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Sublethal Doses of Clove Oil Deplete the Energy Reserves and Downregulate the Carbohydrate Metabolizing Enzymes in the 4th Instar Larvae of *Trogoderma granarium*

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FRS and TR designed and supervised the study, and prepared final draft. AI performed experimental work. SR, AHB and UR performed statistical analysis. SR prepared the first draft.

Key words
***Trogoderma granarium*, Amylase, Clove oil, Glycogen, Trehalase, Stored grains**

ABSTRACT

The main objective was to search for a new chemical insecticide to control the stored grain insect pest *Trogoderma granarium* particularly when this pest developed resistance against the currently used insecticides. Clove oil has been traditionally considered as an effective pesticide. The present study aimed at determining the efficacy of clove oil against the 4th instar larvae of insecticide resistant populations of *T. granarium* collected from different regions of Punjab *i.e.*, Layyah, Okara, Gujranwala and Lahore. The Layyah population of Khapra beetle was identified as the most susceptible population (LC_{50}= 0.1%), and the Lahore population as the resistant one (LC_{50}= 0.78%) after exposure to various oil concentrations. The energy reserves and degradation enzymes needed to breakdown these energy stores were the main focus of this study. The alterations in the levels of carbohydrates, proteins and lipids were remarkable after treatment with clove oil at LC_{20} and LC_{50} doses. In Layyah population, a considerable loss of total protein (28.93%), glucose (84.1%) and glycogen (55.71%) while an increase in trehalose (4.48%) and lipids (264.22%) were recorded after exposure to LC_{50} doses compared with unexposed group. In Lahore population, a reduction in lipid contents (45.97%), total protein (67.67%), glucose (50.45%), glycogen (34.36%) and trehalose (31.59%) were observed after treatment with LC_{50} doses in comparison with untreated group. In case of LC_{20} doses, a decline in all the tested energy stores of both populations were observed in comparison with their respective unexposed groups. The total activities of degradation enzymes *i.e.*, trehalase, amylase and invertase in Layyah population decreased (56.25, 21.10 and 84.62%, respectively) and in Lahore population reduction in activities were 14.62, 87.45 and 75%, respectively after exposure to their respective LC_{20} doses as compared to their respective unexposed groups. The activities of all tested enzymes were decreased after exposure to LC_{50} doses except for invertase activity that increased after exposure to LC_{50} dose in Lahore population. The trends observed at LC_{20} doses were milder compared to that of LC_{50} dose. The clove oil can be used effectively against khapra beetle to control its damage.

INTRODUCTION

Approximately, 1660 insect pest's species invade the agricultural commodity after harvesting (Hagstrum and Subramanyam, 2009). The losses due to the attack of insect pests might be about 30% in storage phase (Haubruge *et al*., 1997). Wheat is the most popular cereal crop in the world. Due to the vast variety of species, assortments, and flexibility, wheat is grown both in the winter and the spring in various countries (McKevith, 2004; Kumar *et al*., 2011). Approximately, 3.72% of the world's wheat-growing regions are in Pakistan and it's the eighth-largest producer of wheat, which accounts for roughly 3.17% of global wheat production (Shuaib *et al*., 2007). Post-harvest wheat loss contributes to its quantitative and qualitative damage mainly caused by insect pests (Riaz *et al*., 2022). Among these insect pests *Trogoderma granarium* is reported to be the most dangerous and destructive insect pest of stored commodities across the globe (Lowe *et al*., 2000). The uncontrollable invasion of khapra beetle cause

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huge commercial loss and cause enormous damage to bulk commodities (Hafiz *et al*., 2017). Larval stages have a three-year resilience to deprivation and a limited supply of nutrient moisture. Human health suffers greatly as a result of swallowing larval spiky hairs that are cut off and remain in product piles (Ahmedani *et al*., 2007).

Synthetic insecticides like organophosphates, pyrethroids and carbamates and fumigants like methyl bromide and phosphine were the most commonly used strategies. Fumigants were considered applicable and economical due to their broad-spectrum activities as well as rapid penetration without leaving any residue on the grains (Varma and Dubey, 2001; Ogendo *et al*., 2008). Methyl bromide was completely pushed out because it causes ozone depletion (Shaaya and Kostyukovsky, 2006; Tayoub *et al*., 2012). So, phosphine was the only strategy to control the invasion of insect pests during storage (Varma and Dubey, 2001). With increase in fumigation frequency, certain other problems like to effect non target organism, safety concerns for human health and environment and development of resistance in insect pests were noticed (Ogendo *et al*., 2008). Now, almost all major pests of stored products have developed resistance against phosphine (Lorini *et al*., 2007; Nayak *et al*., 2012).

In this scenario, some biodegradable, viable, safe, environment friendly and effective alternative to these conventional insecticides and fumigants are the dire need of time (Sheng *et al*., 2020). Several reports are in favor of fumigant/insecticidal potential of various botanical extracts (Tripathi *et al*., 2000; Isman, 2006, 2008; Ebadollahi *et al*., 2013; Sagheer *et al*., 2013; Tarigan and Harahap, 2016). Moreover, a variety of plant essential oils were investigated for their insecticidal efficacy against various insects and these plant essential oils are proved to more effective to control insect pests of storage facilities (Tripathi *et al*., 2000; Opolot *et al*., 2006; Ebadollahi *et al*., 2013; Tarigan and Harahap, 2016; Ramachandran *et al*., 2022; Wanna and Wongsawas, 2022).

Essential oils are synthesized as secondary metabolites by various families like Myrtaceae, Meliaceae, Asteraceae and Umbelliferae etc., of aromatic plants in their different vegetative organs (Ebadollahi *et al*., 2013; Koul *et al*., 2013; Sheng *et al*., 2020; Kheloul *et al*., 2020). Essential oils are the mixtures of highly volatile organic lipophilic components that includes terpenes, aromatic and aliphatic compounds with different functional groups (Brito *et al*., 2021).

Clove (*Syzygium aromaticum* L.) essential oils with great efficacy against storage facility insect pests like *Sitophilus zeamais* and *Acanthoscelides obtectus* are well documented (Huang *et al*., 2002; Li *et al*., 2011; Viteri Jumbo *et al*., 2014; Gonzales Correa *et al*., 2015). However, no attention is paid to towards alterations induced by clove oil essential oils in energy stores and carbohydrase activities in insects. The estimation of energy stores and carbohydrase activities after sublethal exposure is essential because these parameters play an important role in development of resistance, pest revival with secondary pest outbreak leading to failure in management strategy (Desneux *et al*., 2007; He *et al*., 2013; Guedes and Cutler, 2014; Santos *et al*., 2015).

Keeping in view all these challenges, the current study aimed at determining the efficacy of clove oil against the 4th instar larvae of insecticide resistant populations of *T. granarium*. Moreover, an effort was made to document the contents of energy reserves and activities of degradation enzymes after exposure to sublethal LC_{20} and LC_{50} doses.

MATERIALS AND METHODS

Trogoderma granarium *culture and its maintenance*

Four populations of the Khapra beetle were collected from the wheat storage godowns located in Layyah, Okara, Gujranwala and Lahore. Each population had already been exposed to phosphine and deltamethrin for decades while in stores. For propagation of culture, an optimum temperature 35±2°C and relative humidity 60±5% (Riaz *et al*., 2014) was maintained in the insect culture room of Institute of Zoology, University of the Punjab, Lahore. Male and female beetles were allowed to breed in hygienic, microbe-free glass jars of 300 mL capacity. A proper diet comprising of broken wheat and flour were provided. Different growth stages of beetles were developed but 4th instar larvae were chosen in current studies.

Determination of toxicity of clove oil

Clove oil ($C_7H_{12}ClN_3O_2$) was purchased from a departmental store in Lahore. It was pale yellow in color with spicy odor. Absolute acetone was used as an organic solvent. To determine the toxicity of clove oil, various concentrations (0.06, 0.08, 0.1, 0.3, 0.5, 0.7, 0.9, 1.1, and 1.3%) were prepared by using 0.6, 0.8, 1, 3, 5, 7, 9 and 11µL/mL of clove oil in acetone, respectively. The mixtures were stirred thoroughly in test tubes covered with aluminum foil.

The glass petri plates (90 mm diameter) were labelled according to their doses and were uniformly covered with 1 mL solution of respective concentration of clove oil. Petri plates were set to air dry until only oil remained on the surface and volatile acetone had disappeared. Twenty 4th larval instars were introduced in each petri plate and these were incubated at 35±2°C, 60±5% rh for 24 h. Control was also prepared by spreading 1 mL acetone instead of clove oil and proceeded like treatment. A completely randomized design with three replicates for each concentration were used. After 24 h exposure, the larvae showing any response after touch with camel hair brush was considered alive, in case of no response was assumed dead (Tayoub *et al*., 2012). The number of dead larvae in each petri plate was recorded and mortality was subjected to correction according to Abbot (1925). Minitab 16 was used to carry out Probit analysis (Finney, 1971) to estimate the LC_{20} and LC_{50}.

Biochemical assay

About 1000 4th instars larvae of each Layyah and Lahore populations were exposed to their respective LC_{20} and LC_{50} doses for 24 h. Control/unexposed groups were also prepared by exposing the larvae with acetone. All analysis were carried out in triplicates. About twenty-eight 4th instar larvae were crushed in 2 mL phosphate-saline buffer using a motor-driven teflon glass homogenizer. The crushed larvae were centrifuged at 13500xg for 15 min at 4°C. The supernatants were used for estimation of contents of glucose according to Hartel *et al*. (1969), trehalose according to Carroll *et al*. (1956) and proteins (soluble and total) according to Bradford (1976). While the activities of trehalase, amylase, and invertase were calculated according to Dahlaqvist (1966), Wootton and Freeman (1982), respectively. The 4th instar larvae were homogenized in a 30% potassium hydroxide solution (KOH) for estimation of glycogen contents according to Consolazio and Lacono (1963) and lipid contents according to Zöllner and Kirsch (1962).

Statistical analysis

The statistical analysis was done using Minitab 16. Probit analysis gave insight about toxicity data while the data obtained on effect of LC_{20} and LC_{50} doses on energy stores and degrading enzymes was proceeded through un-paired t test to determine the significant differences at $p< 0.05$.

RESULTS

Toxicity assessment of clove essential oil

The LC_{50} values of clove oil for Layyah, Okara, Gujranwala and Lahore populations were 0.1, 0.4, 0.58 and 0.78%, respectively. Based on LC_{50} values, the Lahore population seemed the most resistant and Layyah population least resistant among all the tested populations. Similarly, LC_{20} values for Layyah, Okara, Gujranwala, and Lahore populations were 0.001, 0.08, 0.24 and 0.42%, respectively.

Effect of LC_{20} and LC_{50} on energy reserves

The amount of energy stores (µg/mg) in 4th instar larvae of Lahore and Layyah populations are presented in Table I while the percent change in these tested contents are shown in Figure 1. The total proteins and glucose contents in both populations were downregulated after exposure to both sublethal doses. The glycogen contents were downregulated in Lahore population after exposure to each sub lethal dose but in case of Layyah population glycogen contents were upregulated after exposure to LC_{20} but downregulated after LC_{50} exposure. Trehalose contents in Layyah population were down regulated at LC_{20} but when lethal dose exposure increased up to LC_{50}, these contents were surprisingly increased as compared to LC_{20} as well as control group. In case of Lahore population, the trehalose and lipid contents downregulated after each sublethal dose as compared to control group but surprisingly the trehalose and lipid contents decreased after LC_{20} exposure but when exposure increased upto LC_{50} these contents increased in comparison with LC_{20}. The lipid contents in Layyah population declined after LC_{20} and when exposure dose increased the decline was more prominent.

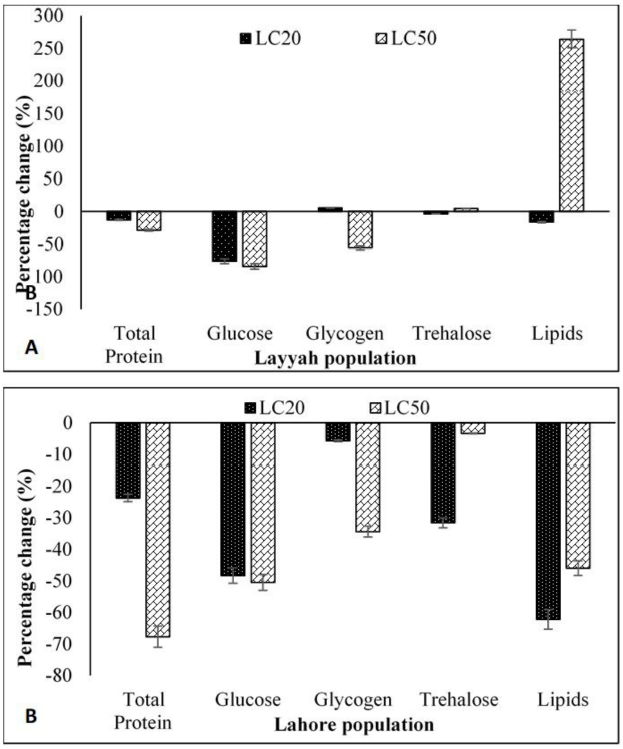

Fig. 1. Effect of clove oil at LC_{20} and LC_{50} on energy reserves of Lahore (A) and Layyah (B) populations *Trogoderma granarium,* as depicted by percent increase or decrease in the concentration of various macromolecules.

Table I. Toxic effects of LC_{20} and LC_{50} of clove oil on the energy reserves of 4th instar larvae of *T. granarium* after 24 h exposure.

Energy stores (µg/mg)	Strains	Unexposed control	Exposure to clove oil	
			LC_{20}	LC_{50}
Total protein contents	Layyah	45.83±1.16^{A}	39.92±0. 2^{B}	32.57±1.31^{C}
	Lahore	35.35±1.15^{A}	26.97±1.55^{B}	11.43±0.83^{C}
Glucose contents	Layyah	94.92±2.31^{A}	22.82±0.87^{B}	15.18±0.32^{C}
	Lahore	81.45±0.18^{A}	42.12±1.12^{B}	40.36±0.73^{B}
Glycogen contents	Layyah	13.75±9.44^{A}	14.50±0.98^{A}	60.92±9.48^{B}
	Lahore	40.84±4.51^{A}	38.49±7.26^{A}	26.81±1.36^{B}
Trehalose contents	Layyah	55.59±1.68^{A}	53.56±2.17^{A}	58.08±3.55^{A}
	Lahore	52.09±4.86^{A}	35.64±5.98^{A}	50.35±4.10^{A}
Lipids contents	Layyah	50.22±2.32^{A}	41.98±2.29^{A}	18.29±1.50^{B}
	Lahore	26.17±4.65^{A}	98.95±3.46^{B}	14.14±3.28^{C}

Mean ± standard error of mean; n = 3 (No. of replicates used in each experiment); Means followed by the same letter in each row for each parameter are not significant from each other at $P < 0.05$with 95% confidence limit (Dunnett's Test).

Table II. Effect of clove oil at of LC_{20} and LC_{50} on the total activity of carbohydrates metabolizing enzymes of 4th instar larvae of *T. granarium*

Strains	Enzymes	Unexposed control	Exposure to	
			LC_{20}	LC_{50}
Layyah	Amylase	63.73±8.46^{A}	50.28±2.09^{B}	38.01±1.27^{C}
	Trehalase	1.12±2.86^{A}	0.49±10.6^{B}	0.76±87.1^{B}
	Invertase	0.01±1.27^{A}	0.002±0.12^{B}	0.001±0.109^{B}
Lahore	Amylase	1.49±3.17^{A}	2.13±6.45^{B}	1.66±358^{A}
	Trehalase	1.71±70.8^{A}	1.46±38.3^{A}	1.15±66.4^{B}
	Invertase	0.004±0.09^{A}	0.001±0.02^{B}	0.009±0.03^{B}

Mean ± standard error of mean; n = 3 (No. of replicates used in each experiment); Means followed by the same letter in each row for each parameter are not significant from each other at $P < 0.05$ with 95% confidence limit (Dunnett's Test). Amylase: SU/mg body wt; Trehalase: IU/mg body wt; Invertase: IU/mg body wt.

Effect of LC_{20} and LC_{50} on carbohydrate degrading enzymes

The amylase and trehalase activities were decreased after both levels of exposure in Layyah and Lahore populations with reference to their respective unexposed group. The percent decrease in these activities was more prominent at LC_{50} as compared to LC20 doses. The invertase activities decreased in both populations after each exposure except in Lahore population where a significant increase was observed after exposure to LC_{50}, when compared with their control group (Table II and Fig. 2).

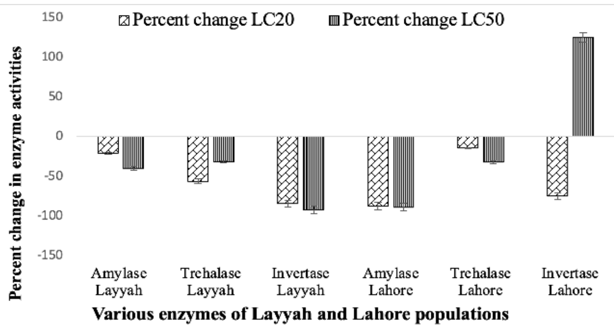

Fig. 2. Effect of clove oil administered at LC_{20} and LC_{50} on carbohydrate degrading enzyme activities of Layyah and Lahore populations of *Trogoderma granarium* as depicted by percent increase or decrease in the concentration of various enzyme activities.

DISCUSSION

Plant essential oils are considered as the most interesting strategy for safer, cheaper and eco-friendly alternatives of conventional insecticides (Stevenson *et al.*, 2017). The insecticidal potential of several plant oils and their extracts against the khapra beetle has been investigated (Asawalam and Onu, 2014). The current study's goal was to assess clove essential oil's efficiency against populations of 4th instar larvae from Layyah, Okara, Gujranwala, and Lahore at 35±2°C and 60±5% R.H. All populations of *T. granarium* exhibited very low

LC_{20} and LC_{50} values of clove oil. These findings were supported by Suma *et al*. (2009) they examined the toxicity of insecticides and a few non-polar plant extracts on the *Coccophagus lycimnia*, *Aphytis melinus*, and *Leptomastix dactylopii*. All populations were said to be sensitive to the lethal and sub-lethal doses of applied treatments.

To suppress the bean weevil, Jumbo *et al*. (2014) used prescribed dosages of clove and cinnamon oil that had lower LC_{50} values. These oils contributed to the pest's eventual repulsing and demise. Clove, eucalyptus, and neem oil extracts were shown to have lower LC_{50} values for controlling the larvae of *C. maculatus*, *T. castaneum* and *T. granarium* as reported by Mostafa *et al*. (2019). The significant impact of oil's LC_{20} and LC_{50} was examined in relation to changes in the energy reserves (total protein, glucose, glycogen, trehalose, and lipids) of *T. granarium* larvae. However, no reports are available on the impact of sub-lethal dosages of clove oil on the energy stores of the Khapra beetle.

Carbohydrates are utilized during metamorphosis and development of insects (Kaufmann and Brown, 2008). They supply energy either to use immediately like glucose, or in the form of reserves like glycogen and trehalose. They may also have some structural role in cuticle synthesis. Protein synthesis play an important role in growth, maintenance, hormonal regulation, reproduction as well as integration in the cell (Cohen, 2010; Sugumaran, 2010). Enzymes, antibodies, and albuminous fractions are referred to as soluble proteins (Shakoori *et al*., 2016). While both soluble and insoluble proteins are present in total proteins. The lipids are considered as an independent reservoir of energy and their mobilization is done through hemolymph from storage site to the other organs where needed especially the vitellogenesis (Zhou and Miesfeld, 2009) and cuticular synthesis (Dapporto *et al*., 2008).

The considerable decline in protein levels as observed in current investigation might be attributed to their breakdown for metabolic need or for supply of amino acids to build polypeptide chains or it might be due to downregulation of protein synthesis. The findings of Khosravi *et al*. (2011) and Valizadeh *et al*. (2013) are in favor of current investigations. They observed decline in total proteins in *Glyphodes pyloalis* larvae exposed to *Artemisia annua* extracts and in elm leaf beetle toxified with neem extract, respectively. Ali and Ibrahim (2018) reported a decline in protein and carbohydrates contents in 4th instar larvae of *Spodoptera littoralis* after exposure to camphor oil while castor oil only decreased the carbohydrate contents.

Tarigan *et al*. (2016) investigated the effect of cinnamon, cardamom and nutmeg essential oils on carbohydrates, lipids and protein contents in various developmental stages of *T. castaneum* and *Callosobruchus maculatus.* They reported that all essential oils decreased all the above-mentioned nutritional components. Dris *et al*. (2017) documented protein, carbohydrate and lipids depletion in 4th larval instar of *Culex pipiens* after exposure with *Ocimum basilicum* essential oils. The drop in lipid contents were attributed to stimulation of adipokinetic hormone, which is involved in lipid metabolism (Kodrik and Socha, 2005). According to various reports, the conversion of lipids into proteins causes decrease in lipids and they are also involved to provide more energy (Omar *et al*., 2005; Shakoori *et al*., 2016).

Amylase, invertase and trehalase are the enzymes responsible for the degradation of carbohydrates to harvest needed energy in insects. The reduction in the total protein contents and reduced activities of degrading enzymes might be due to reduction DNA and RNA synthesis and ultimately leads to inhibition in protein expression are supported by the explanation of El-Barky *et al*. (2008). The current study indicated that stress induced by clove oil causes the inhibition of carbohydrate degrading enzymes and insect was unable to harvest energy from reservoirs and finally died. Similarly, Sohbi (2020) reported reduced activities of trehalase, invertase and amylase in 4th larval instar of *Spodoptera littoralis* after exposure to camphor essential oil. The studies of Yousef *et al*. (2018) also supported the current findings that significant inhibition in the activities of these tested enzymes in *S. littoralis* were observed after exposure to pepper mint oil. In contrast to these reports, El-Gendy and El-Shafiey (2018) reported a significant elevation in the activities of amylase, invertase and trehalase enzymes in *Nezara viridula* after exposure to bottle brush essential oil. They proposed that the decrease in protein contents and elevation in carbohydrate degrading enzymes implies mobilization of amino acids to provide energy which is required during detoxification mechanism (Gamil *et al*., 2011).

CONCLUSION

The clove oil has a great insecticidal potential having very low values of LC_{50} against all tested populations. It can be used as a biopesticide to kill khapra beetle in godowns. These findings are useful and can be incorporated in insect pest management strategy to control *T. granarium.*

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Statement of conflict of interest

The authors have declared no conflicts of interest.

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