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Impact of *Syzygium aromaticum* (L.) essential oil as fumigant against *Tribolium castaneum* (Coleoptera: Tenebrionidae)

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Abstract
Use of synthetic chemicals is a great hazard for the environment and consumers. In the present study, essential oil from buds of *Syzygium aromaticum* was isolated by hydro-distillation methods and tested their insecticidal activity against *Tribolium castaneum* (Herbst). The results showed that the essential oil of *S. aromaticum* 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 *S. aromaticum* essential oil at 48 h was 13.871 and 15.551 µl against larvae and adults of *T. castaneum*, respectively. The essential oil significantly reduced oviposition ($F_{3,20}$=303.983) in adults and also reduced pupation ($F_{3,20}$=54.716) and adult emergence ($F_{3,20}$=101.309) in larvae when fumigated with sub-lethal concentration. The per cent grains infection was reduced 54.69% at 60% of sub-lethal concentration of 24 h LC_{50}. Fumigation of insect with sub-lethal concentration of *S. aromaticum* essential oil inhibited AChE activity. Reduction in AChE activity was 9.32.48 and 10.93% of the control, after 24 h of fumigation with sub-lethal concentration. In conclusion, this essential oil probably induces toxicity in insect by inhibiting AChE activity.

Keywords: Insecticidal activity, *Syzygium aromaticum, Tribolium castaneum,* toxicity, AChE activity

1. Introduction
Red flour beetle, *Tribolium castaneum* (Herbst) (Coleoptera: Tenebrionidae) is one of the most destructive beetle species to several grains and flours. The progeny production rate of *T. castaneum* is so high. The fourth instars larvae are highly active in rainy season and cause very high infestation. This insect pest had a long association with human stored food and has been found in association with a wide range of commodities including grain, flour, peas, beans, cacao, nuts, dried fruits, and spices, but milled grain products such as flour appear to be their preferred food [1, 2]. Control of these insects relies heavily on the use of synthetic insecticides and fumigants, which has led to problems 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 [3-6]. Fumigation was being the most effective method for controlling stored grains insect pests. Used of plants for pest control on stored grains seems to offer desirable solutions, especially in the developing tropical countries where plants are found in abundance everywhere throughout the year. Moreover, there has been growing interest in the use of both plant extracts and their essential oils since they exhibit low mammalian toxicity and low persistence in the environment [7, 8]. Essential oils produced in various external and internal glands of these plants are a very complex mixture of terpenes, sesquiterpenes, their oxygenated derivatives and other aromatic compounds [9]. Many studies of the fumigant activity of such natural substances have been undertaken to establish new control practices with lower mammalian toxicity and low persistence in the environment [10-13]. The essential oil isolated from the clove buds, *Syzygium aromaticum* is widely used and well known for its medicinal properties [14-16]. The biological activity of *S. aromaticum* oil has been investigated against several pests. It was shown to inhibit the emergence of *Culex pipiens* larvae [17] and to display insecticidal activity against *Pediculus capitis* [18], *Anopheles dirus* mosquitoes [19]. Park and Shin [20] has found that the clove oil could be used as a novel fumigant against Japanese termites. The objective of present study was to explore the fumigant activity of *S. aromaticum* (L.) essential oil for protection of stored-grain from insect infestation.

2. Materials and Methods

2.1 Plant collection and isolation of essential oil

For the extraction of essential oil, cloves of *S. aromaticum* were purchased 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 cloves were dried in absence of sun light at room temperature (30±5 ˚C) and grounded using a mixer. The essential oil was extracted by hydro-distillation using a modified Clevenger apparatus with distilled water. Distillation was done continuously for five hours to yield essential oil. Anhydrous sodium sulphate was used to remove water after extraction. The superior phase was collected from the condenser in glass containers and stored in appendorff tube at 5 ˚C until their use for further experiments.

2.2 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 ^{0}C, 75±5% RH and at photoperiod of 10:14 (L:D) without exposure to any insecticides. *T. castaneum* adults were reared on wheat grain (*Triticum aestivum* L.) and flour 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 *S. aromaticum* essential oil.

3. Toxicity

3.1 Larval Toxicity

A serial dilution of four concentrations of essential oil (15, 20, 25 and 30 µl) was prepared in acetone (100 µl). A whatman filter paper strip (diameter 2cm) was impregnated with diluted essential oils (115, 120, 125 and 130 µl for each concentration) and pasted on the inner surface of the cover of each petri dish (height 15mm, radius 45mm). The solvent was allowed to evaporate for 30 seconds and then petri dishes were covered tightly. Acetone was used as a control. Ten 4th instars larvae taken from the laboratory culture were placed with 1 gm. of wheat flour in the petri dishes. Flour was spread uniformly along the whole surface of the petri dishes. All the closed petri dishes were kept in dark and six replicates were set for all treatment and controls, and they were incubated for 48 h. After 24 and 48 h, mortality was recorded. The treated 4th instars larvae were then transferred to fresh petri dishes for determination of end-point mortality.

3.2 Adult Toxicity

The toxic effect of *S. aromaticum* essential oil was tested against adults of *T. castaneum* by fumigation action. The methodology used was the same as that used in determining the toxic effect of larval mortality of *T. castaneum*.

3.3 Oviposition inhibitory activity of essential oil

Oviposition inhibitory activity of *S. aromaticum* essential oil was tested against *T. castaneum* by fumigation. Twenty, 1 - 2 week old adults of mixed sexes were placed in 1 gram of wheat flour in petri dishes. Flour was spread uniformly along the whole surface of the petri dishes. 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. All the closed petri dishes were kept in dark and six replicates were set for each concentration. After 72 h of fumigation, the treated adults were transferred to fresh petri dishes having fresh wheat flour. After 10 days of treatment, the adults were removed and discarded. The number of the larvae hatched was counted for the treated as well as for control groups. The counting was done for four days continuously.

3.4 Determination of developmental inhibitory activity of essential oil

Developmental inhibitory activity of *S. aromaticum* essential oil was tested against 4th instars larvae of *T. castaneum*. Twenty larvae of *T. castaneum* were places in 2 gm 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 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 hour 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.

3.5 Feeding deterrence activity

The Chronic activity of *S. aromaticum* essential oil was tested against adults of *T. castaneum* by contraction method with two Sub-lethal concentrations. Whatman no. 1 filter papers were cut according to the shape and size of petri dishes and treated with sub-lethal concentrations of essential oil prepared in acetone (30 and 60% of 24h LC_{50}) [20, 21] 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 millimeter × radius 45 millimeters). Ten adults taken from the laboratory culture (1-2 week old) were placed with 5 gram 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 per cent 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 Isman *et al*. [23].

$$\text{Feeding deterrent index (FDI) [\%]} = \frac{C-T}{C+T} X100$$

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

3.6 Acetylcholinesterase (AChE) activity determination

Acetylcholinesterase activity of *T. castaneum* was measured by the method of Ellman *et al*. [24]. Adults of *T. castaneum* were fumigated with two sub-lethal concentrations (40% and 80% of 24 h LC_{50}) of *S. aromaticum* essential oil as done in toxicity assay. Six replicates were set up for each concentration. Controls 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 protein^{-1}.

3.7 Data analysis

The 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*. [25]. Correlation and linear regression analysis were conducted to define all dose-response relationships Sokal and Rohlf [26]. Analysis of variance was performed to test the equality of regression coefficient Sokal and Rohlf [26]. Student t-test was performed to test the significant changes in enzyme activity with respect to control Armitage *et al*. [27].

4. Result

Fumigant toxicity of the essential oil from cloves of *S. aromaticum* against larvae and adults of *T. castaneum* gradually increased with increasing exposure time and concentration (P<0.01). The essential oil from *S. aromaticum* killed the larvae and adults of *T. castaneum* by fumigation action. The Medium lethal concentration (LC_{50}) was 17.556 µl and 13.871µl against larvae, whereas 18.465 µl and 15.551 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, 99%.

The regression analysis showed a concentration dependent significant correlation of the oil with larval mortality of *T. castaneum* ($F_{3,20}$=35.490, P<0.01), ($F_{3,20}$=22.754, P<0.01) and adults of *T. castaneum* ($F_{3,20}$=20.728, P<0.01), ($F_{3,20}$=24.412, P<0.01) at 24 and 48 h exposure, respectively (Table 5).

The oviposition was reduced to 69.18, 53.64 and 45.83% 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 ($F_{3,20}$=303.983) when fumigated with sub lethal concentration of *S. aromaticum* (Table 5).

Pupation was reduced to 84.59, 69.24 and 58.62% whereas adult emergence was reduced to 76.78, 57.57 and 48.48% when 4th instars larvae were fumigated with 40, 60 and 80% sub lethal concentrations of essential oil, respectively (Table 3). The decrease in pupation ($F_{3,20}$=54.714) and adult emergence ($F_{3,20}$=101.309) was increased significantly with increased the concentration of essential oil (Table 5).

The per cent grains infection was reduced by *S. aromaticum* essential oil against *T. castaneum* was 30.23% and 54.69% at 30% and 60% of sub-lethal concentration of 24 h $LC_{50,}$ respectively (Table 4).

The regression analysis indicated that per cent damage grain reduction of adults of *T. castaneum* by essential oil showed a significant negative correlation with concentration when fumigated with *S. aromaticum* essential oil (F=362.93) (Table 5).

Fumigation of *T. castaneum* adults with two sub lethal concentration (40 and 80% of 24 h LC_{50}) of *S. aromaticum* essential oil significantly reduced AChE activity. Inhibition in AChE activity was 9.32% and 10.93% of control with 40 and 80% of 24 h LC_{50}, respectively (Table 6).

Table 1: Summary of *Syzygium aromaticum* essential oil toxicity assays against larvae and adult of *T. casteneum*

Parameters	Exposure period (h)	LC_{50}[a] (µl)	LC_{90}[b] (µl)	LCL-UCL[b]	g-value[c]	t- ratio[c]	Heterogeneity[c]	Chi square
Larval mortality	24	17.556	66.657	12.498-20.369	0.211	3.467	0.138	3.030
	48	13.871	48.724	7.663-16.854	0.245	3.463	0.196	4.311
Adult mortality	24	18.465	53.581	14.636-21.010	0.225	3.916	0.247	5.424
	48	15.551	45.352	10.724-18.134	0.223	3.851	0.182	4.009

[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%, 99%).

Table 2: Effect of fumigation of *Syzygium aromaticum* essential oil on oviposition of stored-grain insect pest *T. castaneum*

Essential Oil	24h LC_{50} (µl)	Treatment	Number of eggs/ larvae produced per ten insects (Mean ± SE)	% eggs/ larvae produced per ten insects	Hatchability (%)[a]
Syzygium aromaticum	18.465	**Control**	**265.83 ± 4.61**	**100%**	**100%**
		40% of 24 h LC_{50}	183.33 ± 4.73	69.18%	18.36%
		60% of 24 h LC_{50}	142.16 ± 2.18	53.64%	27.53%
		80% of 24 h LC_{50}	120.83 ± 2.67	45.83%	37.50%

%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 *Syzygium aromaticum* essential oil on development (pupation and adult emergence) of stored-grain insect pest *T. castaneum*

24h LC_{50} (µl)	Treatment	Pupation (Number of pupa transformed per twenty fumigated larvae)	Adult emergence (Number of adults emerged per twenty fumigated larvae)
18.465	Control	17.33 ± 0.331 (100)	16.50 ± 0.34 (100)
	40% of 24 h LC_{50}	14.66 ± 0.49 (84.59)	12.67 ± 0.33 (76.78)
	60% of 24 h LC_{50}	12.00 ± 0.57 (69.24)	9.50 ± 0.42 (57.57)
	80% of 24 h LC_{50}	10.16 ± 0.30 (58.62)	8.00 ± 0.57 (48.48)

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

Table 4: Effect of fumigation of *Syzygium aromaticum* essential oil on damage caused by stored-grain insect pest *T. castaneum*

Essential oil	24h LC_{50} (µl)	Treatment	Grain damage reduction (Mean ± SE) (%)
Syzygium aromaticum	18.465	30% of 24h LC_{50}	30.23 ± 0.47
		60% of 24h LC_{50}	54.69 ± 0.41

Table 5: Regression parameters of lethal, sub lethal and chronic activity on stored-grain insect pest *T. castaneum* with *Syzygium aromaticum* essential oil by fumigation method

Parameters	Exposure time (h)	Intercept	Slope	Regression Equation	Regression coefficient	F-value ($P < 0.01$)
% Adult mortality	24	-3.1764	4.434	Y=-13.018+6.002X	0.9948	20.728*
	48	-9.9560	4.897	Y=-15.672+5.727X	0.9981	24.412*
% Larval mortality	24	-6.9050	4.971	Y=-1.307+4.328X	0.9971	35.490*
	48	-14.5481	5.357	Y=-2.778+4.183X	0.9993	22.754*
% Oviposition	72	122.841	0.475	Y=122.841+0.475X	-0.996	303.983*
% Pupation	72	148.746	-8.356	Y=-148.746+-8.356X	-0.991	54.716*
% Adult emergence	72	119.502	-7.268	Y=119.502+-7.268X	-0.996	101.309*
% FDI	720	-1.783	0.703	Y=-1.783+-0.703X	0.990	362.93**

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 *Syzygium aromaticum* essential oil on acetylcholinesterase enzyme (AChE) activity in *T. castaneum*

Essential oil	Control	AChE activity	
		40% of 24 h LC_{50}	80% of 24 h LC_{50}
Syzygium aromaticum	0.081±0.005	0.069±0.002	0.052±0.005
	(100%)	(89.25 %)	(58.43%)
	t= 8.61	t= 9.32#*	t= 10.93#*

Note. Enzyme activity was expressed as µ mol of 'SH' hydrolyzed min^{-1} mg $protein^{-1}$. Values are mean± SE of six replicates. A value in parentheses indicates the percentage of enzyme activity with untreated control taken as 100%. #Paired t-test was applied. *Significant ($P < 0.01$).

5. Discussion

Certain plants essential oils or their constituents have a broad spectrum of activity against insect pests. As such, they have considerable potential as stored product protectants and for pest management in other situation. Potential of plants essential oils as a source of insecticides has been reported with references to various pests [28-34]. Previous research by Mishra *et al* [35] demonstrated that *S. aromaticum* essential oil has more insecticidal activity against *S. oryzae* than the other essential oils. Contact toxicity of the clove oil and the safety of this oil on non-target organism including man also discussed by Yang *et al* [18] and Formisano *et al* [36]. Plant volatile essential oils are a group of botanical insecticide that has recently been commercialized in the United States Isman [37]. In the present investigation, the essential oil of *S. aromaticum* was found to be effective against larvae and adults of *T. castaneum*. The present investigation was supported with the result of Mishra and Tripathi [22] who investigated the repellent activity of *S. aromaticum* essential oil against stored-grain insect pests. In Gas Chromatography Mass Spectrophotometry (GC/MS) analysis, the essential oil of *S. aromaticum* it was found that eugenol was the major component of the oil *S. aromaticum* [38] and it may be responsible for fumigant toxicity, oviposition deterrent, inhibition of adult development of test insect. 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 [39].

Eugenol is widely used in agricultural applications to protect food from microorganisms during storage, which might have an effect on human health, and as a pesticide and fumigant [40]. Mahdi and Rahman [41] tested the insecticidal effect of some spices on *Callosobruchus maculatus* (Fabricious) in black gram seed. They found that in eleven essential oils, clove oil was more effective than the other. Clove oil could be used as protectant of black gram seed against *C. maculatus*. Moreover, *S. aromaticum* has been used as a pesticide against harmful pests [42].

Earlier it had been shown that sub-lethal treatment with eugenol causes a significant inhibition of AChE activity in the nervous tissue of [43]. The high anti-AChE activity was 81.48% of control with 40% of 24 h LC_{50}. The mode of toxicity for essential oils is believed to be via competitive inhibition of acetylcholinesterase. The rapid action of essential oil against insect pest is indicative to their neurotoxic mode of action interfering with neuromodulator octopamine [44] or with GABA-gated chloride channels [45]. 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 [46]. The rank correlation coefficient applied between the 40 and 80% of 24 h LC_{50} values and the corresponding inhibition of enzyme activity indicate a positive correlation between the LC_{50} and the inhibition of AChE.

6. Conclusion

In summary, *S. aromaticum* 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 *S. aromaticum* 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 *S. aromaticum* 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. Therefore, one can conclude that the potent essential oil might be useful for management of stored grain insect pest.

7. References

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