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Research Article
Investigation of Natural Plant *Aegle marmelos* Essential Oil Bioactivity on Development and Toxicity of *Tribolium castaneum* (Coleoptera: Tenebrionidae)

Bhuwan Bhaskar Mishra, S.P. Tripathi and C.P.M. Tripathi

Entomological Research Laboratory, Department of Zoology, D.D.U. Gorakhpur University, 273009 Gorakhpur, Uttar Pradesh, India

Abstract

Background: The uncontrolled use of synthetic chemicals is a great hazard for the environment and consumers. **Methodology:** In the present study, essential oil from leaves of *Aegle marmelos* was isolated by hydro-distillation and tested their insecticidal activity against *Tribolium castaneum* (Herbst). **Results:** The results showed that the essential oil of *A. marmelos* have fumigant toxicity, oviposition and developmental inhibitory activity against *T. castaneum*. The percentage mortality increased with increasing exposure time and concentration. The median lethal concentration (LC_{50}) of *A. marmelos* essential oil at 48 h was 14.172 and 17.752 µL against larvae and adults of *T. castaneum*, respectively. The essential oil significantly reduced oviposition ($F_{3,20}$ = 304.734) in adults and also reduced pupation ($F_{3,20}$ = 137.442) and adult emergence ($F_{3,20}$ = 225.619) in larvae when fumigated with sub-lethal concentration. The percent grains infection was reduced 83.66% at 60% of sub-lethal concentration of 24 h LC_{50}. Fumigation of insect with sub-lethal concentration of *A. marmelos* essential oil inhibited AChE activity. Reduction in AChE activity was 81.48 and 54.32% of the control, after 24 h of fumigation with sub-lethal concentration. **Conclusion:** In conclusion, this essential oil probably induces toxicity in insect by inhibiting AChE activity.

Key words: Essential oil, *Tribolium castaneum*, toxicity, *Aegle marmelos*, chronic exposure, AChE activity

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Corresponding Author: Bhuwan Bhaskar Mishra, Entomological Research Laboratory, Department of Zoology, D.D.U. Gorakhpur University, 273009 Gorakhpur, Uttar Pradesh, India Tel: +91 551 2202187

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Stored-grain insect pests have been damaging economy by infesting agricultural products. The red flour beetle; *Tribolium castaneum* (Herbst) is one of the major pests of stored grains and grain products in the tropics. Synthetic chemical fumigants are commonly used to control stored product pests throughout the world, but these products caused adverse affect on the environment such as disturbances of the environment, increasing costs of application, pest resurgence, pest resistance to pesticides and lethal effects on non-target organisms in addition to direct toxicity to users and are hazardous to human health (Lee *et al*., 2004; Tapondjou *et al*., 2002). Therefore, considerable amount of investigations have been carried out in the last three decades to find alternative control methods of stored-grain insect pests (Morimoto *et al*., 2002; Park *et al*., 2002). Plant essential oils and their components have been shown to possess potential for development as new fumigants and they may have advantages over conventional fumigants in terms of low mammalian toxicity, rapid degradation and local availability (Isman, 2008). Essential oils derived from more than 75 plant species have been evaluated for fumigant toxicity against stored product insects so far (Rajendran and Sriranjini, 2008). *Aegle marmelos* (L.) Correa (Rutaceae), commonly known as Bael, is a sacred tree for Hindu religon, native to northern India, but is found widely throughout the Indian peninsula and in Ceylon, Burma, Thailand and Indo-China (Bailey, 1963). All parts of the tree namely, root, leaf, trunk, fruit and seed are used for treatment of many different diseases. The constituents of *A. marmelos* are used in heart diseases (Kakiuchi *et al*., 1991), inflammatory and wound healing (Udupa *et al*., 1994). Leaves of *A. marmelos* have been reported as hypoglycemic effect (Santhoshkumari and Devi, 1990; Sharma *et al*., 1996). The essential oil from the leaves of *A. marmelos* is known to exhibited antifungal properties (Rana *et al*., 1997). The repellent effect of *A. marmelos* essential oil has already determined against stored-grain pest *T. castaneum* and *Sitophilus oryzae* (Mishra and Tripathi, 2011).

Keeping these facts in mind, in the present investigation, essential oil of *A. marmelos* was tested as a fumigant to protect stored grain from insect pest *Tribolium castaneum*. The effect of *A. marmelos* oil applications on toxicity, chronic toxicity, oviposition and adult emergence of *T. castaneum* has also been determined.

MATERIALS AND METHODS

Plant collection and isolation of essential oil: Leaves of *A. marmelos* were collected from the local area of Gorakhpur districts of Uttar Pradesh, India. The specimens were identified and authenticated by the expert in the Department of Botany, D. D. U. Gorakhpur University, Gorakhpur. The leaves was dried in absence of sun light at room temperature (30±5°C) and grounded using a mixer. The essential oil was extracted by the method of Mishra *et al*. (2013).

Insect rearing: Larvae and adults of *T. castaneum* were obtained from Entomological Laboratory, D.D.U. Gorakhpur University, Gorakhpur laboratory stock cultures maintained in an incubator at 30±2°C, 75±5% RH and at photoperiod of 10:14 (L:D) without exposure to any insecticides. *Tribolium castaneum* adults were reared on mixed grain and flour of *Triticum aestivum* (L.) at 12-13% moisture content. Ten days old unsexed adults and 4th instars larvae of *T. castaneum* were used to determine the insecticidal property of *A. marmelos* essential oil.

Toxicity

Larval and adults toxicity: A serial dilution of four concentrations of essential oil (15, 20, 25 and 30 µL) was prepared in acetone (100 µL) and treated with larvae and adults of *T. castaneum* by the method of Mishra *et al*. (2012).

Oviposition inhibitory activity of essential oil: Oviposition inhibitory activity of *A. marmelos* essential oil was tested against *T. castaneum* by fumigation method of Mishra *et al*. (2014).

Determination of developmental inhibitory activity of essential oil: Developmental inhibitory activity of *A. marmelos* essential oil was tested against 4th instars larvae of *T. castaneum*. Twenty larvae of *T. castaneum* were places in 2 g of wheat flour and grains in glass petri dish. A paper strip (2 cm^2) treated with 40, 60 and 80% sub-lethal concentration of 24 h LC_{50} of essential oil in acetone was pasted on the inner surface of the cover of each petri dish. Another paper strip (2 cm^2) was treated with absolute acetone only was used as control. All the closed petri dishes were kept in dark and six replicates were set for each concentration. After fumigation of glass petri dishes for 72 h as was done in larvicidal assay and then the treated larvae were transferred to fresh wheat flour in other petri dish. Number of survived larvae, transformed pupae from treated larvae and emerged adults from transformed pupae were recorded. Six replicates were set for each concentration.

Feeding deterrence activity: Chronic activity of *A. marmelos* essential oil was tested against adults of *T. castaneum* by

contraction method with two sub-lethal concentrations of essential oil prepared in acetone (30 and 60% of 24 h LC_{50}) (Mishra and Tripathi, 2011; Mishra *et al*., 2012) by using micropipette. The treated filter papers were dried to evaporate the solvent completely. The treated filter paper placed at the bottom in glass petri dish (height 15 mm × radius 45 mm). Ten adults taken from the laboratory culture (1-2 week old) were placed with 5 g of wheat flour and grain in petri dish. Flour and grains were spread uniformly along the whole surface of the petri dish. All the closed petri dishes were kept in dark and six replicates were set for each concentration. After 30 days, percent grains damage by stored-grain insect pests were recorded. The grain damage was determined by counting feeding injuries and emergence holes on the surface of the grains. Feeding deterrent was calculated using the feeding deterrent index following farmula:

$$\text{Feeding Deterrent Index (FDI) (\%)} = \frac{\text{C-T}}{\text{C+T}} \times 100$$

where, C and T is the weight loss in the controls and in the fumigated sets, respectively.

Acetylcholinesterase enzyme (AChE) activity determination: Acetylcholinesterase activity of *T. castaneum* was measured by the method of Ellman *et al*. (1961). Adults of *T. castaneum* were fumigated with two sub-lethal concentrations (40 and 80% of 24 h LC_{50}) of *A. marmelos* essential oil as done in toxicity assay. Six replicates were set up for each concentration. Control animals were taken as such without any treatment. After 24 h of fumigation, adults were utilized for the measurement of AChE activity in the treated as well as the control group. Enzyme activity has been expressed as µmol "SH" hydrolyzed min^{-1} mg^{-1} protein.

Data analysis: Lethal concentration (LC_{50}), lower and upper confidence limits (LCL-UCL), slope value, t-ratio, g-value, heterogeneity factor and chi-square value were calculated using computer software of Robertson *et al*. (2007). Correlation and linear regression analysis were conducted to define all dose-response relationships (Sokal and Rohlf, 1973). Analysis of variance was performed to test the equality of regression coefficient (Sokal and Rohlf, 1973). Student t-test was performed to test the significant changes in enzyme activity with respect to control (Armitage *et al*., 2001).

RESULTS

Fumigant toxicity of the essential oil from leaves of *A. marmelos* against larvae and adults of *T. castaneum* gradually increased with increasing exposure time and concentration ($p<0.01$). The essential oil from *A. marmelos* killed the larvae and adults of *T. castaneum* by fumigation action. The medium Lethal Concentration (LC_{50}) of larvae of *T. castaneum* was 16.870 µL at 24 h and 14.172 µL at 48 h whereas, 19.656 and 17.752 µL against adults of *T. castaneum* at 24 and 48 h, respectively (Table 1).

The t-ratio values were greater than 1.96, indicating a significant regression of each dose response line. The heterogeneity factor was less than 1.0, demonstrating that the log-dose-probit lines are within the 95% confidence limits and thus the model fittest the data. Value of g less than 0.5 indicated that mean was within the limit at all probability levels of 90, 95 and 95%.

The regression analysis showed a concentration dependent significant correlation of the oil with larval mortality of *T. castaneum* ($F_{3,20}$ = 15.432, $p<0.01$), ($F_{3,20}$ = 22.667, $p<0.01$) and adults of *T. castaneum* ($F_{3,20}$ = 24.583, $p<0.01$), ($F_{3,20}$ = 26.958, $p<0.01$) at 24 and 48 h exposure, respectively. The oviposition was reduced to 60.96, 49.43 and 40.15% when adults were fumigated with 40, 60 and 80% sub lethal concentration of essential oil, respectively (Table 2). The reduction in oviposition potential of *T. castaneum* was significant ($F_{3,20}$ = 304.734) when fumigated with sub lethal concentration of *A. marmelos*. Pupation was reduced to 78.88, 62.49 and 45.18% when 4th instars larvae were fumigated with 40, 60 and 80% sub lethal concentrations of essential oil, respectively. Similarly, adult emergence was reduced to 66.67, 46.48 and 35.33% when 4th in stars larvae were fumigated with different sub lethal concentration of essential oil (Table 3). The decrease in pupation ($F_{3,20}$ = 137.442) and adult emergence ($F_{3,20}$ = 225.619) was increased significantly with increased the concentration of essential oil.

The percent grains infection was reduced by *A. marmelos* essential oils against *T. castaneum* was 51.82 and 83.66% at 30 and 60% of sub-lethal concentration of 24 h LC_{50}, respectively (Table 4). The regression analysis indicated that percent damage grain reduction by adults of *T. castaneum* by essential oil showed a significant negative correlation with concentration when fumigated with *A. marmelos* essential oil (F = 659.63) (Table 5).

Fumigation of *T. castaneum* adults with two sub lethal concentration 40 and 80% of 24 h LC_{50} of *A. marmelos* essential oil significantly reduced AChE activity. Reduction in

Table 1: Summary of *Aegle marmelos* essential oil toxicity assays against larvae and adult of *Tribolium casteneum*

Parameters	Exposure period (h)	LC_{50}[a] (µL)	LCL-UCL[b]	g-value[c]	t- ratio[c]	Heterogeneity[c]	Chi-square
Larval mortality	24	16.870	9.393-20.180	0.232	2.913	0.184	4.038
	48	14.172	6.198-17.384	0.218	3.155	0.163	3.578
Adult mortality	24	19.656	16.239-22.336	0.278	3.957	0.211	4.637
	48	17.752	14.100-20.097	0.376	4.180	0.227	5.004

[a]LC_{50} represent the median lethal concentration, [b]UCL and LCL represent upper confidence limit and lower confidence limit. [c]g-value, t-ratio and heterogeneity were significant at all probability levels (90, 95 and 99%)

Table 2: Effect of fumigation of *Aegle marmelos* essential oil on oviposition of stored-grain insect pest *Tribolium castaneum*

Essential oil	24 h LC_{50} (µL)	Treatments	No. of eggs/larvae produced per ten insects (Mean±SE)	Eggs/larvae produced per ten insects (%)	ODI[a] (%)
Aegle marmelos	19.656	Control	265.00±4.61	100	100
		40% of 24 h LC_{50}	179.00±2.87	67.54	19.36
		60% of 24 h LC_{50}	142.00±3.97	53.89	30.22
		80% of 24 h LC_{50}	111.83±3.60	42.20	41.96

ODI[a] (%) was calculated as 100 (C-T)/(C+T), where C and T represent the number of eggs/larvae produced in the control and in the test, respectively

Table 3: Effect of *Aegle marmelos* essential oil on development (pupation and adult emergence) of stored-grain insect pest *Tribolium castaneum*

24h LC_{50} (µL)	Treatments	Pupation (No. of pupa transformed per twenty fumigated larvae)	Adult emergence (No. of adults emerged per twenty fumigated larvae)
19.656	Control	17.33±0331 (100)	16.50±0.34 (100)
	40% of 24 h LC_{50}	13.67±0.42 (78.88)	11.00±0.36 (66.67)
	60% of 24 h LC_{50}	10.83±0.307 (62.49)	7.67±0.21 (46.48)
	80% of 24 h LC_{50}	7.83±0.307 (45.18)	5.83±0.30 (35.33)

Values in parentheses represent percent with respect to control taken as 100%

Table 4: Effect of fumigation of *Aegle marmelos* essential oil on damage caused by stored-grain insect pest *Tribolium castaneum*

Essential oil	24 h LC_{50} (µL)	Treatments	Grain infected (Mean±SE) (%)
Aegle marmelos	19.656	30% of 24 h LC_{50}	51.82±0.71
		60% of 24 h LC_{50}	83.66±0.31

Table 5: Regression parameters of lethal, sub lethal and chronic activity on stored-grain insect pest *Tribolium castaneum* with *Aegle marmelos* essential oils by fumigation method

Parameters (%)	Exposure time (h)	Intercept	Slope	Regression equation	Regression coefficient	F-value (p<0.01)
Adult mortality	24	-13.0189	6.0023	Y = -13.018+6.002X	0.9999	15.432*
	48	-15.6722	5.7272	Y = -15.672+5.727X	0.9971	22.667*
Larval mortality	24	-1.3077	4.3286	Y = -1.307+4.328X	0.9899	24.583*
	48	-2.7786	4.1834	Y = -2.778+4.183X	0.9948	26.958*
Oviposition	72	122.841	0.475	Y = 122.841+0.475X	-0.996	223.635*
Pupation	72	148.746	-8.356	Y = -148.746+-8.356X	-0.991	137.442*
Adult emergence	72	119.502	-7.268	Y = 119.502+-7.268X	-0.996	225.619*
FDI	720	-1.783	0.703	Y = -1.783+-0.703X	0.990	659.63**

Regression analysis was performed between different concentrations of essential oil and responses of the insect pest. *Significant at 99% probability level. *F-values were significant at all probability levels (90, 95 and 99%), *df: 3,20; **df: 2,20

Table 6: Effect of 40 and 80% of 24h LC_{50} of *Aegle marmelos* essential oil on acetylcholinesterase enzyme (AChE) activity in *Tribolium castaneum*

Essential oil	Control	40% of 24 h LC_{50}	80% of 24 h LC_{50}
Aegle marmelos	0.081±0.005	0.066±0.003	0.044±0.006
	(100%)	(81.48%)	(54.32%)
	t = 8.61	t = 8.61#**	t = 11.17#**

Enzyme activity was expressed as µ mol of 'SH' hydrolyzed min^{-1} mg^{-1} protein. Values indicate Mean±SD of four replicates. Values in parentheses indicate per cent change with respect to control taken as 100%. #Paired t-test was applied. *Significant (p<0.01)

AChE activity was 81.48 and 54.32% of the control after 24 h fumigation with 40 and 80% of 24 h LC_{50}, respectively (Table 6).

DISCUSSION

Essential oils are the best known plant products tested against insects pests (Papachristos and Stamopoulos, 2002; Formisano *et al*., 2008; Chaubey, 2013). Plant volatile essential oils are a group of botanical insecticide that has recently been commercialized in the United States (Isman, 2008). Many essential oils and their constituents have been studied to possess potential as alternative compounds to currently used insect-control agents (Shaaya *et al*., 1997; Huang *et al*., 2000; Lee *et al*., 2004; Batish *et al*., 2008; Sahaf *et al*., 2008; Cosimi *et al*., 2009; Nerio *et al*., 2009). In the present

investigation the essential oil of *A. marmelos* was found to be effective against larvae and adults of *T. castaneum*. The present investigation was supported with the result of Mishra and Tripathi (2011) who investigated the repellent activity of *A. marmelos* essential oil against stored-grain insect pests. In Gas Chromatography Mass Spectrophotometry (GC/MS) analysis, the essential oil of *A. marmelos* show different chemical components. The leaf essential oil of *A. marmelos* contain 15 compounds, including seven monotorpene hydrocarbons, three oxygenated monoterpenes, four sesquiterpene hydrocarbons and one phenolic compound in all of this limonene was the main constituent (Kaur *et al*., 2006). The insecticidal constituents of many essential oils are mainly monoterpenoids (Coats *et al*., 1991; Regnault-Roger and Hamraoui, 1995; Kim *et al*., 2003). Monoterpenoids have been reported to inhibit reproduction of stored insects at several steps of the cycle. The mode of toxicity for monoterpenoids is believed to be via competitive inhibition of acetylcholinesterase (Ryan and Byrne, 1988). On other hand the volatile oil of *Pyrenacantha staudtii* also contains limonene which is reported to be an active insecticidal constituent that exerts a toxic effect on coleopterans (Tapondjou *et al*., 2002). In the present study limonene was the major component of the oil of *A. marmelos* and it may be responsible for fumigant toxicity, oviposition deterrent, inhibition of adult development of test insect. *Aegle marmelos* oil was mixture of a number of components that would reduce the chances for the development of insect resistance, which could be due to the diffusion of the gene selection process (Davies, 1992; Begon *et al*., 1999). However, concerns for residue of essential oil pesticides on food grains should be mitigated by the growing body of evidence that many essential constituents acquired through the diet are actually beneficial to human health (Huang *et al*., 1994). The insecticidal activity of the essential oil would be dependent on the active chemical constituents and the gross sensitivity of the target pest to the active chemical principles (Obeng-Ofori *et al*., 1997). Most of the essential oil components including limonene inhibiting AChE activity have been reported as constituents of *A. marmelos* essential oils and probably might attribute AChE inhibitory activities to the oils under investigation. The rapid action of essential oil against insect pest is indicative to their neurotoxic mode of action interfering with neuromodulator octopamine (Kostyukovsky *et al*., 2002) or with GABA-gated chloride channels (Priestley *et al*., 2003). The mode of action of this essential oil is yet to be confirmed but it appears that death of the adults, larvae may be due to the suffocation and inhibition of different biosynthetic processes of the insect metabolism (Don-Pedro, 1989). Therefore, one can conclude that the potent essential oil might be useful for management of stored grain insect pest.

CONCLUSION

In conclusion, *A. marmelos* oil may be used as botanical insecticide against different stored grain insect pests causing infestation in stored wheat and pulses. On the basis of results of present study, it can be concluded that the insecticidal nature of *A. marmelos* against *T. castaneum* might be due to its AChE inhibitory activities. Moreover, because of the use in traditional medicine in cure of different human diseases, the *A. marmelos* oil may be used as semiochemicals mediating phytopesticide to protect stored food commodities in developing countries, for which some farmers may not have easy access to chemical insecticides.

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