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Eco-Friendly Control Strategies of Green Stink Bug, *Nezara viridula* L. (Hemiptera: Pentatomidae): Repellency and Toxicity Effects of *Callistemon citrinus*, Bottle Brush Essential Oil

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ABSTRACT

The bioactivity of *Callistemon citrinus* leaves essential oil against green stink bug, *Nezara viridula* was evaluated as alternative eco-friendly pesticide. The tested essential oil have high insecticidal activity to *N. viridula* adult where, the lethal concentrations of LC_{10}, LC_{25} and LC_{50} were recorded (105.22, 536.80 and 3274.72 ppm), respectively. The repellent effects on *N. viridula* adult of the three tested concentrations were 48.01, 56.35 and 81.33 % after one hour of exposure time, respectively. The chemical composition analysis of bottle brush essential oil by GC/MS revealed 24 compounds represented 99.47% of the whole amount oil. The major constituent 1,8-cineole represented (73.81%) followed by α pinene (13.13%), D-limonene (5.50%), β-pinene (2.49%) and α-Terpineol (1.10%) assumed 96.03% of the total oil composition, while the minor components were 3.97%. Treatment with all concentrations of *C. citrinus* essential oil exhibited some physiological defects that cleared in the reduction in total soluble protein levels and elevation in carbohydrate hydrolyzing enzymes, nonspecific estrases and acid & alkaline phosphatases activities. Moreover, the LC_{50} inhibited acetyl cholinesterase activity in treated adults. Therefore the oil proved high potency in controlling the *N. viridula*.

Keywords: *Callistemon citrinus*, *Nezara viridula*, repellent effect, essential oil, enzymes activities

INTRODUCTION

There are many species of stink bugs, characterized by shield-shaped bodies and producing a strong disagreeable odor when handled. Stink bugs are consider an economic pest of most crops as cotton, soybean, tomato therefore, the control of this pest is more difficult. Their damage varies depending on the crop phenology when attacked, species of plant and the stage of development (Gore *et al*., 2006 and Mollah *et al.*, 2017). Adults and nymphs have piercing sucking mouthparts, which they use to pierce the peel or hull of fruiting structures and feed on the inner contents as in butter beans, green beans and soybean during pod formation which induce deformed seeds and reduce oil yield (Panizzi, 1997). Seed that are damaged when small usually fail to develop while larger seed will have sunken white or discolored areas where the stink bugs fed Willrich *et al*. (2004).

The *C. citrinus*, red bottle brush plant, family Myrtaceae is native from Australia now is widespread in many countries due to its very attractive appearance especially in mild climates countries Cornes (2006). The use of natural pesticides to protect crops because of their low toxicity to human beings and minimal environmental impact in comparable of most synthetic pesticides Isman (2000) and overcome the problem of development of resistance in insects Viswan *et al.* (2014). Recently many plants subjected to further investigation and their secondary metabolites have been formulated as botanical pesticides in plant protection and biological control. Essential oils, which are secondary metabolites used for defense against herbivorous, have been extracted and utilized as natural pesticides, antifeeding, growth regulators inhibitors and repellents, Suthisut *et al.*(2011). Previous studies have reported antifungal Nguefack *et al.* (2007), antibacterial Seyydnejad *et al.* (2010) and insecticidal activity (Ndomo *et al.* 2010) of volatile compounds from *Callistemon species*. Therefore, the current study made an attempt to determine the chemical composition and insecticidal activity of *C. citrinus* essential oils from leaves on the sting bug, *N. viridula* and establishing eco-friendly control strategy.

Nowadays natural pesticides based on plant extracts and essential oils represent alternative crop protectants and used in the control of several pests. The aim of this study is throw light on the deficiency in information on the effectiveness of repellents against *N. viridula* and other phytophagous hemipterans.

MATERIALS AND METHODS

Tested insect:

The sting bug, *N. viridula* subjected to bioassay studies were obtained from Sharkia province fields and reared on Okra leaves, *Abelmoschus esculentus* under constant conditions 25±5 °C and 65±5% RH and 12:12 h photo phase in physiology department, Branch of Plant Protection Research Institute at Zagazig, Sharkia.

Essential oil Extraction.

The plant under investigations was collected from various localities in Sharkia province. The *C. citrinus* fresh leaves of (500 gm.) were subjected to hydro distillation in two liters of distilled water until all essential oil was extracted afterword dried over anhydrous sodium sulfate and stored at 4°C until use.

Identification of oil components

The identification of oil components analysis was carried out in National research Center, Cairo, Egypt according to the method of Likenes and Nickerson (1966).

The bottle brush essential oil were analyzed on Gas chromatography Mass Spectrometry HP 6890 Series A (Agilent) equipped with column (Thermo Scientific (TR-5MS), 5% Phenyl Polysil Phenylene Siloxane; 30 m x 0.25 mm i.d.; 0.25 µm film thickness). The column flow rate was 1.00 ml He /min. Temperature program: initial temperature 50^0C for 5 minutes , temperature rate 4 °C/min, final temperature 250 °C. The injector temperature was 250^0C; injection volume 1µL. Empirical identification of the essential oil components were conducted by comparison of their relative retention times (RT) and their relative retention index (RRI) to a series of n-alkanes. Mass spectrum matching was aided by commercial libraries; NIST, Replib, wiley9, and mainlib.

Bioassay

Toxicity evaluation:

Serial concentrations of aqueous solutions of the tested essential oil were prepared. Okra leaves were dipped

in each mentioned concentration for 10 seconds then left to dry at room temperature, treated leaves were put separately in glassy jars covered with muslin. Three replicates of each concentration were prepared used 20 adult of *N. viridula* were transferred to each one. The mortality percentages were recorded after 24 h. Mortality data were corrected according to Abbott's formula (1925). The LC_{10}, LC_{25} and LC_{50} values were determined by using Probit-analysis of Biostat version 5 Analystsoft program.

Repellency assay

The repellent activity of *C. citrinus* oil was performed in multichannel olfactometer, which consisted of a circular central arena with five protruding tubes on the sides orthogonal to each other. These tubes connected to the five odor-chambers. The okra leaves treated with 105.22, 536.80 and 3274.72ppm of bottle brush essential oil putted in 3arms, respectively and the untreated one putted in other arm, the rest 1 arm closed with cotton. 20 individual of adult sting bugs released in the central arena under lab conditions 25±5 °C and 65±5% RH and 12:12 h photo phase three replicates were occurred. After 1, 12 h. the number of insects in each arm, and the number that remained in the central arena, were counted. Percentage of repellency (PR) was calculated according to the following equation: PR= [(NC-NT)/ (NC+NT)] × 100, where NC was the number of *N. viridula* adult on the untreated tube and NT was the number of insects on the treated tubes after the exposure intervals Akhtar *et al*., (2012).

Biochemical studies:

Samples of 3 replicates of *N. viridula* adult were collected after 24h. of treatments with LC_{10}, LC_{25} and LC_{50}. The untreated one was used as control. Samples were homogenized in distilled water using a Teflon homogenizer surrounded with a jacket of crushed ice. The homogenates were centrifuged at 6000 r.p.m. for 10 minutes at 5°C. The supernatant were stored at -20°C until analysis.

Total soluble protein

Colorimetric determination of total soluble protein was carried out as described by Gornall *et al.* (1949).

Carbohydrases enzymes activity:

The carbohydrases enzymes; invertase, amylase and trehalase activities were determined based on the digestion of sucrose, starch and trehalose, respectively, according to (Ishaaya and Swirski, 1976).

α and β estrases

the activeties of α estrases and β estrases, as non-specific estrases, were colorimetrically measured by Van Asperen (1962) method by using substrate α-naphthyl acetate and β-naphthyl acetate, respectively.

Acid and alkaline phosphatases

The acid and alkaline phosphatase activities were estimated by using Powell and Smith (1954) method as the amount of phenol that released by enzymatic hydrolysis from disodium phenyl phosphate (substrate).

Acetyl cholinesterase determination

The activity AchE (Acetyl cholinesterase) was determined by the Simpson *et al.* (1964) method using acetyl choline bromide (AchBr) as substrate.

Statistical analysis

The repellency test results were subjected to (ANOVA) variance analysis in addition to the Tukey's least significant difference to estimate significance differences between samples by Costat (2005) version 6.311.

RESULTS

Susceptibilty of *N. viridula* to *Callistemon citrinus*, essential oil.

Regarding Figure (1) the insecticidal action of four serial concentrations of *C. citrinus* essential oil on adult *N. viridula* after 24hr of treatment were evaluated. The high toxic effect (70%) mortality recorded at 10000ppm while the lowest concentration (625ppm) caused 30% mortality. The observed data indicate that the volatile oil of *C. citrinus* have toxic effect on *N. viridula* adult.

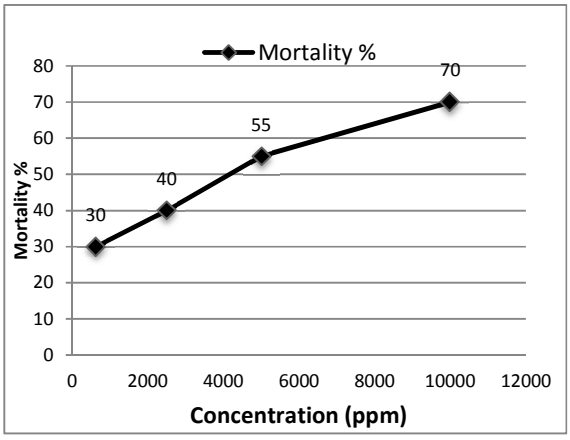

Fig. 1. Effect of *Callistemon citrinus* essential oil on adult mortality of *Nezara viridula*.

Toxicity bioassay:

The toxic effect of *C. citrinus* were listed in table (1) the LC_{10}, LC_{25} and LC_{50} were (105.22, 536.80 and 3274.72 ppm), respectively with slope 0.8585 and Chi-square 0.2811 for *N. viridula* adult after 24h of treatment.

Table 1. Toxic effect of different concentration of *Callistemon citrinus* essential oil.

	Concentration ppm	Confidence limits		slope	Chi-square (χ2)
		Lower	upper		
LC_{10}	105.22	0.0026	503.03		
LC_{25}	536.80	1.906	1403.49	0.8585	0.2811
LC_{50}	3274.72	1115.16	11406.19		

Repellency assay

The *C. citrinus* essential oil exhibited repellant effect against adults of *N. viridula*, dependent on the dose ($P<0.05$). Furthermore, the repellent action increased slightly when the insects were exposed for more time (Table 2) at 3274.72 ppm (LC_{50}) from 81.33% after 1h. to 82.66% after 12h. While, at concentrations of LC_{25} (536.80) and LC_{10} (105.22), the repellency changed from 56.35% to 49.16% and from 48.01 % to 42.66% after 1 and 12 h exposure times, respectively.

Table 2. Repellency effect of *Callistemon citrinus*, essential oil on *N. viridula*

Concenterations of *Callistemon citrinus*	1h.	12h.
LC_{10}	$48.01^{b}\pm1.01$	$42.66^{b}\pm1.76$
LC_{25}	$56.35^{b}\pm0.45$	$49.16^{b}\pm3.87$
LC_{50}	$81.33^{a}\pm9.49$	$82.66^{a}\pm10.10$
L.S.D.	19.09	21.90
P	0.0127 *	0.0089 **

L.S.D. means low significance differences at P< 0.05

Chemical compounds of *C. citrinus* essential oil

The obtained analysis results of GC-MS showed that the essential oil of *C. citrinus* contains 24 constituents represented by 99.47% of the whole amount oil (Table 3). 1,8- cineole (73.81) and α pinene (13.13) are the major components followed by D-limonene (5.50), β-pinene (2.49) and α-Terpineol (1.10) percentages with 96.03% of the total oil composition. The other components are traces compound (3.97%). The main component of the tested oil is1,8- cineole.

Biochemical assay

The biochemical responses of supernatant obtained from adult *N. viridula* homogenates were assessed after 24h. of treatments with the LC_{10}, LC_{25} and LC_{50} of *C. citrinus* essential oil. The changes in the amount of total soluble protein and the carbohydrate hydrolyzing enzymes activities; (trehalase, invertase, amylase), acid & alkaline phosphatases, α & β esterase and acetylcholinesterase enzymes were observed.

The total soluble protein:

Data in Fig (2a) cleared that the three concentrations of *C. citrinus* essential oil caused reduction in the amount of total soluble protein, in general. The highest reduction (5.35) observed at LC_{50} followed by LC_{25} (8.43) while LC_{10} caused slightly decrease (13.96) as compared to control (14.54 mg/gm. b. wt.).

Carbohydrate hydrolyzing enzymes:

Data presented in Fig (2b) show the effect of the tested oil at different concentrations on carbohydrate hydrolyzing enzymes in the homogenate adult of *N. viridulla*. It is explained that all concentration induced significant elevation in the activity of amylase, invertase and trehalase. The maximum elevation of invertase (28.17) and trehalase (15.70) exhibited at 536.80 ppm (LC_{25}), while the maximum activity of amylase (6.56) detected at 105.22 ppm (LC_{10}) as compared to the control activity of amylase, invertase and trehalase (2.07, 0.82 and 3.15 mg glucose /g. b. wt./min), respectively.

Table 3. The main compounds of the *Callistemon citrinus* essential oil from leaves.

Rt	Compound	Area%	CAS number
3.87	3-Pentanone, 2,4-dimethyl-	0,05	565-80-0 Wiley9
5.50	Isopentyl acetate	0.07	123-92-2 Wiley9
6.77	α-Thujene	0.12	2867-05-2 Wiley9
7.04	α-Pinene	13.13	80-56-8 Wiley9
8.56	β-Pinene	2.49	127-91-3 Wiley9
9.48	Pseudolimonene	0.08	499-97-8 Wiley9
9.58	α-Phellandrene	0.33	99-83-2 Wiley9
9.96	α-Terpinene	0.16	99-86-5 Wiley9
10.33	p-Cymene	0.04	99-87-6 Wiley9
10.45	D-Limonene	5.50	5989-27-5 Replib
10.64	1,8-Cineole	73.81	470-82-6 Mainlib
11.62	γ-Terpinene	0.62	99-85-4 Wiley9
12.74	α-Terpinolene	0.09	586-62-9 Wiley9
13.44	Linalool	0.22	78-70-6 Wiley9
14.44	Fenchol	0.04	1632-73-1 Wiley9
15.42	trans-Pinocarveol	0.04	547-61-5 Wiley9
16.86	Borneol	0.06	507-70-0 Replip
17.17	Terpinen-4-ol	0.74	562-74-3 Wiley9
17.91	α-Terpineol	1.10	98-55-5 Wiley9
27.46	trans-Caryophyllene	0.37	87-44-5 Wiley9
29.04	Humulene	0.05	6753-98-6 Wiley9
30.47	Virdiflorene	0.04	21747-46-6 Wiley9
34.40	Caryophyllene oxide	0.18	1139-30-6 Replib
34.56	(-)-Globulol	0.14	489-41-8 Wiley9
Total identified			99.47
Other compound			0.53

Rt = Retention time

Nonspecific estrases (α and β):

It is worthy to mention that the activity of α and β estrases Fig (2c) increased at all tested concentrations of *C. citrinus* essential oil. The highest increase recorded at LC_{50}, 0.37 and 14.58 followed by 0.14 and 9.66 at LC_{25} than control 0.05 and 0.16 µg α-βnaphthol/min/g.b.wt for α and β estrases, respectively.

Acid and Alkaline phosphatases (ACP, ALP):

The obtained results in Fig (2d) illustrated that all concentrations of tested oil recorded an elevation in ACP activity in the supernatant of the treated homogenate of adult *N. viridulla*. The abnormal activation (134.44) caused at LC_{50} (3274.72 ppm) while the ACP activity of control (1.05 µg phenol/min/g.b.wt.). All the treatments caused slightly elevation of ALP activity in homogenate *N. viridula* adult.

Acetylcholinesterase activity:

It is obvious from Fig. (2e) that the level of cholinesterase activity in the treated adult of *N. viridula* increased as compared with the level of untreated one in exception with LC_{50} which caused inhibition of this enzyme. The cholinesterase activities in treated adult were 1.73, 1.29 and 0.15 µg AChBr /min/ gm b. w. at LC_{10}, $LC_{25}$and LC_{50} , respectively, while it was 0.53 in control adult.

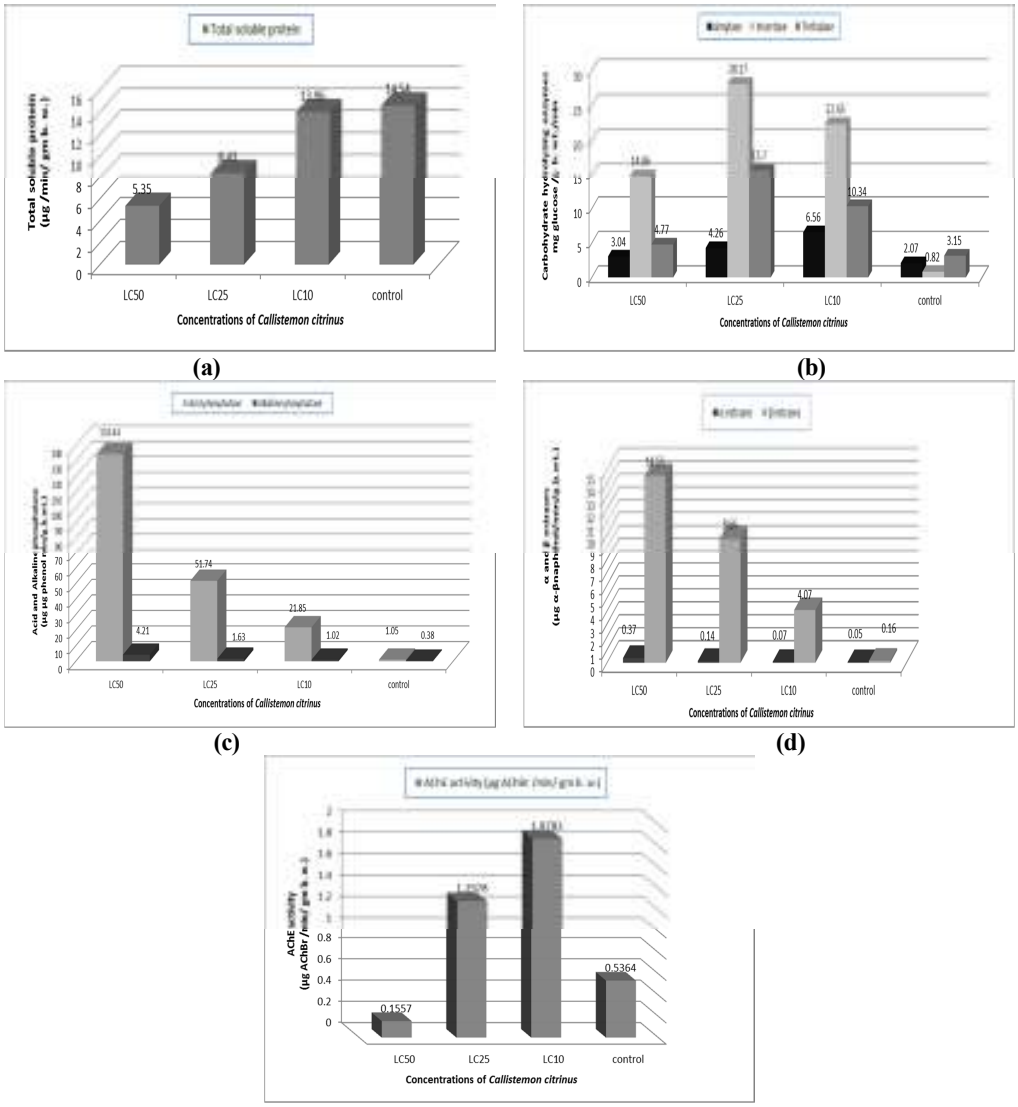

Fig. 2. Biochemical parameters in homogenate *N. viridula* adult treated with *C. citrinus* leaves essential oil.

DISCUSSION

The obtained results from GC-MS analysis demonstrated that the essential oil of *C. citrinus* leaves contains 1,8- cineole (73.81) and α pinene (13.13) are the major components followed by D-limonene (5.50), β-pinene (2.49) and α-Terpineol (1.10) percentages with 96.03% of the total oil composition. The high repellent activity may regarded to the presence of 1,8-cineole.

The composition of the essential oils from these studies compared with previous one emphasized that the major components of the *C. citrinus* essential oil were similar with different percentages. These results are in harmony with those studies by (Oyedeji *et al.* 2009 and Fall *et al*. 2017) indicated that the major compounds of *C. viminalis* oil were 1.8-cineole (58.12%), limonene (9.72%), α-terpineol (9.56%) and α-pinene (2.49%). Salem

et al. (2013) stated that the essential oil of *C. viminalis* leaves contain 14 identified constituents. The major component were 1,8- cineole and α pinene (64.53 & 9.69 %). Moreover, Jazet *et al.* (2009) reported the identified component of *C. citrinus* essential oil from Cameroon were 1,8-cineole (73.8%), α-pinene (16.3%) and α-terpineol (4.8%). Many studies elucidated that 1,8- cineole is the major compound in *C. citrinus* essential oil (Shrestha *et al*., 2015; Aweke and Yeshanew; 2016 and Andola *et al*., 2017).

The family myrtaceae has major compounds characterized by insecticidal properties, such as 1.8-cineole, limonene, α-terpineol and α-pinene. The insecticidal action of 1.8-cineole has been evaluated against several insects. The repellent effect of this compound against *Sitophilus granarius* and *Sitophilus zeamais* were detected (Liska *et al*, 2010). In addition, α-pinene showed insecticidal activity against *Tribolium confusum* (Chaubey, 2012). Moreover Zandi-Sohani *et al.* (2013) revealed that the *C. citrinus* essential oil has the repellent and insecticidal activity against the adult males of *Callosobruchus maculatus* (F.) and the essential oil exhibited that the oil was toxic in the fumigation test at LD50 values 12.88 and 84.4 μL·L−1 to males and females, respectively. Both the essential oils from leaves and flowers of *C. viminalis* exhibited a repellent activity against the *Maize persicae*. It can be inferred that the principle compounds (1.8-cineol and α-pinene) in the essential oil are related to its insecticidal activity (Sales *et al*., 2017). The LC_{50} of 57.4 μg/mL essential oil against fruit fly with, while LC_{50} of 38 μg/mL for worker termites (Shrestha *et al*., 2015). The insecticidal activity of *C. citrinus* leaf oil can be attributed to the major components 1,8-cineole , α-terpineol, and eugenol (Bora and Khanikor, 2011).

The essential oils play an effective role in disrupting insect physiology in different manners. Moreover, the essential oils affect the insect nervous system by inhibiting (AChE) as insecticides (Abdel-Tawab, 2016). The most important compounds of insect are protein that binds with foreign compounds. The decrease of total protein in treated insect can affect the activity of various enzymes. In the same trend, protein is the major components which necessary for development, growth and performance of vital activities (Rashwan, 2013).

The essential oil of *C. citrinus* caused deficiency in the amount of total soluble protein at all concentrations used so the reduction of total soluble protein of supernatant of treated adult *N. viridulla* may be due to increased breakdown of proteins to detoxify the active principles. The explanation of these results supported by (El-barky *et al.,* 2008) stated that inhibition of DNA and RNA Synthesis may lead to the reduction in protein content.

Amylase, trehalase and invertase enzymes involved in carbohydrates metabolism play an important role in the digestion and utilization of carbohydrates for production of needed insect energy. In this study the treatments cause elevation in amylase, trehalase and invertase enzymes. The reduction in total protein content and the increase in carbohydrate hydrolyzing enzymes could indicate mobilization of amino acids to supply energy which needed in detoxification process. (Gamil *et al*., 2011).

The highest elevation of α & β estrases, ACP, ALP caused by the treatments of *C. citrinus* essential oil were observed. Esterases are a large group of hydrolases enzymes which hydrolyze numerous substances including esters and certain non-ester compounds, which split ester compounds with the addition of water to yield alcohol and acids (Shaurub *et al.,* 1999) also, esterases play an important role in conferring or contributing to insecticide detoxifications in insect and arthropod species. In this respect Cai *et al*., 2009 suggesting that esterase enzymes are involved in detoxification of plant chemical defenses. Furthermore, Azab *et al*. (2011) concluded that, alkaline and acid phosphatase enzymes activities increased significantly in, *Bemisia tabaci* and *Aphis gossypii* after treated with damaseia extract.

The increase in the gut enzymatic activity after treatment may be related to the destructions of the mid-gut epithelial cell this may lead to intensive release of acid and alkaline phosphatases. Subsequently when the insect suffer from loss of weight and lake of feeding, the insect may try to compensate these pathological features by excess production of these enzymes for faster growth and development.

Apparently, increased activity of detoxification enzymes in adult of *N. viridula* indicated that the insect trial response to body intoxication with oil components of insect tissue degradation.

The LC_{50} of bottle brush essential oil caused inhibition of (AChE) while the induction action occurred at LC_{10} and LC_{25}. (AChE) is a key enzyme in the nervous system which plays vital role in neurotransmission at cholinergic synapses by rapid hydrolysis of the neurotransmitter acetylcholine to choline and acetate. In general, many workers emphasized that essential oil induce mortality by inhibiting AChE activity in insect. In the same trend Yu *et al*. (2011) stated that the anti-AChE activity of essential oils is strongly dependent on the interaction of different terpenoid contents. In contrast the presence of oxygenated functional groups in bicyclic terpenes caused decrease its potent to inhibit AChE also the α-pinene has been demonstrated as a potent AChE inhibitor (Miyazawa *et al*., 2005).

CONCLUSION

Based on the chemical constituent's properties of *C. citrinus* essential oil have a strong insecticidal and repellent activity against *Nezara viridula*. This oil has high potency in controlling the target pest. These findings are important and useful to incorporate *C. citrinus* essential oil into insect programs management.

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إستراتيجيات المكافحة الصديقة للبيئة لحشرة البقة الخضراء: التأثيرات الطاردة والسميه لزيت فرشاة الزجاج الأساسي

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معهد بحوث وقاية النباتات – مركز البحوث الزراعية- الدقي- الجيزة

تم تقييم النشاط الحيوي للزيت العطري لاوراق اشجار فرشاه الزجاج ضد حشرة البقة الخضراء كمبيد بديل صديق للبيئة. قد اظهر الزيت المختبر درجة عالية من النشاط الابادي علي الحشرات الكاملة حيث سجلت التركيزات المميتة لل10 و25و50 ٪ من التعداد (105.22 و 536.80 و 3274.72 جزء في المليون) على التوالي كما كان التاثيرالطارد للحشرات الكاملة للتركيزات الثلاثة التي تم اختبارها 48.01 و 56.35 و 81.33٪ بعد ساعة من وقت التعرض على التوالي. وقد بين التحليل الكيميائي للزيت العطري بواسطة جهاز الفصل الكروماتوجرافي الغازي المقترن بجهاز تحليل طيف الكتلة عن وجود 24 مركب تمثل 99.47٪ من كمية الزيت الكلية.حيث كان المكون الاساسي هومركب 1،8- سينول ويمثل (73.81٪) يليه الفا بينين(13.13٪) ودي- ليمونين(5.50 ٪) ،بيتا- بينين(2.49٪) و الفا تيربينول (1.10) ٪بنسبه 96.03٪ من إجمالي تركيب الزيت الكلي ، في حين كانت المكونات الثانوية (3.97 ٪). وقد أظهرت المعاملة باستخدام جميع تركيزات الزيت العطري بعض الخلل الفسيولوجي الذي اتضح في تقليل كمية البروتين الكلي الذائب ، والارتفاع في نشاط الإنزيمات المحللة للكربوهيدرات، وانزيمي الاستيريز غير المتخصص وانزيمي الفوسفاتيزالحامضي والقلوي . علاوة على ذلك ، ثبط التركيز نصف المميت نشاط الكولين استيريز في الافراد البالغة . بناء علي ذلك أثبت الزيت العطري كفاءة عالية في مكافحة حشرة البقة الخضراء.