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EVALUATION OF VOLATILE CONSTITUENTS OF NEEM (*AZADIRACHTA INDICA* A. JUSS.) LEAF EXTRACTS AGAINST *CALLOSOBRUCHUS MACULATUS* (F.)

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Abstract: The effect of neem (*Azadirachta indica* A. Juss.) leaf volatiles was investigated against the cowpea bruchid, *Callosobruchus maculatus* (F.) to test fumigant toxicity, contact toxicity and repellency using olfactometer and choice chamber bioassays. *Callosobruchus maculatus* is more susceptible to contact toxicity than to fumigant toxicity. During fumigant and contact toxicity bioassays, the percentage mortality increased, while oviposition and the number of F_1 adults that emerged were significantly inhibited by neem leaf volatiles at concentrations higher than 23.0 g/l and 3.8 g/l respectively at 3 days after treatment. The LC_{50} values for contact and fumigant toxicity were 1.14 g/l and 8.45 g/l respectively. The results for the toxicity bioassays revealed that the contact effect of leaf volatiles on bruchids is higher than the fumigant effect. Choice chamber and olfactometer bioassays demonstrated the repellent effect of neem leaf volatiles against cowpea bruchids. At a dose of 160 mg, the mean number of bruchids that responded in the olfactometer and choice bioassays were 3.83 and 0.20 respectively. These findings indicate the suitability of neem leaf volatiles as a botanical insecticide and a repellent in the control of cowpea bruchids. Further studies on the active compounds in the volatiles and their effect on bruchids need to be investigated before recommending neem leaf volatiles as a suitable commercial product.

Key words: *Azadirachta indica*, Bruchid, *Callosobruchus maculatus*, cowpea, insect repellent, neem leaf.

INTRODUCTION

Cowpea [*Vigna unguiculata* (L.) Walp] is one of the major sources of protein in many developing countries, including Sri Lanka. Cowpea is a seasonal crop and farmers store the harvest for considerable periods of time.

The major pests encountered in stored cowpea in Sri Lanka are the bruchids, *Callosobruchus maculatus* (F.) (Southern cowpea weevil) and *Callosobruchus chinensis* (L.) (Adzuki bean weevil). Pulses stored for prolonged periods under high moisture and relative humidity become infested with the above two bruchid species. Infestation by bruchids is evident from the characteristic emergence holes in seeds.[1] Damage to seed by bruchids cause quantitative and quality losses,

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reducing the economic value and the viability of seeds. Caswell reported a 50 % loss in cowpea stored for 3-4 months due to infestation by *C. maculatus.*[2]

At present, in Sri Lanka and in many other countries, pulses such as cowpea and mungbean stored in warehouses are fumigated using chemicals such as methyl bromide and phosphine. Methyl bromide, now recognized as a potent ozone depletor, is most likely to be banned or at least be highly restricted by the year 2005. Phosphine (PH_3) is the most widely used gas fumigant for disinfecting stored grains; however, there are disadvantages such as aluminium hydroxide residues in grain and the need for sophisticated equipment to circulate the phosphine gas.[3]

Therefore, it is essential to develop safe, effective and simple alternative methods to control insect pests in stored grains. Plant extracts such as *Azadirachta indica, Zingiber perfurium* (Roscoe), *Eucalyptus camaldulensis, Tridax procumbens, Lantana camara* and *Cinnamomum camphora* have been used in the recent past to develop natural, biodegradable, environmentally friendly pesticides.[4,5,6] Although the efficacy of plant extracts are sometimes inferior to those of toxic, broad spectrum, synthetic insecticides, the degree of the reduction of the insect population is quite sufficient.[7]

It is a traditional practice of rural farmers in developing countries to mix dried leaves of neem with stored cowpea for protection against insects. Neem oil, neem seed kernel, neem fruit dust, neem seed powder and neem leaves are known to control several stored grain pests including *Callosobruchus* spp.[8] Neem seed oil at 10 % w/w protects cowpea seeds from bruchid infestation for at least 4 months.[9, 10] Effects of neem seed extracts against insects, include antifeedancy, reduced oviposition, egg hatch, emergence and direct lethality.[11,12] The use of powdered neem leaves in controlling *Rhyzopertha dominica* (F.) has been demonstrated.[11]

Even though insecticidal properties of the powder or the volatile constituents of neem seeds, and the powder of neem leaves have been tested against different stored grain pests, the insecticidal/ repellent nature of neem leaf volatiles have not been investigated against stored grain pests. The present study was conducted with the aim of identifying toxicity and insect repellant properties of volatile constituents of neem leaves against *Callosobruchus maculatus* in stored cowpea. Once the efficacy of neem leaf volatiles is identified, they could subsequently be formulated and marketed to manage weevils and other insect pests in cowpea, mungbean and other stored grains.

METHODS AND MATERIALS

Source of cowpea: Newly harvested, non-fumigated cowpea (*Vigna unguiculata*) seeds were collected from the Anuradhapura district in Sri Lanka. Cowpea thus obtained

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METHODS AND MATERIALS

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was used in the experiments and also for rearing bruchids.

Laboratory rearing of bruchids: Adults of *Callosobruchus maculatus* were obtained from the Entomology Division, Horticultural Crop Research and Development Institute, Gannoruwa, Sri Lanka, and the rearing of bruchids was carried out at the Department of Chemistry, University of Kelaniya, under laboratory conditions of 28 ± 3 °C and 75 ± 5 % r. h. using the method described by Bandara and Saxena.[13]

Clear, transparent 1 L plastic bottles (with 8-cm diameter screw lid) were used to breed bruchids. A hole (3 cm diameter) was made in the lid of each bottle and a fine gauze (25 mesh/ cm^2) was pasted over the hole from the inside of the lid to provide sufficient ventilation for the bruchids. Twenty-five pairs of adult bruchids were placed in each container, with 250 g of seeds. Under the above laboratory conditions, a new generation of bruchids emerged after 3-4 weeks from eggs laid by the introduced females. Five to ten hour old adults were obtained from the laboratory cultures.

Plant materials: Fresh leaves of *Azadirachta indica* A Juss. (Neem) were collected from plantations in the Anuradhapura and Gampaha districts. Seven hundred grams of fresh neem leaves were steam distilled for 3 hours and the aqueous distillate (500 ml) was extracted with CH_2Cl_2 (200 ml) to obtain the volatile constituents. The extract was concentrated to 20 ml using a rotavapour (BUCHI, Rotavopor, R-114, Waterbath, B-480, Beckman Instruments Inc, Westbury, N.Y. 11590) at 35 °C and dried with anhydrous Na_2SO_4. The solvent was evaporated by passing a N_2 steam. The Clavenger arm was not used to collect volatiles from neem leaf, as the percentage yield was very low (0.15 %) and there was no separable amount of volatiles collected after the distillation of 700 g of neem leaves. The modified Lickens and Nickerson apparatus was used with a cold-water condenser to check whether highly volatile constituents in neem leaf are lost during the steam distillation process. The GC analysis of the two extracts indicates that the chemical constituents of neem leaf were not significantly different from each other.

Toxicity of neem leaf volatiles on Callosobruchus maculatus

Bioassay for contact toxicity: Bioassays were performed using a modified method Huang *et al.*[14] Varying concentrations of neem leaf extracts (0.35 - 3.80 g/l) were prepared using ethanol as the solvent (100 µl). Extracts of known concentration were evenly applied to the inner surfaces of a glass vial (6.5 ml). The solvent was evaporated using N_2 gas. Five pairs of 5-10 hour old adult weevils were introduced into each vial and the caps were tightly screwed. After 24 hours, the weevils were transferred into clean vials with 50 untreated, fresh cowpea seeds and kept in the insect room under prevailing environmental conditions of 28 ± 3 °C and 75 ± 5 % r. h. The same procedure was carried out with vials coated with equal amount of

ethnol and vials without any treatment were considered as controls. Percentage mortality of bruchids and the number of eggs laid were recorded daily during the incubation period of 10 days. When the first generation adults (F_1) emerged, the numbers of F_1 adults were also recorded. Experimental design was a completely randomised design (CRD) with six-replicates.

Bioassay for fumigant toxicity: Fumigant toxicity was tested using a modified method described by Bandara and Seneviratne.[4] Filter paper discs (1.5 cm diameter) were impregnated with each concentration of neem volatiles dissolved in ethanol (100 µl) and the ethanol was allowed to evaporate for 10 minutes. The filter paper was then attached to the inner surface of the screw cap of a glass vial. Five pairs of 5-10 hour old bruchids were introduced, and the neck of the vial was blocked with a piece of metal mesh to prevent bruchids coming in contact with the treated filter paper. The vials were tightly screwed and kept for 24 hours at 28 ± 3 °C and 75 ± 5 % r. h. Thereafter, the bruchids were transferred into clean vials with 50 untreated, fresh cowpea seeds. Six different concentrations (0.75 - 23.00 g/l) of extracts were used to coat the vials. The same procedure was carried out with vials coated with equal amount of ethnol and vials without any treatment were considered as controls. The experimental design was a CRD with six replicates per treatment.

Percentage mortality of bruchids and the number of eggs laid were recorded daily during the experimental period of 10 days. When the first generation of adults (F_1) emerged, the numbers of F_1 adults were recorded.

Repellent action of volatiles

Bioassay with olfactometer: The repellent action of volatiles was tested using a modified Y-shaped olfactometer. The olfactometer consisted of 3 glass arms each 10 cm long having a diameter of 1 cm. At the point where the three arms meet, a fourth tube was connected (0.5 diameter and 3 cm long), which was directed to the vacuum pump through a 1 m long rubber tube (Figure 1).

Two perforated plastic containers (300 ml) were fixed to the ends of two arms of the Y-tube. A filter paper strip (2.5 cm x 5.0 cm) held by copper wire was placed in the center of each container. A known volume (10 - 160 µl) of the test sample was placed on the strip; the solvent was allowed to evaporate and the strip was then placed in the container (baited arm). A filter paper strip treated with an equal volume of ethanol was placed in the non-baited arm. The airflow through the olfactometer was regulated using a vacuum pump (Model DYNAX, Charles Austen Pump Limited, England). Subsequently, a round-bottomed flask (100 ml) with 50 unsexed adult bruchids was connected to the third arm of the olfactometer.

The experimental apparatus was kept in an upright position with the baited and non-baited arms directed towards a 40 W fluorescent light source. The arm connected to the round-bottomed flask was kept in the dark by placing it inside a cardboard box. The sequence of placing the test samples and ethanol in the two arms was interchanged randomly after replication. The bioassays were conducted between 7 a.m. and 11 a.m. After a duration of half an hour, the number of bruchids that had moved into each container was counted.

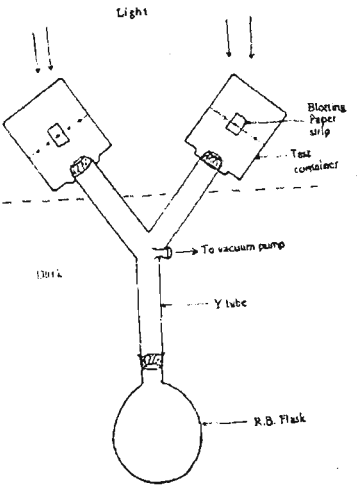

Figure 1: Set- up for the olfactometer bioassay

Five different doses (10 - 160 mg) of volatiles were tested separately with 6 replicates. The olfactometer was thoroughly cleaned using CH_2Cl_2 and detergent, and dried in subsequent replicates.

Bioassay with choice chamber: The choice chamber consisted of eight transparent plastic bottles (300 ml), placed equidistant to each other. The 8 plastic bottles were connected to a large transparent bottle (1 L) placed in the center of the chamber using glass tubing (diameter 1 cm and length of 8 cm). The experimental set-up was placed in a plastic basin (diameter of 42 cm and depth of 18 cm) and the sides covered with black paper.

In each experiment, five different doses (10 mg - 160 mg) of neem leaf volatiles and the solvent were used. Each volatile sample was placed on a filter paper strip (2.5 cm x 5.0 cm) and the solvent allowed to evaporate. Strips having different concentrations were placed in different bottles, each containing 50 cowpea seeds. Two of the bottles each containing 50 cowpea seeds without any treatment were considered as the controls. Two hundred and fifty adult bruchids (unsexed, 1-3 days old) were introduced into the central bottle, and the chamber was placed in a dark room. After 24 hours, the number of bruchids that

moved through the arms into the baited bottles and the number of eggs deposited in each bottle was recorded. Each experiment with 5 doses of the extract, ethanol and the control were replicated 5 times. The choice chamber apparatus was thoroughly cleaned using CH_2Cl_2 and detergent and dried in subsequent replicates.

Statistical analysis: Data obtained following bioassays for toxicity and repellency were analysed statistically using One-way ANOVA and the means compared using Tukey's Multiple Range Comparison tests. The results of the olfactometer and choice chamber experiments were analysed using Chi Squared tests. The LC_{50} values of neem leaf volatiles were calculated using Probit Analysis.

RESULTS

Contact and fumigant toxicity of neem leaf volatiles against *Callosobruchus maculatus*

Contact toxicity

The mortality of bruchids increased in the different treatments with the increasing concentrations of volatiles. Figures 1, 2 and 3 illustrate the effect of contact toxicity of neem leaf volatiles on *C. maculatus*. Percentage mortality in the solvent treated and control groups was 1.70 %. When the extract concentration was increased to 3.80 g/l, the mortality reached 100 %. The LC_{50} value for contact toxicity was 1.14 g/l. As shown in Figures 2 and 3, the mean number of eggs laid and the F_1 adults were significantly reduced with the increasing volatile concentrations ($p<0.05$). Egg laying and F_1 generation were completely inhibited at a concentration of 3.80 g/l. The results revealed that neem leaf volatiles significantly inhibited the number of F_1 adults ($p<0.05$) produced, than the number of eggs laid. The highest number of eggs and F_1 adults were observed in solvent treatments and controls and

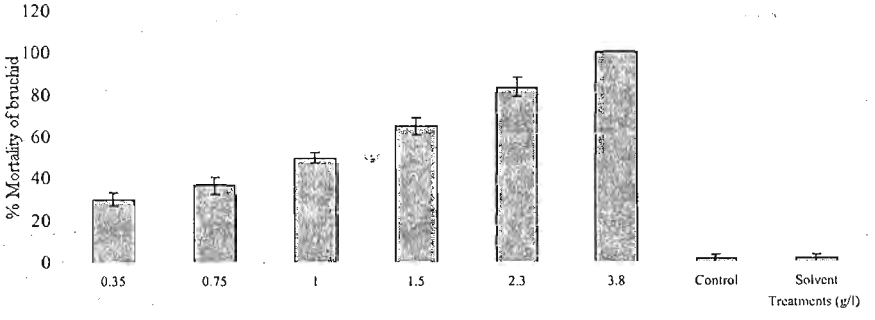

Figure 2: Percentage mortality of *Callosobruchus maculatus* on 3rd day after treatment following contact toxicity bioassay. Each bar represents the mean ± S.E. of 6 replicates (F= 118.93)

there was no significant difference between these two treatments ($p>0.05$), indicating that the use of ethanol as a solvent has no significant effect on bruchid behaviour.

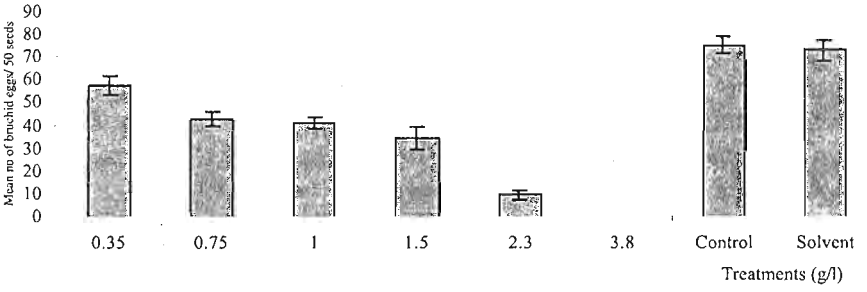

Figure 3: Number of eggs by *Callosobruchus maculatus* on 3rd day after treatment following contact toxicity bioassay. Each bar represents the mean ± S. E. of 6 replicates (F = 59.34)

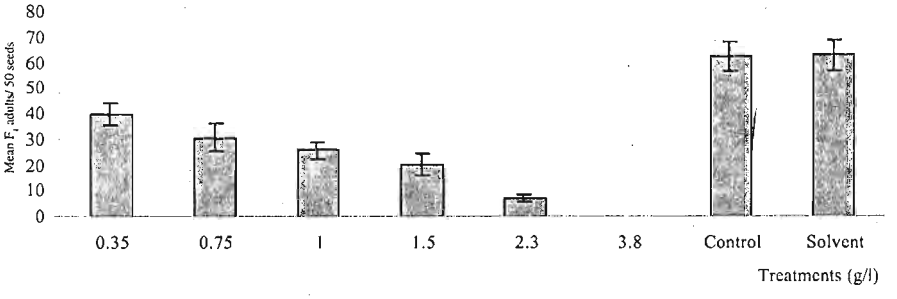

Figure 4: Number of F_1 adults of *Callosobruchus maculatus* following contact toxicity bioassay. Each bar represents the mean ± S.E. of 6 replicates (F= 27.98)

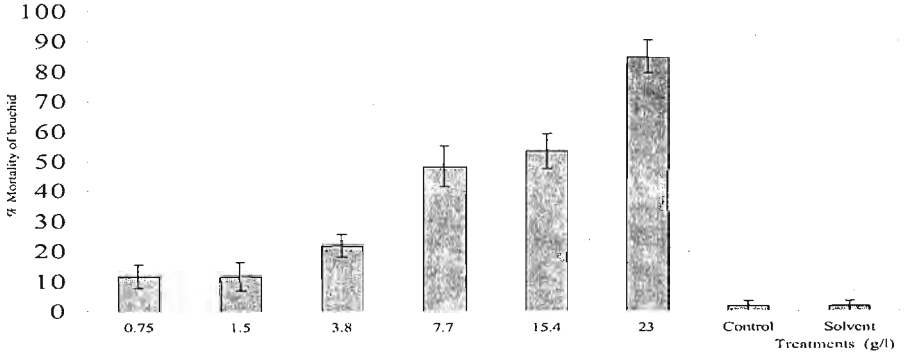

Figure 5: Percentage mortality of *Callosobruchus maculatus* on 3rd day after treatment following fumigant toxicity bioassay. Each bar represents the mean ± S. E. of 6 replicates (F= 46.04)

Fumigant toxicity

The mortality of bruchids increased with increasing volatile concentrations. Bruchid mortality reached 85 % at a concentration of 23.00 g/l (Figure 4). Figures 5, 6 and 7 show percentage mortality, the mean number of eggs laid and the mean number of F_1 adults. At concentrations higher than 1.50 g/l, the number of eggs laid and the number of F_1 adults were significantly low compared to the control and solvent treatment ($p<0.05$). The LC_{50} for fumigant toxicity test was 8.45 g/l. Significantly low mortality of bruchid (1.70 %) and a significantly higher number of eggs (73.0) were recorded in both the control and solvent treated samples.

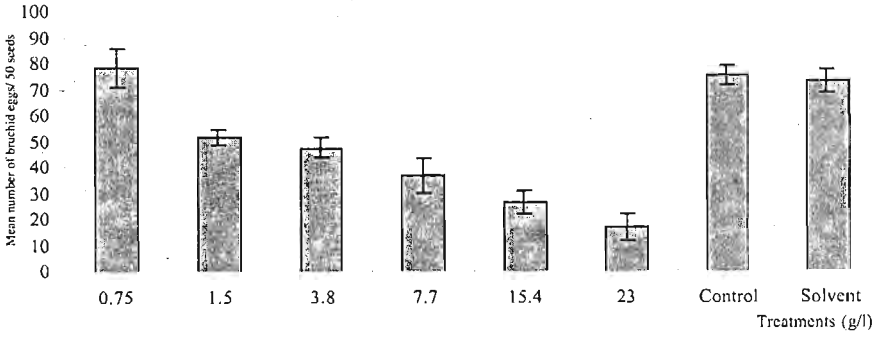

Figure 6: Number of eggs laid by *Callosobruchus maculatus* on 3rd day after treatment following fumigant toxicity bioassay. Each bar represents the mean ± S.E. of 6 replicates (F= 20.70).

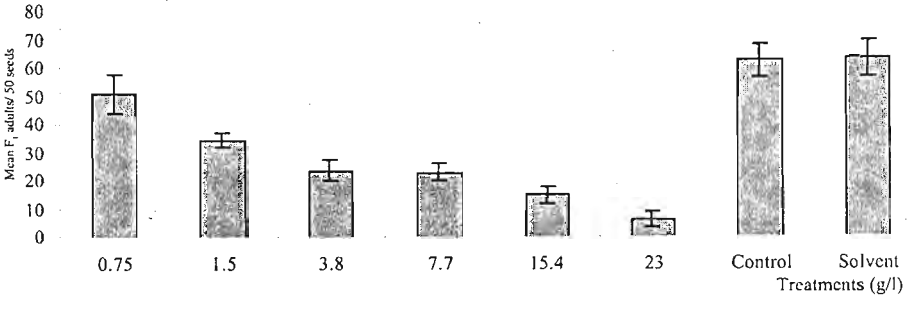

Figure 7: Number of F_1 adults of *Callosobruchus maculatus* following fumigant toxicity bioassay. Each bar represents the mean ± S.E. of 6 replicates (F= 21.94).

Table 1: Response of *Callosobruchus maculatus* to leaf volatiles of neem following olfactometer bioassay

Extract dose		Responding (mean ± S. E.)		
(mg)	N	Baited arm	Non-baited arm	Σ^2
10	6	8.50±1.92	7.33±1.49	0.08
20	6	9.33±1.58	10.83±1.91	0.11
40	6	8.66±1.20	18.16±1.46	3.40
80	6	6.33±1.08*	17.00±3.03*	4.88
160	6	3.83±1.01*	21.00±0.96*	11.87

Fifty insects were used in each experiment. Insects in each arm were counted 30 min after introduction. Except at 10, 20 and 40 mg doses, the mean numbers of insects in baited and non-baited arms were significantly different ($p<0.05$, 3.84* Significant at 5 %, Chi square test).

Table 2: Response of *Callosobruchus maculatus* to leaf volatiles of neem following choice chamber bioassay

Extract dose		Responding (mean ± S. E.)		Eggs laid in baited arm
(mg)	N	Baited container	Σ^2	(mean ± S.E.)
10	5	17.0±1.7	6.90	21.5±0.7[a]
20	5	8.4±1.5	17.11*	8.0±0.7[ab]
40	5	3.4±1.1	25.22	2.2±1.1[b]
80	5	1.2±0.9	29.29	0.0+0.0[b]
160	5	0.2±0.2	31.25*	0.0±0.0[b]
Solvent	5	30.4±8.4	0.42	135.6±11.3[c]
Control	5	34.8±8.2	0.81	141.6±8.0[c]

Two hundred and fifty insects were used in each experiment. Insects in each container were counted 24 hours after introduction. Mean response of the test insects was analyzed using Chi square tests ($p< 0.05$,14.07, Significant at 5%)

The mean number of eggs laid was analysed using ANOVA and Tukey's Multiple Range Tests. The number of eggs laid in each treatment (denoted by similar letters) was not significantly different ($p<0.05$).

Repellent effect of neem leaf volatiles against bruchids

Olfactometer

The movement of bruchids towards the baited arm was considered a positive response. Results presented in Table 1 show that the number of bruchids that moved to the treated arm was significantly low in the neem leaf volatiles treated arm compared to the non-baited arm. A higher number of bruchids had moved into the solvent treated arm (non-baited arm) indicating that use of ethanol has no repellent effect on bruchid behaviour. The number of bruchids that responded decreased from 8.50 to 3.83 when the concentrations of the neem leaf volatiles were increased from 10 to 160 mg. At doses higher than 40 mg, the repellent effect of neem volatiles was significantly higher compared to the control ($p<0.05$).

Choice chamber bioassay

Table 2 gives the results of the choice chamber bioassay where different doses of neem leaf volatiles, solvent and a control were used. Some degree of repellency was shown in all the treatments. The number of bruchids in the neem treatment was significantly different from the control and the solvent treatments ($p<0.05$). The response to the neem volatile treatment (40-160 mg) revealed that the number of bruchids that responded was 3.4-0.20, whereas in the solvent and the control treatments it was 30.4 - 34.8 % (not significantly different, $p>0.05$). When the volatile concentration was increased from 10 mg to 40 mg, the number of eggs laid decreased from 21.5 to 2.2 (at doses higher than 80 mg the number of eggs laid was zero). The number of eggs laid in the solvent treatment and control was not significantly different (135.6 and 141.6 respectively) ($p>0.05$) and these results were significantly different ($p<0.05$) from neem treated samples.

DISCUSSION

Semiochemicals such as plant volatiles have been used in the past as fumigants to control stored grain pests.[4,6,11] Several authors have reported the use of volatile constituents containing plant materials and vegetable oils to control *Callosobruchus spp.* in stored cowpea.[4,6]

Traditionally, for the control of stored grain pests, Indo-Pakistan farmers mixed 2 - 5 kg of dried neem leaves/100 kg grain[15] or soaked empty sacks overnight in water containing 2 - 10 kg of neem leaves / 100 L and dried these sacks before filling them with grain.[16] A variety of biologically active constituents, including triterpenoids azadirachtin, salanin and meliantriol are found in neem leaf, fruit, bark and seed.[17] These compounds reportedly control more than 100 species of insects, mites and nematodes.[18] Modes of control include antifeedant, growth regulatory, repellent or pesticidal action on larva and / or adult stages of these pests.[19] Neem leaves,[16] water

extract of crushed neem seeds,[20] neem oil,[21] and neem "cake,"[22] appear particularly promising for pest-control use in developing countries. Powdered neem seed kernels have been tested against adult *Rhyzopertha dominica,* adult *Sitophilus oryzae* and larva of *Trogoderma granarium* in wheat seeds. The results revealed that powdered neem seeds at the rate of 1 to 2 parts per 100 parts of seeds protect wheat seeds, from these insects for at least 10 - 12 months.[20]

During the present survey, toxic effects of neem leaf volatiles were investigated by monitoring the mortality, oviposition and F_1 adults of *C. maculatus*. The results of the bioassay clearly indicate that the contact toxicity of neem leaf volatiles is significantly high when compared to the untreated control. During the present study, the egg laying capacity and F_1 adults in the neem leaf volatile treatments decreased significantly. Over 85 % mortality was observed at concentrations higher than 2.30 g/l and 23.00 g/l during contact and fumigant toxicity bioassays. The present study also revealed that the neem leaf volatiles bring about contact and fumigant toxicity in bruchids at low LC_{50} values such as 1.14 g/l and 8.45 g/l.

The study of repellent effects of leaf volatiles using a Y shaped olfactometer and the choice chamber bioassay indicated that the repellent effect of neem leaf volatiles varies with the dose. The number of insects in all treatments was significantly low when compared to the control and the solvent treatments. At a dose of 160 mg, the number of bruchids that responded to the olfactometer bioassay and the choice chamber bioassays was 3.83 and 0.20 % respectively, indicating that the effect of the volatile is more pronounced after 24 hours than after 30 minutes. Jilani and Su and Jilani *et al.*, studied extracts of neem leaves and seeds against *Tribolium castaneum* (Herbst.), *Sitophilus granaries* (L.) and *Rhyzopertha dominica* (F.).[23,24] Stored rice and maize insects were used in their study and the findings are in agreement with the present study, although different insect species had been used.

Neem extracts have generally been found to be safe for mammals and the environment. For example, the incorporation of 20 % neem cake in their diet resulted in a higher growth rate in sheep.[25] Hence, contact, fumigant and repellent properties of neem leaf volatiles could be used to develop environmentally friendly pesticides from neem leaf volatiles.

Neem is used for medicinal purposes in Sri Lanka and in many other Asian countries. Botanicals with insecticidal or repellent properties are, therefore, an alternative to the expensive synthetic pesticides and fumigants. Volatile neem based repellent products, if developed and introduced to the local market, would no doubt be adapted by consumers owing to the environmentally friendly, medicinal properties of the products. It is evident by the present study, that the volatile constituents of neem leaf make it a safe control method against insect pests in stored cowpea and similar grains. Experiments are being conducted to identify the bioactive components

in neem leaf volatiles using gas chromatography. Thereafter, GC-EAG studies will be carried out to assess the response of bruchids to selective bioactive components of neem volatiles.

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