°C and pressure rate of 56 kPa. The injector temperature was 300°C; the injection value was 2 µL and it occurred in split mode (20:1). The transfer line was kept at 285°C and the ionization and quadrupole sources were at 230°C and 150°C, respectively. The detection system was mass spectrometry in "scan" mode in the range of mass:charge (m/z) of 40-550, with "solvent delay" of 3 min. The essential oil samples were diluted in a 1:10 proportion with dichloromethane. The essential oil chemical compounds were identified by comparing their mass spectra with mass spectra from Wiley 275 libraries and also based on the comparison of their retention index (RI) obtained from a homologous n-alkane standard series (C8-C30) (Adams, 2017).

Essential oil insecticidal activity

A. aegypti third-stage larvae and pupae were obtained from the Department of Sanitary Surveillance of Umuarama, Paraná, Brazil. The essential oil from fruits, leaves, and flowers were diluted in aqueous 2% polysorbate-80 solution at concentrations from 1.500 to 0.00074 µg/mL. Ten *A. aegypti* larvae were collected with a Pasteur pipette and placed in 25-mL flasks with 1.0 mL essential oil at different concentrations for 24 h (Fernandes *et al*., 2005); those without movement and without response to stimuli were considered dead. The same procedure was used for *A. aegypti* pupae. The negative control was an aqueous 2% polysorbate-80 solution (volume/volume) and the positive control was the organothiophosphate temephos at the concentration of 400 µg/mL (Camargo *et al*., 1998). Lethal concentration (LC_{50} and $LC_{99.9}$) of fruit, leaf, and flower essential oils were calculated by probit analysis.

Essential oil anticholinesterase activity

The anticholinesterase activity was determined by bioautographic method described by Yang *et al*. (2009). The methanolic solutions of the essential oil from fruits, leaves, flowers, and positive control were tested at concentrations ranging from 61500 to 0.00023 µg/mL. Samples of each solution were applied onto the thin-layer aluminum chromatography plates (10 x 10 cm, 0.2 mm-thick 60 F254 silica gel) and after drying, they were sprayed with an acetylcholinesterase enzyme solution (500 U) in a buffer solution of tris (hidroximetil) amino metano hidrocloridrato (0.05M, pH 7.8); next, they were sprayed with an α-naftyl acetate solution (0.15%). The plates were kept at 37°C for 20 min. After this period, the chromo plates were sprayed with fast blue B salt colorimetric reagent (0.05%), resulting in a purple color surface. The anticholinesterase activity of *G. integrifolia* essential oil was determined by the emergence of white stains after 10 min, showing the inhibitory action of the evaluated concentrations on the enzyme activity, contrasting with the purple color of the colorimetric reagent.

Statistical analysis

The experimental design was completely random. The data were submitted to analysis of variance (ANOVA) and the differences between the arithmetical averages and the standard deviation were determined by Tukey test at 5% significance. The lethal concentrations that killed 50% (LC_{50}) and 99.9% ($LC_{99.9}$) of larvae or pupae and the respective CI (α = 0.05) were calculated by probitos analysis (ED_{50} Plus version 1.0). All the tests were carried out in triplicate.

Major component analysis

A multivariate analysis was also done to determine the principal component analysis (PCA) which allowed the evaluation of the major chemical compounds and chemical class of all compounds found in the essential oil from leaves, fruits, and flowers. The analysis result was graphically presented (biplot), helping the characterization of the analyzed variable groups (Moita Neto & Moita, 1998).

For each sample of the essential oil from leaves, fruits, and flowers, the identified major chemical compounds and their respective chemical classes (Table No. 1) were plotted. Data were transformed into orthogonal latent variables called principal components, which are linear combinations of original variables created with the eigenvalues of the data covariance matrix (Hair *et al*., 2009). Kaiser's criterion was utilized to choose the principal components and an eigenvalue preserved the relevant information when it was greater than the unit. This analysis was carried out in two ways: the former contained only data referring to the chemical composition of major compounds obtained in three periods, and the latter analyzed the grouped chemical classes to which those compounds belong to (Camacho *et al*., 2010). Both analyses were performed using Statistica 13.3 software (Statsoft, 2017).

RESULTS

Essential oil yield and chemical composition

The yield of *G. integrifolia* essential oils extracted from fruits, flowers and leaves are shown in Table No. 1.

Table No. 1
Determination of yield (%) of *Gallesia integrifolia* fruits, flowers and leaves essential oil

Gallesia integrifolia EO	Yield (%)	CI (95%)
Fruits	$0.1444^{b} \pm 0.0027$	(0.1402 – 0.1485)
Leaves	$0.0558^{c} \pm 0.0033$	(0.0516 – 0.0599)
Flowers	$0.1952^{a} \pm 0.0028$	(0.1910 – 0.1993)

The values represent the mean ± standard deviation, and confidence intervals (CI) of the essential oil (EO) obtained by Tukey's test ($p \leq 0.05$)

Fifty-six essential oil compounds were obtained by GC-MS, 31 were identified from fruits, 47 from leaves, and 42 from flowers of *G. integrifolia* essential oil; sulfur compounds were the majority class with 99.2, 95.3, and 95.9%, respectively (Table No. 2). PCA evidenced the major essential oil compounds such as 2,8-dithianonane (52.6%) in fruits, 3,5-dithiahexanol-5,5-dioxide (38.9%) in leaves, and methionine, ethyl ester (45.3%) in flowers (Table No. 2 and Figure No. 1). However, other major compounds were found such as dimethyl trisulfide (15.3%) and lenthionine (14.7%) in fruits, 1,3,5-trithiane (13.7%) and N-ethyl-1,3-dithioisoindole (12.6%) in leaves, and methyl p-tolyl sulfide (17.1%) and N-ethyl-1,3-dithioisoindole (13.4%) in flowers.

Table No. 2
Chemical composition of essential oil (EO) from fruits, leaves and flowers of *Gallesia integrifolia*

Peak	[a]Compounds	[c]RI_{calc}	Relative area (%)			Methods of identification
			Fruit EO	Leaf EO	Flower EO	
1	Dimethyl disulfide	808	0.89	0.27	3.34	a, b, c
2	3-ethylthiophene	810	-	0.13	0.13	a, b, c
3	2,4-dithiapentane	847	0.04	3.96	0.03	
4	Methyl (methylsulfinyl) methyl sulfide (FAMSO)	885	0.11	1.99	2.22	a, b, c
5	*α*-pinene	937	-	t	t	a, b, c
6	*α*-thujene	937	-	0.05	t	a, b, c
7	*β*-pinene	949	-	0.17	t	a, b, c
8	1,2,4-trithiolane	973	0.84	1.08	1.75	a, b, c
9	*α*-terpinene	1022	t	t	0.09	a, b, c
10	limonene	1037	0.15	0.05	t	a, b, c
11	*α*-fenchone	1057	-	t	0.01	a, b, c
12	Linalool	1094	-	-	0.06	a, b, c
13	*δ*-2-carene	1099	0.06	t	-	a, b, c
14	*trans*-*β*-ocimene	1100	-	t	t	a, b, c
15	*δ*-3-carene	1100	-	t	t	a, b, c
16	Camphene	1101	t	t	0.02	a, b, c
17	Dimethyl trisulfide	1110	15.28	1.07	0.07	a, b, c
18	Dimethyl thiosulphonate	1121	-	0.13	-	a, b, c
19	1,3,5-trithiane	1137	0.28	13.74	0.14	a, b, c
20	Methyl *p*-tolyl sulfide	1138	-	-	17.08	a, b, c
21	2,3,5-trithiahexane	1140	0.10	1.62	0.22	a, b, c
22	7-methyl-4-thiaoctane	1167	-	0.08	-	a, b, c
23	4-methylthio-2-oxo-butanoic acid	1171	-	0.17	0.06	a, b, c
24	Allyl phenyl sulfide	1174	-	0.19	-	a, b, c
25	2,7-dithiaoctane	1192	0.15	-	-	a, b, c
26	Methyl-phenethyl sulfide	1218	-	0.10	0.11	a, b, c
27	Trithiometoxymethane	1219	0.35	0.13	0.08	a, b, c
28	1,2,4,5-tetrathiane	1262	5.66	0.36	0.18	a, b, c
29	n.i.	1344	-	0.18	-	a, b, c
30	Dimethyl tetrasulfide	1367	-	5.32	0.48	a, b, c
31	*Trans*-*α*-ionone	1430	0.09	t	-	a, b, c
32	Butanoic acid, 3-(acetylthio)	1457	0.13	-	0.04	a, b, c
33	2,8-dithianonane	1478	52.63	0.12	0.04	a, b, c
34	*Trans*-*β*-ionone	1479	t	0.13	0.15	a, b, c
35	3,6-dithiaoctan-1-ol	1506	0.66	0.14	-	a, b, c
36	n.i.	1559	0.61	-	-	a, b, c
37	3,6-dioxa-8-mercaptooctane-1-ol	1618	0.08	1.79	-	a, b, c
38	3,5-dithiahexanol-5,5-dioxide	1634	0.10	38.93	5.94	a, b, c
39	2,3,5,6-tetrathiapentane	1671	0.14	0.12	0.54	a, b, c
40	Methionine, ethyl ester	1716	0.10	5.32	45.28	a, b, c
41	3,6-dithiaoctan-1,8-diol	1718	0.14	0.32	0.60	a, b, c
42	Lenthionine	1761	14.69	0.63	0.15	a, b, c
43	Ethanol, 2-(octylthio)	1780	0.11	0.63	0.33	a, b, c
44	2-undecanone-6,10-dimethyl	1792	-	0.09	0.05	a, b, c
45	Pentyl, 3-(methylthio) propanoate	1780	0.09	0.10	0.33	
46	Hexathiepane	1879	5.53	0.29	-	a, b, c
47	N-ethyl-1,3-dithioisoindole	1847	0.12	12.58	13.40	a, b, c
48	5-methyl-2-phenylindole	1914	0.56	0.08	0.03	a, b, c

49	Phytol	1916	-	3.90	3.14	a, b, c
50	Eicosane	2024	-	-	0.20	a, b, c
51	1,3-dimethyl-4-azaphenanthrene	2174	-	-	0.20	a, b, c
52	1,3,6,9-dodecatetraene-12-methylthio (Z,Z,Z)	2174	-	0.24	-	a, b, c
53	Propyl-undecenoate	2194	-	2.60	0.11	a, b, c
54	3-[[(methylthio)methy]sulfonyl]-1-phenyl-1-propanone	2321	0.18	0.81	-	a, b, c
55	S-phenyl-p-toluene-thiosulfonate	2325	0.23	0.18	3.00	a, b, c
56	n.i.	2328	-	0.21	-	a, b, c
	Total identified		**99.49**	**99.61**	**99.58**	
	Oxygenated diterpenes		-	3.90	3.14	
	Ionone compounds		0.09	0.13	0.15	
	Linear hydrocarbon		-	-	0.20	
	Monoterpene hydrocarbons		0.21	0.27	0.12	
	Oxygenated monoterpenes		-	-	0.06	
	Sulfur compounds		99.19	95.31	95.91	

[a]Compounds listed in order of elution in column HP-5MS; [c]$RI_{calc.}$= Identification based on retention index (RI) using a homologous series of *n*-alkane C_8-C_{30} on Agilent HP-5MS column. [d]MS = identification based on comparison of mass spectra using Wiley 275 libraries. Relative area (%): percentage of the area occupied by the compounds in the chromatogram. n.i. = not identified. t = traces. (-) = without compound.

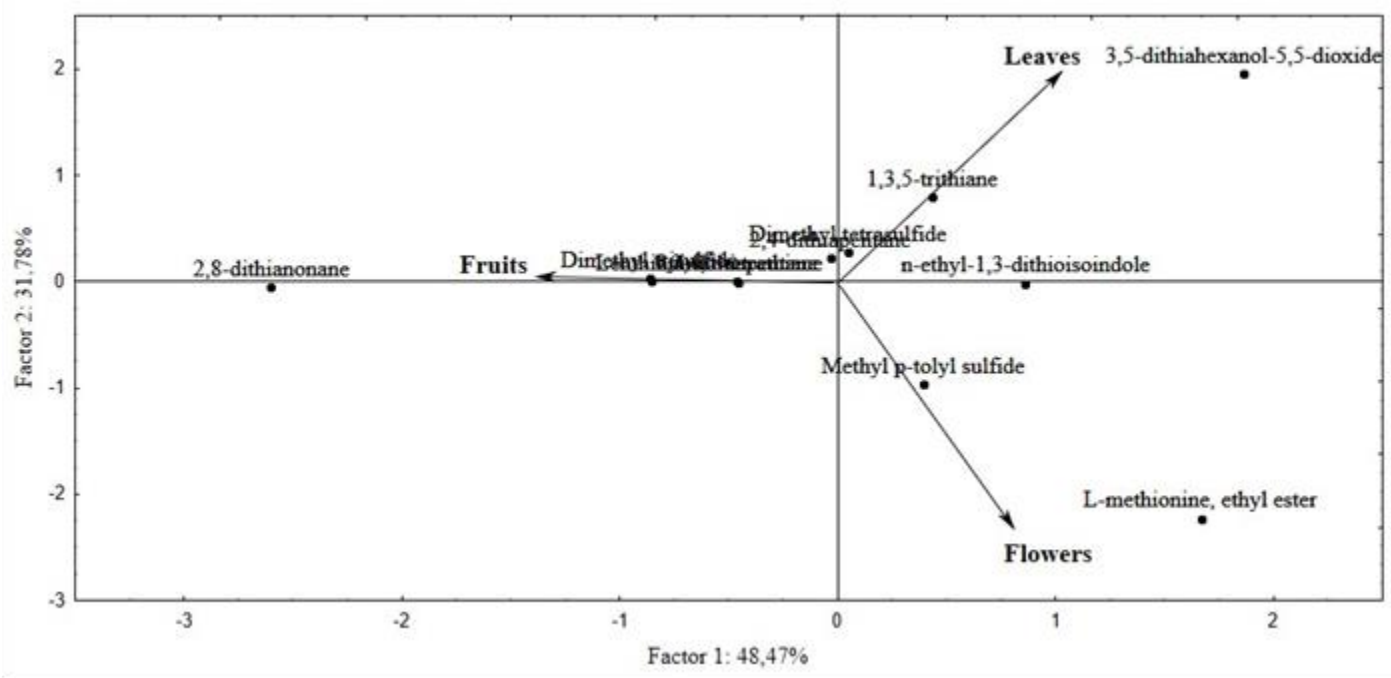

Figure No. 1
Biplot projection of the major chemical compounds of *Gallesia integrifolia* essential oil from leaves, fruits, and flowers

Essential oil insecticidal activity against A. aegypti

G. integrifolia leaf essential oil against *A. aegypti* larvae had $LC_{99.9}$ 2.2 fold more efficient than flower essential oil and 611 fold more efficient than fruit essential oil (Table No. 3). $LC_{99.9}$ of flower essential oil was 280 fold more efficient than fruit essential oil (Table No. 3). However, essential from leaves and flowers were equal ($p \leq 0.05$), both were more efficient ($p \leq 0.05$) than fruit essential oil. LC_{50} was proportionally similar to $LC_{99.9}$ data of *G. integrifolia* essential oil from leaves, flowers, and fruits (Table No. 3).

G. integrifolia leaf and flower essential oil against *A. aegypti* pupae had equal ($p \leq 0.05$) $LC_{99.9}$ values and were four fold more efficient than fruit essential oil (Table No. 3). LC_{50} was proportionally similar to $LC_{99.9}$ data of *G. integrifolia* essential oil from leaves, flowers, and fruits (Table No. 3).

Despite the great differences of values, the lethal concentration to kill *A. aegypti* larvae was very low for fruit essential oil, mainly from leaf and flower essential oil, indicating high efficiency of the insecticidal activity against larvae. However, the essential oil lethal concentration was much higher to kill *A. aegypti* pupae than *A. aegypti* larvae.

The lowest essential oil concentrations that inhibited acetylcholinesterase enzyme were 0.00023 µg/mL from leaves and 0.0073 µg/mL from flowers, followed by 1.87 µg/mL for fruits (Table No. 4). These values were lower or equal to that obtained for organothiophosphate control temephos with inhibition concentration of 0.0073 µg/mL (Table No. 4). Therefore, the essential oil from leaves and flowers showed greater activity to inhibit acetylcholinesterase enzyme. In addition, these results suggest that the insecticidal action of the essential oil may be related to the inhibition of acetylcholinesterase enzyme.

Table No. 3
Lethal concentration of *Gallesia integrifolia* essential oil (EO) from fruits, leaves, and flowers against *Aedes aegypti*

G. integrifolia essential oil	Larvae		Pupae	
	LC_{50} (mg/mL) (CI)	**$LC_{99.9}$** (mg/mL) (CI)	**LC_{50}** (mg/mL) (CI)	**$LC_{99.9}$** (mg/mL) (CI)
Leaves	$0.0041^{(a)} \pm < 0.01$ (0.0031 – 0.0042)	$0.0096^{(A)} \pm 0.01$ (0.0093 – 0.0097)	$90.16^{(a)} \pm 0.01$ (70.12 – 111.83)	$348.33^{(A,B)} \pm 0.02$ (323.00 – 395.00)
Flowers	$0.0083^{(a)} \pm < 0.01$ (0.008 – 0.009)	$0.0209^{(A)} \pm < 0.01$ (0.019 – 0.021)	$131.36^{(a,b)} \pm 0.01$ (118.73 – 138.60)	$342.84^{(A)} \pm < 0.01$ (339.25 – 349.25)
Fruits	$2.90^{(b)} \pm 0.85$ (2.05 – 3.75)	$5.87^{(B)} \pm 0.94$ (4.93 – 6.81)	$820.00^{(c)} \pm 0.05$ (730.00 – 890.00)	$1476.67^{(C)} \pm 0.03$ (1430.00 – 1530.00)
Positive Control	$0.398^{(a)} \pm 0.05$ (0.309 – 0.486)	$1.14^{(A)} \pm 0.06$ (1.051–1.228)	$234.37^{(b)} \pm 30.1$ (204.20–264.60)	$443.64^{(B)} \pm 14.87$ (413.46 – 473.82)

EO: essential oil; LC_{50}: 50% lethal concentration; $LC_{99.9}$: 99.9% lethal concentration; (CI): confidence interval; Positive control (organothiophosphate temephos). Means ± standard deviation followed by lower case letters or capital letters in the same row does not differ from each other by the Tukey test ($p \leq 0.05$). Negative control (aqueous 2% polysorbate-80 solution) = zero adult female and larval mortality

Table No. 4
Inhibiting activity of acetylcholinesterase enzyme of *Gallesia integrifolia* essential oil (EO) from leaves, flowers, and fruits by bioautographic method

Inhibition of acetylcholinesterase enzyme									
Concentration (µg/mL)	**Leaf EO**	**Flower EO**	**Fruit EO**	**PC**	**Concentration (µg/mL)**	**Leaf EO**	**Flower EO**	**Fruit EO**	**PC**
61500	+++	+++	+++	+++	1.87	+	+	+	+
30750	+++	+++	+++	++	0.94	+	+	-	+
15370	+++	++	+++	+	0.47	+	+	-	+
7680	++	++	++	+	0.23	+	+	-	+
3840	+	+	++	+	0.12	+	+	-	+
1920	+	+	++	+	0.058	+	+	-	+
960	+	+	+	+	0.029	+	+	-	+
480	+	+	+	+	0.015	+	+	-	+
240	+	+	+	+	0.0073	+	+	-	+
120	+	+	+	+	0.0037	+	-	-	-
60	+	+	+	+	0.0018	+	-	-	-
30	+	+	+	+	0.0009	+	-	-	-
15	+	+	+	+	0.00045	+	-	-	-
7.5	+	+	+	+	0.00023	+	-	-	-
3.75	+	+	+	+	-	-	-	-	-

PC: positive control (organothiophosphate temephos). (+++): strong inhibition of acetylcholinesterase enzyme; (++): moderate inhibition; (+): weak inhibition; (-): absence of inhibition. EO: essential oil

DISCUSSION

A greater yield of the essential oil was found in flowers (0.1952%), followed by (0.1444 %) and leaves (0.0558 %), and up to the present date, there are no reports in the literature on the EO from *G. integrifolia* fruits, flowers and leaves. Arunachalam *et al*., (2017) found a yield of 2% (w/w) of the essential oil from fresh bark material of *G. integrifolia*. Neves *et al*., (2014) measured the yield of the essential oils from *Petiveria alliacea* L. (Phytolaccaceae), leaves, stems, flowers and roots and found 0.09%, 0.01%, 0.12% and 0.07%, respectively. According to the European Pharmacopoeia, the minimum extraction essential oil yield is 2 mL/kg for the development of applications (Nemeth & Bernath, 2008). The yields found in our study indicate that the EO from flowers point our 1.95 mL/Kg of fresh flowers, considering that the flowers are in the limit to be utilized in the development of products recommended by the European Pharmacopoeia. Another part of *G. integrifolia* that is deserving of pointing out is the fresh bark, which presented a yield of 2 mL/Kg of fresh bark. However, in our research team is focused on the biological investigation of leaves, flowers and fruits of this still little studied species.

The presence of sulfur in the molecules of compounds found in essential oil from fruits, leaves, and flowers of *G. integrifolia* (Table No. 2) could be responsible for the strong insecticidal activity against *A. aegypti* larvae. Sulfur is one of the chemical compounds most commonly used as an insecticide and fungicide. Some organic compounds containing sulfur structures as flubendiamide that act through the ryanodine receptor and sulfoxaflor which acts on insect nicotinic acetylcholine receptors (Li *et al*., 2019), are promising molecules for reducing the insect resistance to sulfur pesticides.

Regarding the amount of sulfur atoms in the molecules, studies reported that the presence of trisulfides provides greater insecticide potential than disulfides and monosulfides (Mann *et al*., 2010). Huang *et al*. (2000), evaluated methyl allyl disulfide and diallyl trisulfide against *Sitophilus zeamais* Motschulsky (an insect that attacks corn), two isolated sulfur compounds from essential oil of *Allium sativum* L. For these authors, diallyl trisulfide showed greater insecticidal activity against larvae and eggs than the compound with only two atoms of sulfur in its molecule. This explains the high larvicidal activity of the essential oil from flowers and leaves, followed by fruits (Table No. 3), which presented dimethyl trisulfide (Figure No. 2a), 1,3,5-trithiane (Figure No. 2b) (three sulfur atoms in their molecular structure), 2,8-dithianonane (Figure No. 2c), 3,5-dithiahexanol-5,5-dioxide (Figure No. 2d),

N-ethyl-1,3-dithioisoindole (Figure No. 2e) (with two sulfur atoms in their molecular structure), and methionine, ethyl ester (Figure No. 2f) as major compounds. Also, Liu *et al*. (2014), evaluated the larvicidal activity of dimethyl trisulfide and methyl propyl disulfide against *Aedes albopictus* Skuse (Diptera: Culicidae) and reported that dimethyl trisulfide has strong larvicidal activity with LC_{95} of 49.43 µg/mL.

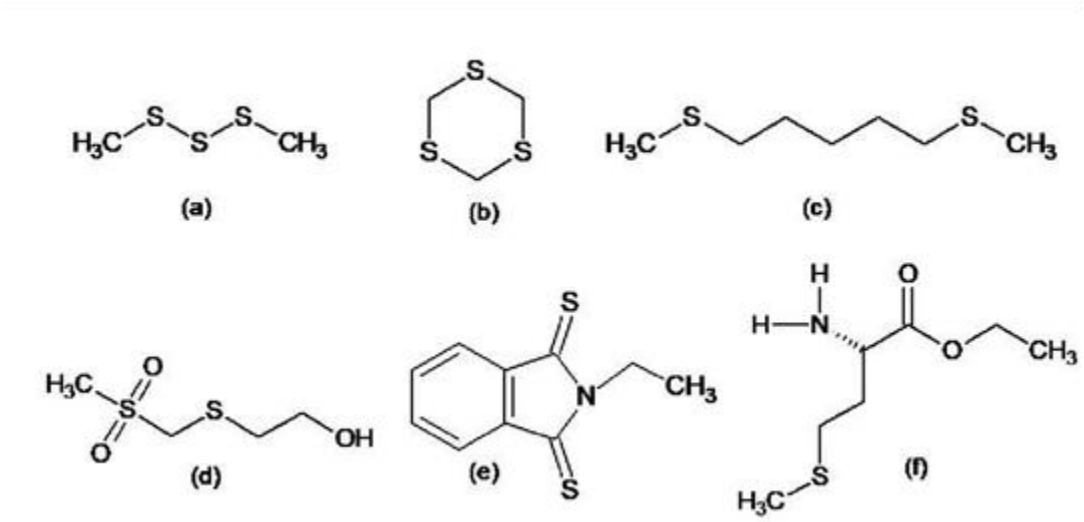

Figure No. 2
Chemical structures of major compounds in *Gallesia integrifolia* essential oil from fruits, leaves, and flowers: (a) dimethyl trisulfide, (b) 1,3,5-trithiane, (c) 2,8-dithianonane, (d) 3,5-dithiahexanol-5,5-dioxide, (e) N-ethyl-1,3-dithioisoindole, and (f) methionine, ethyl ester

Even presenting high concentrations of sulfur compounds, we can observe that there was a difference between $LC_{99.9}$ found in the essential oil from different parts of *G. integrifolia*, indicating greater activity of essential oil from leaves (0.0096 µg/mL) and flowers (0.0209 µg/mL) against *A. aegypti* larvae. Analyzing the chemical composition (Table No. 2 and Figure No. 1), we can verify that the percentage of N-ethyl-1,3-dithioisoindole (Figure No. 3a) is found in greater concentration in the essential oil from leaves (12.58%) and flowers (13.40%), when compared to fruit essential oil (0.12%).

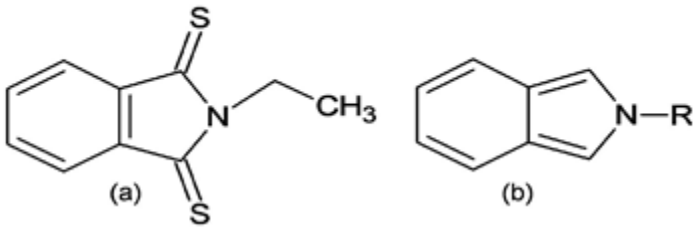

Figure No. 3
Chemical structure of the compound (a) N-ethyl-1,3-dithioisoindole found in *Gallesia integrifolia* essential oil from leaves and flowers, and (b) an isoindole skeleton

The importance of finding high concentrations of N-ethyl-1,3-dithioisoindole in the leaves and flowers of *G. integrifolia* essential oil is that this compound presents isoindole skeleton (Figure No. 3b) in its structure. The isoindole structure can be found in several natural and pharmaceutical compounds with varied pharmachological activities such as antimicrobial, antifungal, antitumoral, anti-inflamatory (Speck & Magauer, 2013). The isoindoles also present a great insecticidal and larvicidal potential, constituting the class of isoindole organothiophosphate insecticides (MacBean, 2012), which has two available products in the market. The former contains O,O-dimethyl S-phthalimidomethyl phosphorodithioate (Figure No. 4a), a non-systemic insecticide applied on plants (cotton, fruits, and potatoes) and animals (cattle, pig, sheep, and dog) (Sinderhauf & Schwack, 2003), and the latter contains phosphorodithioic acid, S-(2-chloro-1-phthalimidoethyl) O,O-diethyl ester (Figure No. 4b), a non-systemic fungicide and insecticide for treatment of soil and for controlling insects and mites in fruits and vegetables (Morais *et al*., 2005).

Isoindole organothiophosphate has neurotoxicity by binding to and phosphorylating the acetylcholinesterase enzyme in the central and peripheral nervous systems (Mascini *et al*., 2005). The essential oil, mostly from leaves and flowers, can inhibit the acetylcholinesterase enzyme at a lower concentration than control temephos (Table No. 4). The essential oil from leaves and fruit has higher N-ethyl-1,3-dithioisoindole concentration than from fruits. These results indicate that the probable insecticidal action mechanism of the essential oil is related to the acetylcholinesterase enzyme inhibition and could explain the greater insecticidal activity of *G. integrifolia* essential oil from leaves.

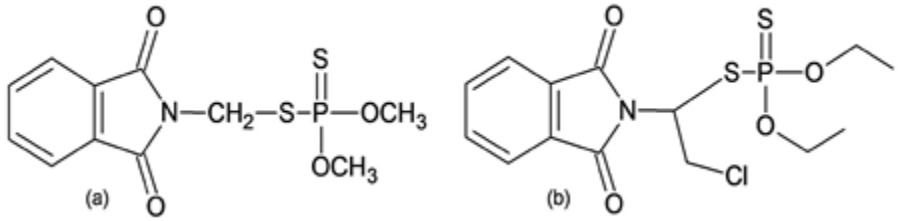

Figure No. 4
Chemical structure of isoindole organothiophosphate insecticides: (a) O,O-dimethyl S-phthalimidomethyl phosphorodithioate and (b) phosphorodithioic acid, S-(2-chloro-1-phthalimidoethyl) O,O-diethyl ester

Another important observed point in *G. integrifolia* essential oil from leaves, flowers, and fruits is related to the structural characteristics of sulfur compounds in the larvicidal activity. In essential oil from flowers, the major compound is methionine, ethyl ester (45.3%). Ethyl ester may be somehow inserting themselves in the plasmatic membrane and contributed sinergically to potentialize the larvicidal effect of the essential oil (Silva *et al*., 2016).

Also, it could lead to higher membrane fluidity and subsequent increase in the Na^+/K^+-ATPase activity, causing cellular extravasation and death of larvae. In essential oil from leaves, the presence of 3,5-dithiahexanol-5,5-dioxide (38.9%) stands outs because it contains a lateral hydroxyl chain and, according to Martins *et al*. (2013), a greater number of OH groups in a carbon chain implies in an increment of hydrogen bindings between the solute and water; therefore, the solubility of these molecules is increased. It suggests that the presence of hydroxyl in 3,5-dithiahexanol-5,5-dioxide may have contributed to the high insecticidal activity found in leaf essential oil. It was shown in our assay that the essential oil presented better results against *A. aegypti* larvae than pupae and it is probable related that larvae have a digestive system and feed themselves with possible toxic compounds found in the water, whilst pupae do not feed themselves. It could explain the smaller effect of the essential oil against pupae when compared to the larval stage. In addition, the presence of essential oil substances in the water cause difficulties in the breathing mechanisms of larvae and may result in their death (Consoli & Oliveira, 1994).

CONCLUSION

Gallesia integrifolia essential oil from fruits, leaves, and flowers have mainly sulfur compounds ranging from 95 to 99%. The main major compound of the essential oil from fruit is 2,8-dithianonane (52.6%), from leaf is 3,5-dithiahexanol-5,5-dioxide (38.9%), and from flower is methionine, ethyl ester (45.3%). Other major compounds are dimethyl trisulfide (15.3%) and lenthionine (14.7%) in fruit, 1,3,5-trithiane (13.7%) and N-ethyl-1,3-dithioisoindole (12.6%) in leaf, and methyl p-tolyl sulfide (17.1%) and N-ethyl-1,3-dithioisoindole (13.4%) in flower. The essential oil concentration from fruit (5.87 µg/mL), leaf (0.0096 µg/mL), and flower (0.0209 µg/mL) kill 99.9% of the population of *A. aegypti* larvae. The greatest insecticide action is with the essential oil from *G. integrifolia* leaves and flowers and they are the lowest essential oil concentration reported in the literature. Similarly, the $LC_{99.9}$ values against *A. aegypti* pupae are much lower than the ones reported in the literature. The essential oils have acetylcholinesterase inhibition, mainly the leaf essential oil that is effective even though it is 32 times less concentrated than the positive control organothiophosphate. N-ethyl-1,3-dithioisoindole, from isoindole organothiophosphate class, found in greater amount in the essential oil from leaves and flowers, may probably be the main compound with insecticide activity. The reduced concentration of *G. integrifolia* essential oil with insecticide activity against *A. aegypti* larvae and pupae suggests new and promising perspectives of applications of this product as a natural insecticide.

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