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Control potentials of *Hyptis suaveolens* L. (Poit.) extracts against *Artemia salina* L. Nauplii and *Tribolium castaneum* (HBST.) adults

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Abstract
The insecticidal activity, insect repellency and brine shrimp lethality tests of Petroleum ether (Pet. ether), $CHCl_3$ and CH_3OH extracts of *Hyptis suaveolens* L. (Poit.) were assessed against *Tribolium castaneum* (Herbst) adults and *Artemia salina* L. nauplii under laboratory conditions. Pet. ether and CH_3OH extracts of leaves offered mortality to the test beetles and gave LD_{50} values 4.322, 3.487, 2.653, 2.647 and 2.647mg cm^{-2}, and 5.587, 4.122, 3.269, 3.075 and 2.727 mg cm^{-2} both after 12, 24, 36, 48 and 60h of exposure; and the $CHCl_3$ extract of roots gave LD_{50} values 6.494 and 5.311mg cm^{-2} after 48 and 60h of exposure respectively. The Pet. ether, $CHCl_3$ and CH_3OH extracts of stem and the CH_3OH extracts of root showed repellency at 5% level ($p<0.05$) of significance and the $CHCl_3$ extract of roots showed repellency at 1% level ($p<0.01$) of significance. Except the Pet. ether extract of leaves and the CH_3OH extracts of root all the extracts showed lethality against *Artemia salina* L. nauplii at different time exposures.

Keywords: Dose mortality, repellency, Brine shrimp lethality, *Hyptis suaveolens*, *Tribolium castaneum*, *Artemia salina*

Introduction
Insects are amongst major pests of stored cereals and they often cause an important economic damage that amount to 5–10% in the temperate zone and to 20–30% in the tropical one [1]. The modern storage techniques have been introduced where grain pests are usually controlled either by contact insecticides or by fumigation with an insecticidal gas. However, residual toxicity, resistant insect strains, worker's safety, and high cost of the treatment call for new systems for their control [2]. Bio-insecticides, based on the plant components may represent an alternative to traditional insecticides. In fact, components extracted from various spices and herbs can act as contact or fumigant insecticides against stored product pests [3-4] and could be evaluated in more detail as potential alternatives [5-6]. Several aromatic plants such as Lamiaceae have traditionally been used in the developing countries to protect stored grain and legumes from pests [7-9]. Dichloromethane extracts and infusions from some Lamiaceae species were tested against *T. castaneum* (Herbst) and higher lethal effect was achieved with *Mentha rotundfolia* L. [10]. Plants belong to the *Hyptis* Genus, which included more than 400 species, are highly aromatic. The test plant *Hyptis suaveolens*, known as Wilayati tulsi belongs to the family Lamiaceae and is an ethno-botanically important medicinal plant and considered as an obnoxious weed. Almost all parts of this plant are being used in traditional medicine to treat various diseases. Leaves and twigs are considered to be antispasmodic and used in anti-rheumatic and antisuporific baths, anti-cancer, anti-inflammatory, antifertility agents [11-12] and also applied as an anti-septic in burns, wounds, and various skin complaints. The decoction of the roots is highly valued as appetizer and is reported to contain urosolic acid, a natural HIV-integrase inhibitor [13]. Flowers are small, blue in axillary racemiform cymes or cymes collected into thyrsiform almost leafless panicles. The plant is stimulant, carminative, sudorific and lactagogue; infusion is used in catarrhal conditions, affections of the uterus and parasitical cutaneous diseases. Leaves are used against cancer, tumor, colic, stomachaches and fever. The seeds allay thirst; in case of habitual constipation, in internal piles and also used for the remedy of urinary complications by the Chakma people. The main chemical constituents obtained are 1,8-cineole and caryophyllene; and it is widely recognized that this genus

possesses insecticidal properties [14-17] reported that the essential oils of *H. suaveolens* were sabinene, limonene, bicyclogermacrene, phellandrene and 1,8-cineole. After that Ziegler *et al.* [18] isolated dehydroabietinol from this plant. A relatively recent literature reports that essential oils of *H. spicigera* and *H. suaveolens* could be a valid option to chemical insecticides for the control of many stored-food pests [19-26], suggesting the necessity of further investigations. The present investigation was carried out to find out the potentials of this plant as of insecticidal and insect repellent activity against the red flour beetle, *Tribolium castaneum* (Herbst), and lethality against the brine shrimp, *Artemia salina* L. nauplii.

The red flour beetle is reddish-brown in color and its antennae end in a three-segmented club [27]. Although small beetles, about ¼ of an inch long, the adults are long-lived and may live for more than three years [28], and thus became a suitable lab insect. The *Artemia salina* belongs to a genus of very primordial crustacean (crawfish - crayfish) the *Anostraca* (Fairy Shrimps). Crawfish of this Genus just have a divided exoskeleton made of chitin enhanced protein, no usual crust of chitin (escutcheon) as other crawfish has. There are many species within the Genus of *Anostraca*, but the *A. salina* is very nice to grow, since the rate of successful hatches is very high.

2. Materials and Methods

2.1 Collection and preparation of test materials

H. suaveolens were collected from Kurigram District of Northern Bangladesh in August 2015, (identified by the Department of Botany, University of Rajshahi, Bangladesh) and the study was accomplished in June 2017. Leaves and stems were collected separately and excess soil was removed from the roots without washing. Collected plant parts were chopped into small pieces, dried under shade and powdered using an electric blender (Excess heat during grinding was avoided). Weighed and placed in separate conical flasks to add Pet. ether, $CHCl_3$ and CH_3OH (Merck, Germany) (100gm × 300ml × 2 times) and placed on a shaker for 48h. Filtration was done by Whatman filter paper (made in USA) at 24h interval in the same flask followed by evaporation until the extracts were left. The extracts were then removed to glass vials and preserved in refrigeration at 4°C with proper labeling.

2.2 Collection and culture of the testinsect:

T. castaneum, used in the present experiment were taken from the stock cultures of the Crop Protection and Toxicology Laboratory, Department of Zoology, University of Rajshahi, Rajshahi-6205, Bangladesh; and reared as subcultures to be used in the experimentation. Adults of *T. castaneum* were reared in glass beakers (500ml) in a standard mixture of whole-wheat flour with powdered dry yeast (19:1) [29-30] in an incubator at 30°C ±0.5°C without light and humidity control for getting continuous supply of adults for the experiments.

2.3 Dose-mortality test on *T. castaneum* adults

The dose-mortality assay was conducted by the surface film method. Doses of Pet. ether and CH_3OH extracts of leaf and $CHCl_3$ extracts of root were selected through *Ad Hoc* experiments by putting 50mg of each of the extracts diluted separately in 1ml of solvent (as Pilot doses) to apply in 50mm Petri dishes and by increasing or decreasing the amount of extracts in repeated manner until a suitable mortality range was obtained. The concentrations used were 4.074, 3.565, 3.056, 2.547 and 2.037mg cm^{-2}. Each of the doses were diluted in 1ml of solvent, poured into a Petri dish and allowed to dry. Ten adult beetles were released in each Petri dish, and the experiment of all the doses for each of the extracts were set in 3 replicates. The mortality of the beetles was assessed after ½, 12, 24, 36, 48 and 60h of exposure.

2.4 Brine shrimp nauplii lethality test

Brine shrimp cysts were purchased from Dhaka and kept in aerated seawater at room temperature (25-30°C) and took 30-48h to give fresh nauplii. The series of concentrations were 33, 16.5, 8.25, 4.13 and 2.07ppm for all the extracts of *H. suaveolens*. Ten freshly hatched nauplii were added to each of the test tubes with different concentrations and observed mortality after 6, 12, 18, 24 and 30h of exposure. The data was then subjected to probit analysis [32, 33].

2.4.1 Statistical Analysis

The mortality (%) was corrected using Abbott's formula [31]:

$$P_r = \frac{P_o - P_c}{100 - P_c} \times 100$$

Where, P_r = Corrected mortality (%), P_o = Observed mortality (%), P_c = Control mortality (%). The data were then subjected to probit analysis by using a software developed at the University of Newscastle Upon Tyne, UK.

2.5 Repellent activity test against *T. castaneum* adults

The repellency test was adopted from the method (No. 3) of McDonald *et al.* [34] with some modifications [35-36]. Half filter paper discs (Whatman No. 40, 9cm diam.) were treated with the selected doses of 0.314, 0.157, 0.079, 0.039 and 0.019 mg cm^{-2} of all extracts and then attached lengthwise, edge-to-edge, to a control half-disc with adhesive tape and placed in the Petri dishes. Ten adult insects were released in the middle of each of the filter paper circles. The orientation was changed in the two remaining replicates to avoid the effects of any external directional stimulus affecting.

2.5.1 Observation and analysis of repellency data

Each concentration of each of the solvents was tested for five times. Insects that settled on each of the non-treated half of the filter paper discs were counted after 1h and then observed repeatedly at hourly intervals for five hours. The average of the counts was converted to percent repellency (*PR*) using the formula of [35, 37] : $PR = (N_c - 5) \times 20$, where, 'N_c' is the percentage of insects on the untreated half of the disc.

3. Results

3.1 Dose mortality effects:

The dose mortality assay of Pet. ether, $CHCl_3$ and CH_3OH extracts are represented in Table 1. The CH_3OH extracts of leaf offered highest mortality giving LD_{50} values ranged between 5.587 to 2.727 mg cm^{-2} followed by the Pet. ether extracts ranged between 4.322 to 2.647 mg cm^{-2} and $CHCl_3$ extracts of root ranged between 6.494 to 5.311 mg cm^{-2}.

Table 1: LD_{50} values of Pet. ether and $CHCl_3$ extracts of *H. suaveolens* against *T. castaneum* adults.

Plant parts	Solvents	LD_{50} (mg cm^{-2}) at different hours				
		12h	24h	36h	48h	60h
Leaf	Pet. ether	4.322	3.487	2.653	2.647	2.647
	CH_3OH	5.587	4.122	3.269	3.075	2.727
Root	$CHCl_3$	-	-	-	6.494	5.311

3.2 Lethality against Brine shrimp nauplii

The lethality against *A. salina* nauplii of the Pet. ether, $CHCl_3$ and CH_3OH extracts of leaf, stem and root of *H. suaveolens* are represented in Table 2. The highest and the lowest lethality have been observed for the Pet. ether extract of root (LC_{50} 0.620ppm) and the $CHCl_3$ extracts of root (LC_{50} 11.193ppm) after 30h of exposure. For the Pet. ether extracts of root the LC_{50} values ranged between 43.288 to 10.631ppm; followed by the $CHCl_3$ extracts of leaf the LC_{50} values ranged between 29.678 to 0.779ppm and for the $CHCl_3$ extracts of stem ranged between 47.594 to 4.362ppm; and in case of CH_3OH extracts of leaf and stem the LC_{50} values ranged between16.052 to 2.245 ppm and 31.794 to 12.044ppm respectively.

Table 2: LC_{50} values of Pet. ether, $CHCl_3$ and CH_3OH extracts of *H. suaveolens* against *A. salina* nauplii.

Plant parts	Solvent	LC_{50} (ppm) at different exposures 12h, 18h, 24h, 30h, Duration of exposure in hours			
		12h	18h	24h	30h
Leaf	$CHCl_3$	29.678	7.124	2.883	0.779
	CH_3OH	16.052	8.935	5.755	2.245
Stem	Pet. ether	-	6.316	3.242	0.620
	$CHCl_3$	47.594	8.016	6.184	4.362
	CH_3OH	31.794	15.645	15.158	12.044
Root	Pet. ether	-	43.288	20.467	10.631
	$CHCl_3$	121.211	21.503	21.063	11.193

3.3 Repellent effects on *T. castaneum*

All the Pet. ether, $CHCl_3$ and CH_3OH extracts of stem offered repellency at 5% level of significance ($P<0.05$) and the $CHCl_3$ and MeOH extract of root of *H. sauveolens* offered repellency at 1% ($P<0.01$) level and 5% level ($P<0.05$) of significance respectively against *T. castaneum* adults while the Pet. ether, $CHCl_3$ and CH_3OH extracts of leaves and the Pet. ether extract of roots didn't show any repellency at all (Table 3 and 4). According to the intensity of repellency the results could be arranged in a descending order of**:** Root ($CHCl_3$) > Stem (CH_3OH) > Root (CH_3OH) > Stem (Pet. ether) > Stem ($CHCl_3$).

Table 3: ANOVA results of repellency against *T. castaneum* adults by the Pet. ether, $CHCl_3$ and CH_3OH extracts of *H. suaveolens* L. (Poit.).

Types of extract		Sources of variation			F- ratio with level of significance		P- value	
Plant Parts	Solvents	Between doses	Between time intervals	Error	Between doses	Between time intervals	Between doses	Between time intervals
Leaf	Pet. ether	4	4	16	3.251	2.151	0.039	0.121
	CHCL3	4	4	16	1.088	0.140	0.396	0.965
	CH3OH	4	4	16	1.900	2.155	0.160	0.121
Stem	Pet. ether	4	4	16	15.908	0.674	1.96E-05	0.619
	CHCL3	4	4	16	12.796	0.946	7.34E-05	0.463
	CH3OH	4	4	16	17.937	7.535	9.21E-06	0.001
Root	Pet. ether	4	4	16	2.887	5.459	0.056392	0.006
	CHCL3	4	4	16	27.782	0.380	5.03E-07	0.820
	CH3OH	4	4	16	17.805	1.372	9.66E-06	0.288

Table 4: Significance level of the repellency against *T. castaneum* adults by the Pet. ether, $CHCl_3$ and CH_3OH extracts of *H. suaveolens* L. (Poit.)

Plant parts	Solvents	Between doses (df=4)		Between time interval	
		F- values	Level of significance	F- values	Level of significance
Leaf	Pet. E.	3.251	-	2.151	-
	$CHCL_3$	1.088	-	0.140	-
	CH_3OH	1.900	-	2.155	-
Stem	Pet. E.	15.908*	P<0.05	0.674	-
	$CHCL_3$	12.796*	P<0.05	0.946	-
	CH_3OH	17.937*	P<0.05	7.535	-
Root	Pet. E.	2.887	-	5.459	-
	$CHCL_3$	27.781**	P<0.01	0.380	-
	CH_3OH	17.805*	P<0.05	1.372	-

** = Significant at 1% level ($P<0.01$) * = Significant at 5% level ($P<0.05$); (-) = Not significant at any level

4. Discussion

The repellency of the essential oil of *H. suaveolens* (L.) leaves evaluated at 0.4-18.3mg/cm^2 concentrations using an area preference test against adults of four stored-product coleopteran pests and the percent repellency ranged from 20.0 to 94.7% at 5h against the test insects at the highest dose tested (18.3 mg/cm^2) [38]. The essential oils *H. suaveolens* showed repellent activity on *S. granarius* adults at the lowest dose 2×10^{-4} µl and highest dose 2×10^{-2} µl/cm^2 oil. *H. suaveolens* essential oil manifested a higher contact toxicity at lower doses (0.1µl/ insect) and determined 30% mortality of adults (Coleoptera: Dryophthoridae) [39]. *H. suaveolens* was also tested on *Callosobruchus maculates* (F.) (Coleoptera: Bruchidae) eggs to test the ovicidal activity, showing 100% of eggs' mortality. The powder of the leaves of *H. suaveolens* has greater insecticidal action against *C. maculates* [23]. Essential oil extracted from *H. suaveolens* was assayed [19] on the Coleopteran species, using kaolin powder as carrier to test the ovicidal activity, obtaining 100% of egg mortality. *H. suaveolens* seed extract also showed significant mortality of

adult beetles at 75mg/ml and 100mg/ml. The petroleum ether extract of *H. suaveolens* seeds showed insecticidal activities on second instar larvae of the Diamond back moth, *Plutella xylostella* (L.) [40-41]. On the contrary, *H. suaveolens* leaf powder was less effective in its insecticidal action against *C. maculatus* [42]. Brine shrimp lethality test is a broad spectrum bioassay capable of detecting cytotoxic effect and bioactive presence in an extract and dimethyl sulfoxide (DMSO) are widely used as dissolving agents in *Artemia salina* lethality test to screen the pharmaceutical properties. Brine shrimp lethality test was reported as a tool for the evaluation of cytotoxicity and pesticide activity of compounds and was considered to be a very useful preliminary tool for isolation of bioactive compounds from plant extracts. Toxicity of three *Hyptis* sp; *H. suaveolens, H. rhomboidea, H. brevipes* were tested while all showed significant toxicity with median lethal concentration (LC_{50}) values of 62.2±3.07µg ml^{-1}, 65.9±6.55µg ml^{-1} and 60.8±9.04µg ml^{-1}, respectively [43-44]. Rebelo [45] experimented that the oil and methanol extract of *H. crenata* showed a significant cytotoxicity while the LC_{50} values were 6.7±0.2µg ml^{-1} and 13.0±3.7µg ml^{-1}, respectively. The seeds of *H. suaveolens* displayed considerable general toxicity towards brine shrimps where the LC_{50} value of the extract was 80µg ml^{-1} [46].

5. Conclusion

The present study depicted that the extracts of *H. suaveolens* has potentials for insect suppression either by killing action or by repellency. The present data provided a support for the traditional use of the plant as an insecticidal remedy. However, further studies will be necessary to isolate and characterize the active principles which are responsible for the insecticidal effect and to understand its mechanism of action.

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