Chemistry and Insecticide activity of *Bougainvillea glabra* Choisy against *Spodoptera frugiperda* Smith

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Abstract

Bougainvillea glabra Choisy var 'Variegata' shows medicinal and insecticidal activities attributed to the chemical content of this botanical variety. The purpose of this work was to identify the metabolites obtained from methanol extracts of leaves of this variety cultivated under greenhouse conditions and determine the biological activity against Spodoptera frugiperda larvae. Secondary metabolites were identified through gas chromatography coupled to mass spectrometry (GC-MS) using ^{13}C and ^{1}H nuclear magnetic resonance. Twenty-three compounds were identified and d-pinitolwas the major compound isolated. The biologic effect of d-pinitol against second instar larvae was tested at concentrations of 0, 25, 50, 75 100, and 125 ppm. The major biological effect observed was at a 75 ppm concentration, showing low weight and interruption of pupae formation after 60 days. Only emerging adults were used in the test.

Keywords: extracts, secondary metabolites, *d*-pinitol, biological effect, bio-insecticide

1.Introduction

Bougainvillea glabra Choisy ('buganvilia') of Nyctaginaceae family (Martínez, 1997) presents diverse varieties such as 'Violet', 'Surprise', 'Golden', 'White' and 'Variegate' which are used as ornamentals and in traditional medicine to treat respiratory diseases, diabetes and stomach acidity (Adebayo et al, 2009; Gupta et al., 2009). For this, Mariajancyrani et al. (2013) isolated a terpenoid soluble in ethyl acetate from the leaves: 3-*O*-acetyloleanolic acid. Sahu and Saxena (2014) isolated one flavonoid from methanolic extract of bracts and identified quercetin-3-O-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside. Also, Hussein (2014) identified five known flavonoids: vitexin, isovitexin, chrysoeriol, apigenin, luteolin and a new flavonoid: luteolin-7-*O*-[2"-*O*-(5'"-*O*-feruloyl)-β-D-apiofuranosyl]-β-D-glucopyranoside from the methanolic extract of the leaves. Martinez (1997) reported that in *B. glaba* leaves there are *β*-sitosterol, glucose, rhamnose, glycine and aspartic acid, along with compounds such as tannins, flavonoids, saponins, steroids, terpenoids and cardiotonic glycosides. And by using GC-MS Rani et al.

(2012) identified: phytol, squalene, 3-*O*-methyl-D-glucose, tetradecanoic acid ethyl ester, 9,12,15-Octadecatrienoic acid, hexanedioic acid bis (2-ethylhexyl) ester, 1,2-benzenedicarboxylic acid diisooctylester and vitamin E. *Bougainvillea glabra* var 'Variegata' is considered valuable because the striking appearance of its leaves which are opaque green color with a white milky outline, short internodes and orange color in three bracts where they embrace the white flowers, which are less striking. The plant could be propagated by a hardwood stake (Evangelista et al., 2005).

In observations carried out on different varieties under nursery conditions, it was observed that not all varieties are attacked by insect pests (Valdés et al, 2004). In addition, there exist reports on the effect of *B. glabra* against the leishmaniosis vector from Egypt; *Phleboromus papatasi* (Diptera: Psychodidae) since the extract killed 16 % of the females and 24 % of the males and reduced their cycle life the rest of individuals (Kaldas et al, 2014). A similar effect was produced in *P. argentipes* adults, which is also a leishmaniosis vector (Sharma and Sing, 2008). In cabbage leaves (*Brassica oleracea* var 'Acephala'), the bougainvillea aqueous extract had dissuasive effects towards 95 % of the eggs of *Plutella xylostella* (Lepidoptera: Plutelliadae) (Medeiros et al, 2005). With the aqueous extract of bougainvillea bracts at 100 % concentration, 80 % of *Shitophilus oryzae* weevils died (Coleoptera: Curculionidae) (Kalirajan et al, 2012). Some species of Nyctaginaceae family contain compounds with insecticidal activity as *d*-pinitol (Poongothai and Sripathi, 2013). Chaubal et al. (2005) reported that *d*-pinitol caused 90 % and 100 % mortality in the fourth instar larvae of *Aedes aegypti* and *Culex quinquefasciatus* at 500 and 50 ppm respectively, while in *Helithis zea* it inhibited larvae develop (Reese et al, 1982). In tropical and subtropical America regions one of the principal pests in grasses, legumes and horticultural crops is *Spodoptera frugiperda* (J. E. Smith) (Lepidoptera: Noctuidae) (García and Tarango, 2009). Because *B. glabra* is a Nyctaginaceae, it is possible that *d*-pinitol exists in some fractions of the methanolic extract. Currently, there are no reports available about the biological activity of this compound against *S. frugiperda* as well as not reports of the botanical variety 'Variegata' containing the compound. Therefore, the objectives of this research were to isolate and identify the secondary metabolites from methanol extracts of leaves of *B. glabra* var 'Variegata' grown in nursey and evaluate its biological effect against the larvae of *S. frugiperda*.

2. Materials and Methods

2.1 Collection of plant material

Ripe leaves of *B. glabra* variety 'Variegata' plants, located in CeProBi – IPN (18.4944278°N, 99.0534296°W; altitude 1064 m.a.s.l.) were collected, rinsed and dried for 24 to 48 h to obtain the dried weight of material.

2.2. Extract recuperation from *B. glabra* leaves

The dried material (270 g dry weight) was extracted by maceration in a 5 l flat bottom flask and immersion in methanol for 3 days. Afterward, the dissolvent was filtered and evaporated in a rotary evaporator. The solvent was placed again in a flask for 3 days and the procedure was repeated two more times. The three organic residues were mixed to provide of 36.3 g of the crude methanolic extract from the *B. glabra* leaves.

2.3. Chromatographic separation of the crude methanolic extracts of *B. glabra* leaves

The methanol extract was fractionated by a gravity column chromatography procedure. 36.3 g of the extract was adsorbed in 34 g of flash silica gel and placed in a column previously packed with 30 cm of clean flash silica gel. The column was eluted with different mobile phases: first hexane: ketone (100:0 to 50:50 v/v), second, ketone (100:0) and finally, methanol (100:0) as gradient of elution. Each fraction was analyzed by High Performance Thin Layer Chromatography (HPTLC) separation performed on a percolated silica gel aluminum plate 60 F254 (0.25 mm thick, Merck, Darmstadt, Germany). The spots in TLC were visualized by UV light (280 and 360 nm UV lamp) after spraying with 1% developer solution of $(NH_4)_4Ce\text{-}(SO_4)_4 \cdot H_2O$ in 2N $H_2SO_{4,\ which}$ was heated prior to use.

2.4. Gas chromatography coupled mass spectrometry (GC-MS)

The main compounds found were also analyzed using GC-MS (Agilent 6890 System Plus, coupled to Agilent 5973 Network Mass selective detector), which was equipped with a silica capillary column (30 m X 0.25 mm, film thickness 0.25mm). The GC oven temperature conditions were: 45 to 250 °C with a gradient temperature of 10 °C/min. A sample volume of 1.0 µL of each fraction at 0.02 g/L of concentration was injected. The

identification of the majority of the chemical compounds was based on the comparison of their index mass fragmentation with authentic compounds of the mass spectra database N-15598 equipment (Falodunet al., 2009).

2.5. ^{1}H and ^{13}C Nuclear Magnetic Resonance (NMR)

The NMR spectra were measured on a Varian Mercury NMR spectrometer (^{1}H NMR, 200 and 400 MHz MHz; ^{13}C NMR, 50 MHz and 100 MHz). Chemical shifts, δ, were expressed in ppm units downfield from TMS and coupling constants *J* in Hertz (Hz). Depending of the solubility of the compounds and groups, deuterated solvents as chloroform, methanol or DMSO were used.

2.6. Evaluation of the fractions of *B. glabra* against *S. frugiperda*

Fall armyworm larvae were obtained from a colony maintained at laboratory conditions of the Department of Entomology, at the Centro de Desarrollo de Productos Bióticos of the Instituto Politécnico Nacional (CEPROBI-IPN) Morelos, Mexico. The larvae were maintained in a Precision model 818 incubation chamber at 27 ± 1 °C, 60-70 % RH and 12:12 h L:D and reared on a meridic diet (Burton & Perkins 1987). Second-generation (F2) larvae were used for all experiments with 4 replicates of n = 100 neonate larvae were used in each treatment.

2.7. Bioassays to assess the toxicity

Individual fractions of *B. glabra* were incorporated into the above meridic diet (Burton y Perkins, 1987) by incorporating the methanol extract and the main compound (*d*-pinitol in fraction K) at six concentrations: 0, 25, 50, 75, 100 and 125 ppm in order to evaluate its effect on the development and survival of the *S. frugiperda* larvae. The control diet was prepared with 1 mL of methanol. Diet ingredients and the fractions were mixed following the protocol of Franco et al. (2006) and the prepared mixture was dispensed at 15 mL per container into cylindrical plastic containers (3 cm high × 3.5 cm diameter). Once the diet had cooled and solidified, one neonate larva was placed in each container with the aid of a fine camel hairbrush. Data were arranged in completely randomized design. Each treatment was performed in 4 replicates with a total of 100 neonate larvae.

Observations were made every seven days considering larval development and mortality, according to Duso et al., (2008), particularly weight (mg) and any visible malformations. The larvae were considered dead when they did not mobilize displace or change position after being pressed down on the abdomen with a dissection needle. The developed adults and larvae were kept in laboratory conditions (27 + 1°C, 60-70 % RH and photoperiod 16:8). The percent mortality was calculated by means of Abbot's formula (Abbot 1925). The resulting data were subjected to ANOVA and mean comparison (i.e., the mean ± standard deviation (MSD) at $P = 0.5$ were also calculated). Prior to ANOVA, the normality and homoscedasticity of the data was verified by the Shapiro-Wilk and Levene tests, respectively (Sigma Plot v.12.5).

3. Results and discussion

3.1. GC-MS of *B. glabra* methanolic crude extract of leaves

The methanolic crude extract of leaves *B. glabra* was green dark color with a 13 % of yield recovered. Twenty-three compounds were identified by GC-MS from groups A, B, C, E and K (Table 1). From group **A**, five compounds were identified: squalene, pentacosane, heptacosane, nonaocosane and hentriacontane. From group **B**: 3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo [4.1.0] hept-1-yl)-, 2(4H)-benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, 1,7-Nonadien-4-ol, 4,8-dimethyl- and stigmasta-5,22-dien-3-ol were identified. And from group **C** there were eight compounds identified: 6, 10, 14-trimehyl-, 2-pentadecanone, palmitic acid methyl ester (palmitic acid),*9,12-Octadecadienoic acid (Z,Z)-, methyl ester (*linoleic acid, methyl ester; *cis-9,cis-12-Octadecadienoic acid (*linoleic acid), (5E, 9E)- 6,10,14-trimethyl-5,9,13-pentadecatrien-2-one, 2,2-Dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-henicosapentaen-1-yl) oxiranand vitamin E. From group E was identified three compounds: linoleic acid, methyl ester, phytol, 3,7,11,15-Tetramethylhexadec-2-en-1-ol. And from group **K**, three other compounds were identified: 3-O-(3H-methyl)-d-glucose, 4-piperidone, 2,2,6,6-tetramethyl- and 2-methoxy-4-vinylphenol.

In-group **K** a compound that was confirmed as *d-pinitol* (3-O-methyl-D-chiro-inositol) was found in greater proportion. This was isolated by TLC and analyzed and identified by ^{1}H and ^{13}C NMR. The chemical shifts by ^{13}C (100 MHz, CD_3OD) δ 84.85 (C-1), 74.24 (C-5), 73.71 (C-3), 73.49 (C-6), 72.53 (C-2), 71.97 (C-4), 60.76 (OMe). And for ^{1}H (400 MHz, CD_3OD) δ 3,9 (d, J = 2.4 Hz, 2H, H-1, H-6), 3.75 (dd, J = 9.6 and 2.4 Hz, 1H, H-2), 3.70 (dd, J = 9.6 and 2.4 Hz, 1H, H-5), 3.61 (s, 3H, OMe), 3.52 (dd, J = 11.2 and 11.2 Hz, 1H, H-4), 3.26 (dd, J = 9.6 and 9.6 Hz, 1H, H-3). The chemical shifts by ^{1}H and ^{13}C NMR was compared with previous reports (Figure 1 and Table 2).

Table 1. Compounds identified by GC-MS from *B. glabra* leaves var 'Variegata'.

TR	Area GC	Compound IPAC name (number)	[m+/Z]	Fragmentation
8.29	3.05	*4-piperidone, 2,2,6,6-tetramethyl*	155	140, 112, 98, 83, 58, 42
10.65	1.74	*1,7-nonadien-4-ol, 4,8-Dimethyl-*	168	109, 86, 69, 43
11.19	1.25	*2-Methoxy-4-vinylphenol*	150	135, 107, 77
13.47	5.19	*3-Buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo [4.1.0] hept-1-yl)-*	208	177, 135, 123, 43
14.12	5.18	*2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-*	180	165, 152, 137, 111, 67, 43
16.17	84.41	*3-O-methyl-D-glucose*	194	144, 116, 103, 87, 73
17.40	7.26	*(2E)-3,7,11,15-Tetramethyl-2-hexadecen-1-ol*	278	137, 123, 109, 95, 82, 68, 57, 43
17.46	1.17	*6,10,14-Trimethylpentadecantrien-2-one*	268	250, 165, 124, 109, 71, 58, 43
18.27	7.92	*Palmitic Acid methyl ester*	270	227, 143, 87, 74, 55, 43
18.73	89.78	*Palmitic Acid*	256	213, 185, 171, 157, 129, 83, 73, 43,
19.93	3.03	*9,12-Octadecadienoic acid (Z,Z)-, methylester*	294	263, 150, 123, 109, 95, 81, 67, 41
20.10	1.99	*Phytol*	296	278, 196, 123, 71, 57, 43
20.36	8.22	cis-*9*, cis-*12-Octadecadienoic acid*	280	264, 149, 123, 109, 95, 81, 67, 55, 41
20.46	75.28	*9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-*	292	149, 135, 121, 108, 95, 79, 67, 55, 41
22.47	58.99	*Stigmasta-5,22-dien-3-ol*	414	397, 369, 300, 271, 255, 107, 81, 69, 55, 43, 18
22.75	0.94	(5E,9E)-*6,10,14-Trimethyl-5,9,13-pentadecatrien-2-one*	247	204, 161, 135, 107, 81, 69, 43
24.13	2.63	*Pentacosane*	340	141, 113, 99, 85, 71, 57, 43
26.97	5.80	*Heptacosane*	380	295, 197, 169, 141, 113, 99, 85, 71, 57, 43
29.12	3.74	*Oxirane, 2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19-heneicosapentaenyl)-*	426	215, 189, 147, 121, 95, 81, 69, 41
29.50	10.15	*Squalene*	410	149, 137, 121, 95, 81, 69
31.20	35.39	*Nonacosane*	408	295, 197, 169, 141, 113, 99, 85, 71, 57, 43
37.86	40.06	*Hentriacontane*	436	253, 225, 295, 197, 169, 141, 113, 99, 85, 71, 57, 43
39.32	60.54	*Vitamin E*	430	414, 388, 358, 316, 288, 274, 219, 165, 121, 91, 43

RT = retention time

Table 2. Data of NMR from *d*-pinitol

Carbon	^{13}C NMR (100 MHz)		^{1}H NMR (400 MHz)	
	Raya et al, 2008 in D_2O	Compound **41** in CD_3OD	Jawla et al, 2013 in D_2O	Compound **41** in CD3OD
1	85.93	84.85	3.85 (1H, m)	3.9 (1H, d, J = 2.4)
5	74.32	74.24	3.61 (1H, dd, $J_{5,4}$ = 9.98, $J_{5,6}$ = 2.6)	3.70 (1H, dd, J = 9.6, 2.4)
3	73.75	73.71	3.19 (1H, dd, $J_{3,2}$ = 9.90, $J_{3,4}$ = 9.53)	3.26 (1H, dd, J = 9.6, 9.6)
6	73.47	73.40	3.85 (1H, m)	3.9 (1H, d, J = 2.4)
2	72.56	72.53	3.66 (1H, dd, $J_{5,4}$ = 9.98, $J_{2,1}$ = 2.6)	3.75 (1H, dd, J = 9.6, 2.4)
4	72.04	71.97	3.50 (1H, dd, $J_{4,3}$ = 9.53, $J_{4,5}$ = 9.98)	3.52 (1H, dd, J = 11.2, 11.2)
OMe	60.75	60.76	3.45 (3H, s)	3.61 (3H, s)

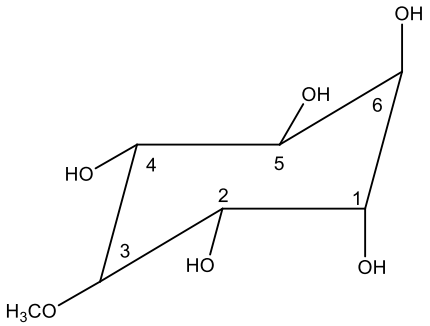

Figure 1.*d*-pinitol structure, compound identified by ^{13}C and ^{1}H NMR from methanolic leaves extract of *B. glabra*var 'Variegata'

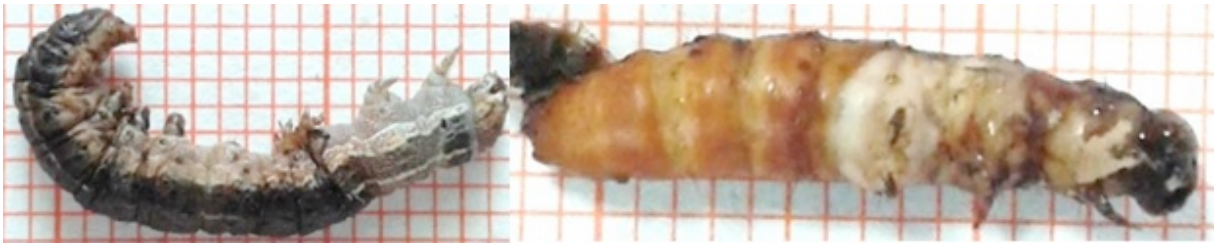

Figure 2. Biological effect of *B. glabra* leaves of var 'Variegata' against *S. frugiperda,* treated with 75 ppm of group K. Larvae showing an incomplete ecdysis (left) and larvae-pupae at intermediate stage (right).

The compounds identified were 30% of terpenoids type and 22% of fatty acids. The terpenoids were *squalene, oxirane2,2-dimethyl-3-(3,7,12,16,20-pentamethyl-3,7,11,15,19,*(2*E*)*-3,7,11,15-tetramethyl-2-hexadecen-1-ol, phytol and 9,12-octadecadienoic acid (Z,Z)-, methyl ester*. The compounds alkanes type were *hentriacontane, nonacosane, heptacosane* and *pentacosane*. The ketones were *3-buten-2-one, 4-(2,2,6-trimethyl-7-oxabicyclo*[*4.1.0*]*hept-1-yl)-, 2,2,6,6-tetramethyl-4-piperidone* and *6,10,14-trimethyl*-5,9,13-*pentadecan-2*-one, the phenolic compound was *2-methoxy-4-vinylphenol* and the lactone type compound was *2*(*4H*)*-benzofuranone, 5,6,7,7a-tetrahydro-4, 4, 7a-trimethyl*.

In the present research, no flavonoids or phenolic acids were isolated, as previously reported from *B. glabra*. This was probably due to the plant variety, origin of the biological material, the collected zone and the agronomic activities. Only the *3-O-methyl-d-glucose, phytol* and vitamin E were previously reported by Rani et al. (2012). Although their compounds were obtained from ethanol extract of *B. glabra* the plant variety was not reported. Sahu and Saxena (2014), Rani et al. (2012) and Napoleon et al., (2013) experimented with leaves, flowers and

bracts from India; Hussein (2014) and Kaisoon et al. (2011) studied flowers from Egypt and Thailand, respectively. None of their studies included the leaves or was comparable to the climatic conditions of this present research.

3.2. Evaluation of the fractions of *B. glabra* against *S. frugiperda*

The compound present in the group **K** was rich in *d*-pinitol and therefore it was evaluated against *S. frugiperda* at different concentrations from 0 to 125 ppm (Table 3). It could be observed that 50 and 75 ppm produced the best biologic effect at 7 days because the larvae did not gain weight and these treatments **K**50 and **K**75 ppm were highly statistically different with respect to the control ($p = 0.001$). In all experiments after 7 and 14 days there were no significant difference among treatments (Kruskal Wallis $H = 55.937$; $df = 10$ and $H = 39.86$, $df = 10$, respectively). In the treatment with 75 ppm of group K, malformations were observed in the larvae after two months and they did not development into pupae. The larvae showed an incomplete ecdysis and 30 % of the population remained in the larvae-pupae intermediate stage (Figure 2).

Figure 3. Pupae of *S. frugiperda*feed with the group K from *B. glabra* leaves of var'Variegata'. Normal pupae (left), discontinue cuticle in the ventral segment (middle); and dorsal (right).

In this study, it could be observed an interruption of the growth in the middle phase of the instar larvae into pupae, the insect died at the beginning of the mude without ending its morphogenesis. Some larvae died with the exuviae adhered to the body (Figure 2, left). The 43 % of the pupae that were fed with 25, 100 and 125 ppm showed discontinued cuticle development in the ventral segment, and 39 % of them had ventral ectodermal invaginations growth (Figure 3). The 5 % of the treated with 125 ppm, presented exploted bags in the encephalic region. 13 % of pupae appeared normal, however, they did not emerge into the adult stage like the control. The percentage of mortality of *S. frugiperda* larvae that were fed with the K fraction of *B. glabra*var 'Variegata' was higher than 50 %.

Table 3. Biological activity of group **K** from *B. glabra* leaves var 'Variegata' against *S. frugiperda* on the second instar larvae.

Fractión K* ppm	*Spodoptera frugiperda* weight (mg) ($\bar{x}$ ± se**)				
	Larvae				Pupae
	d7	d14	d21	d28	
125	9.06 ± 2.99	61.30 ± 18.80	150.30 ± 34.70	199.30 ± 52.10	119.0 ± 14.8
100	11.10 ± 4.67	79.80 ± 29.10	194.00 ± 54.20	161.00 ± 34.80	146.0 ± 21.6
75	3.87 ± 0.82[a]	27.90 ± 5.71[a]	97.70 ± 19.10	156.00 ± 32.30	NFP
50	5.32 ± 1.53[a]	36.70 ± 10.60[a]	146.00 ± 37.80	140.00 ± 48.00	115.0 ± 6.4
25	18.20 ± 3.93	148.00 ± 24.80	225.00 ± 34.80	182.00 ± 44.90	143.0 ± 10.5
0	21.73 ± 4.28	131.90 ± 19.98	245.91 ± 30.7	215.66 ± 36.89	177.0 ± 11.1

*Rich in *d*-pinitol; **se = standard error; NFP = No pupae formation

This is in agreement with Silva *et al.* (2003) who considered that *B. glabra* is insecticidal. Also, Chaubal et al. (2005) reported chronic toxicity of *d*-pinitol against *A. aegypti* and *C. quinquefasciatu*. The same alterations as those in our study were observed such as pupae inhibition formation and no emergence of the adults. The processes for the change to the larva stage in insects begin when the epidermis cell responds to the hormonal change through increasing the protein synthesis. For this, the first step comes with the apolysis, which is the separation of the epidermal cells from the inner surface of the old endocuticle and the formation of the subcuticular space, where a molt gel and enzymes are secreted (Marks, 1980).

During molt, the levels of ecdysteroids increase to stimulate the emergence of the apolysis and the synthesis of the cuticle. Later, the levels decrease so that the eclosion hormone (Truman and Riddford, 2002) and the hormone of the activation of the ecdysis (Zitnan et al., 1999) in the final stage are released. The main component of the leaf methanol extract i.e. *d*-pinitol, altered the ecdysteroid metabolism, resulting in an inhibition of emergence or by reducing the ETH liberation (Hésterlee y Morton, 1996). Chaubal et al. (2005) reported that the crude acetone extract of *Acacia nilotica* showed chronic toxicity against *A. aegypti* and *C. quinquefasciatus* at the 4th instar mosquito larvae at 500 ppm, where the active compound identified was *d*-pinitol.

4. Conclusion

The main compound identified on *B. glabra* var 'Variegata' was *d*-pinitol, which is a representative compound of the *Leguminosae* family. The group K was rich in *d*-pinitol and caused a biological effect against *S. frugiperda* at 75-ppm concentration by reducing larvae weight and preventing the pupae development. Therefore, *d*-pinitol can be considered a compound with insecticidal activity. This work reported for the first time that this compound isolated from *B. glabra* var 'Variegata' has potential to control an insect pest.

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