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Toxicity of Neem seed *Azadirachta indica* Juss (Meliaceae) different solvents extracts against cotton mealybug *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae) under Laboratory conditions

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Abstract
This study investigated the toxicity of neem treatments in comparison with Confidor 20% SL against cotton mealybug *Phenacoccus solenopsis* Tinsley. The experiments were laid out under completely randomized design (CRD) with six main treatments including untreated check under laboratory conditions at Agricultural research Institute Tarnab, Peshawar-Pakistan during 2012/2013. Results indicated that Confidor 20% SL and neem seed extracts (acetone and n-hexane extracts) caused 100 % mortality of *P. solenopsis* after 48 hrs and one week duration respectively. Over all after a week exposure, mortality trend was recorded as 100%, 97%, 88% and 67% by Neem seed n-Hexane extracts, Neem seed Acetone extracts, Neem oil and Neem seed water extracts respectively for 3% concentration. Similarly for 2% concentration it was recorded as 87%, 84%, 74% and 46% and for 1% concentration the mortality trend was noted as 68%, 62%, 56% and 29% in n-Hexane, Acetone, Neem oil and Neem seed water extracts respectively. The neem extracts and oil bioassay revealed that % mortality of *P. solenopsis* was dose and time dependent. These findings provide clear indication that n-Hexane and Acetone are good solvents for neem seed extracts preparation to be used against cotton mealybug in Pakistan. However further study needs to be conducted to know the compatibility of these extracts against the natural enemies in field as well as in laboratory conditions.

Keywords: Cotton mealybug, Neem seed extracts, organic solvents, toxicity

Introduction
Cotton mealybug *Phenacoccus solenopsis* Tinsley (Sternorrhyncha: Pseudococcidae) is a polyphagous insect feeding on a large variety of plant species including fruits, vegetables, crops and a few ornamentals belonging to Malvaceae, Solanaceae and Leguminaceae families [1]. In Pakistan, it was detected for the first time in 2005 on cotton and other cultivated plants in Punjab and Sindh provinces [2]. In addition to the direct losses by sucking the phloem sap, its feeding secretions (honeydew) cause additional losses to the plants by disturbing the photosynthesis activity and inducing fungal contaminations [3, 4].
Farmers mostly rely on insecticides for the control of insect pests including mealybug [5]. However, the frequent use of these fast-acting chemicals against the insects has generated a number of biological and environmental hazards including phytotoxicity, toxicity to non-target organisms, environmental degradation, health hazards to farmers and they also may accelerate development of the pest biotypes resistant to specific pesticidal chemicals [6, 7]. There is therefore a need to search for new materials and innovative approaches for effective and biodegradable pesticides for insect pest control.
This awareness has created worldwide interest in the development of alternative strategies, including the re-examination of using plant derivatives against agriculturally important insect-pests. Plant-derived materials are more readily biodegradable [8]. More than 2,400 plant species have been tested for pest-control properties [9, 10]
Neem tree, *Azadirachta indica* Juss (Meliaceae) is commonly known as neem or Dharak found in Indo-Pak subcontinent. In Pakistan, neem tree is mostly found in southern areas i.e. Sindh and Punjab regions. The tree contains bioactive ingredients and more than 100 chemical compounds have been identified so far, but the most effective bio active compounds are

azadiractin and salamin. The bioactive components are generally extracted in organic solvents particularly methanol, ethanol, acetone, hexane, petroleum ether, diethyl ether, chloroform or methyl chloride [10].

The insecticidal effect of neem has been proved to be efficient to control several insect pests of agricultural importance including more than 540 insect pests' species belonging to Lepidoptera, Diptera, Coleoptera, Homoptera and Hemiptera [11, 12]. In this context, the present study aimed to determine the toxicity of the mealybug *Phenacoccus solenopsis* (Sternorrhyncha: Pseudococcidae) to neem oil and other different solvent extracts under laboratory studies.

Material and Methods

1. Collection of experimental materials

1.1 Mealybug

For culture preparation, the mealybugs were reared according to the procedures of Venilla *et al*., (2010b) [13]. Sprouted potatoes were placed in plastic cages (70 cm length x 40 cm width x 40 cm height). China rose infested plant shoot containing female mealybugs (already confirmed to be *P. solenopsis* Tinsley through taxonomic keys of Hodgson *et al*. (2008)) [14] with nymphs sacs were put in the middle of the cage. After a while all the hatched and active crawlers started moving from the twig to the uninfested potato sprouts. After 40 minutes, the twigs were removed and the cage was fully closed. After 30 days, we obtained a full culture of the mealybug having each developmental stage and were used for all experiments [7]. The insects were kept at 25-32°C, 55-65% R.H. For each bioassay 20 adult mealybugs feeding on okra fruits in Petri dishes were used.

1.2 Plants

Neem seed and its oil were purchased from the local market (Sheikh Pansari Khyber Bazaar, Peshawar-Pakistan). The seeds were grinded and passed through a 25-30 mesh sieve to obtain a fine powder while the oil was directly used.

Okra fruits of 10-12cm length were got from the field of Agricultural research institute Tarnab Peshawar washed by water and air dried then used for experimentation in Petri dishes (14.5 cm dia).

2. Preparation of neem extracts

2.1 Aqueous extracts

Neem seed water extraction was done according to the protocol of Khattak *et al.* (2005) [15], in which about 100 g of grinded neem seed and 3 g detergent were mixed together and kept in 1 liter of water at 85 ^{0}C for 16 hrs. After 16 hrs the solution was squeezed and the rubbish was discarded. This solution corresponded to 10% of neem water extract and was stored in the fridge as stock solution while further concentrations of 1, 2 and 3% (v/v) were prepared for toxicity experimentation. Simultaneously, the neem oil solution was diluted to obtain 1, 2 and 3% (v/v) solutions.

3.2 Extracts in different solvents

Neem seed extraction was carried out in two different solvents: acetone and n-hexane. For each solvent, 50 g of seed powder was mixed with 300 ml of each solvent and stirred for an hour continuously and then each extract was allowed to stand for 72 hrs. After 72 hrs, each extract was mixed vigorously and then filtered, each filtrates was mixed with 100 ml of its respective solvent, shaked for 30 minutes and then filtered. Thereafter all the filtrates were combined and solvents were completely evaporated in a water bath at 65 °C. Then the final crude extracts in the form of a paste were mixed with a known volume of distilled water and mixed 2 ml of 1% detergent solution as emulsifier (Amaugo and Emosairue, 2003) [16] and filtered again and the resultant solutions were preserved as stock solution at 5-10 °C until used for further bioassays. For all experiments, three different concentrations of 1, 2 and 3% (v/v) of the stock solution were prepared by dilution.

4. Toxicity Bioassay

Neem treatments at 1, 2 and 3% (v/v) were applied on 20 adult mealybugs prior to any ovisac formation following the procedure of Rashid *et al*. (2012) [17]. Two okra fruit with mealybugs already feeding on them was fully sprayed by the neem extract solution to test through a hand atomizer in a way so that all the mealybugs could receive equal dose of the extract. While confidor 20% SL was applied as per company recommended dose and on the same above mentioned pattern. After each treatment application, the number of dead *P. solenopsis* was recorded after 1, 2, 3, 5, and 7 days and the % mortality was calculated in comparison with a control (i.e. Petri dishes where only water was sprayed on the mealybugs) by the Abbott's formula (Abbott, 1925) [18].

PM (Mortality (%) = (Mo – Mc)/Mo x 100

Where:

PM = Mortality (%)

Mo = Observed mortality (%) in neem treated Petri dishes

Mc = Control mortality (%) (in Petri dishes where only tap water was sprayed)

For each extract and each concentration, the experiments were replicated five times (n=5).

5. Statistical analyses

Data for all mentioned experiments if needed were log-transformed for normalization and then subjected to ANOVA through a Completely Randomized Design (CRD). Untransformed results are presented in the tables. Means were separated by LSD at 5% level. Statistical tests were done using STATISTIX 8.1 package**.**

Results and Discussion

Results in table-1 indicated that after 24 hrs of exposure among all treatments different concentration caused different mortality, Neem seed n-Hexane extracts 2 and 3 % concentrations caused similar mortality which was significantly different (F=54.6; p=0.000) from the rest of the treatments and untreated check. Similarly Acetone extracts 2 and 3% concentration caused similar effect, however n-Hexane in 1% concentration affected equal number of mealybugs as 3 % Acetone while 2 % Acetone affected similar number of mealybugs as 3 % Neem oil. However, no significant difference was observed between Neem seed water extract in 1 % concentration and control and both caused similar mortality which is the natural mortality.

After 48 hrs of exposure a significantly higher mortality (F=89.5; p=0.000) was recorded in 3 % concentration of n-Hexane followed by 3% concentration in Acetone treatments. While 1 and 2% concentrations of n-Hexane and Acetone caused similar mortality. Similarly, 1 % n-Hexane and Acetone caused similar mortality to 3 % Neem oil followed by Neem seed water extracts. Lowest mortality was recorded in control. Likewise after 72 hrs similar trend (F=102.0; p=0.000) was observed for n-Hexane and Acetone followed by Neem oil and during day 2 and 3, Neem seed water extracts also recorded as active and caused significantly different mortality than the control.

After day 5 again significantly highest mortality (F=128; p=0.000) was recorded in n-Hexane followed by Acetone and then Neem oil. Neem seed water extracts seemed to lose its

toxicity after 5 days because similar mortality was recorded in control as well. Table-1 also showed that after 7 days of exposure 3 % n-Hexane and Acetone caused significantly higher mortality (F=127.00; p=0.000) followed by Neem oil while Neem seed water extract with 1% concentration remained non toxic like control. Similarly, data revealed that n-Hexane extracts, Acetone extracts and Neem oil in 1% concentration acted like 3% Neem seed water extracts in terms of % mortality. Over all after a week exposure mortality trend was recorded as 100 %, 97%, 88% and 67% by Neem seed n-Hexane extracts, Neem seed Acetone extracts, Neem oil and Neem seed water extracts respectively for 3% concentration. Similarly for 2% concentration it was 87%, 84%, 74% and 46% and for 1% concentration this was noted as 68%, 62%, 56% and 29% in n-Hexane, Acetone, Neem oil and Neem seed water extracts respectively (Table-1). Moreover, data also indicated that mostly in all extracts in different concentrations highest % mortality in comparison with control was recorded on day 2 then gradually decreased except Neem oil where % mortality increased day by day and the highest was recorded on day 5 in all three concentrations (Table-2). Overall results indicated that the commercialized insecticide (Confidor) caused highest mortalities in 24 and 48 hrs of duration, which were significantly different from the rest of the treatments. Thereafter, neem seed n-hexane and acetone extracts caused the highest mortalities; and the efficiency of those extracts increased when their concentrations increased because significantly high mortality occurred in 3 % of all concentration used except water extracts. In addition, their efficiencies were optimal at day 2 after treatment and then decreased significantly from day 2 onward.

Azadirachtin is the most important limonoid in neem, which has repellent and insecticidal action against many insect species [19, 20]. Neem products have been reported to have toxic effect on insect pests and to prolong the insect's growth depending on the product concentration and the insect species [21, 17]. Sadre *et al*. (1983) [22] and Satti *et al*. (2010) [12] reported also detrimental effects on the behavior and the physiology of various insect groups including Homoptera and Hemiptera species. Neem oil and water extracts have been also used by various researchers under laboratory and field conditions against various insect species [23, 24]. In this study, we used neem seeds to extract their potential bioactive components using acetone and n-hexane as reported by Khan *et al*. (2015) [9] and Egwunyenga *et al*. (1998) [10].

In our study highest and significant mortality of *P. solenopsis* during 24 hrs and 48 hrs duration was recorded when a commercial insecticides Confidor 20 % SL was tested. Such results were also recorded by Aziz *et al*. (2013) [24] of the insecticide amidacloprid which is the active ingredient of Confidor, against wheat aphid *Sitobion avenae* during a week duration. Our results showed that neem seed extracts as well as neem oil have insecticidal activities against *P. solenopsis*. Neem oil has been shown to act as larvicide, oviposition inhibitor and growth regulator against several insect pests [25, 23]. The mealybug, *Planococcoides njalensis* (Dist.), and its attendant ants, *Pheidole megcephala* and *Camponotus* spp. were also adversely affected by neem extract and the commercial neem formulations [26]. In our study, the *P. solenopsis* mortality significantly increased when the acetone and n-hexane neem seed extracts were used. This is consistent with the results of Aziz *et al*. (2013) [24] who reported the highest mortality of *Sitobion avenae* F. on wheat using neem oil n-hexane. In conclusion neem seed extracts (acetone and n-hexane extracts) and neem seed oil can be used to control *P. solenopsis* in the field but further study needs to be conducted to know the compatibility of these extracts with development and behavior of the natural enemies particularly parasitoid i.e. *Aenasius bambawalei* Hayat used to control *P. solenopsis* in Pakistan.

Table 1: Toxic effect of different extracts used at different concentration on *Phenacoccus solenopsis* (% mortality, means[1] ± SE, n=5).

Treatments	Concentration (%)	% Mortality after (days)				
		1	2	3	5	7
Neem seed (acetone extracts)	1	14.00 ± 1.00gh	36.00 ± 2.45ef	49.00 ± 1.87ef	56.00 ± 1.87e	62.00 ± 2.54e
	2	23.00 ± 1.22de	50.00 ± 1.58d	64.00 ± 2.45c	75.00 ± 2.24c	84.00 ± 1.87b
	3	27.00 ± 1.22cd	60.00 ± 2.24c	73.00 ± 1.22b	91.00 ± 1.00 b	97.00 ± 1.22a
Neem seed (n-hexane extracts)	1	28.00 ± 2.55c	39.00 ± 2.91e	51.00 ± 2.44 e	57.00 ± 2.55 e	68.00 ± 2.55d
	2	39.00 ± 1.87b	51.00 ± 1.87d	67.00 ± 3.00c	76.00 ± 3.32 c	87.00 ± 2.00b
	3	43.00 ± 2.00b	65.00 ± 1.58b	81.00 ± 1.87a	98.00 ± 2.00a	100.00 ± 0.0a
Neem seed (water extracts)	1	11.00 ± 1.00hi	21.00 ± 1.00i	26.00 ± 1.00h	27.00 ± 1.22 h	29.00 ± 1.87h
	2	13.00 ± 1.22gh	23.00 ± 1.22hi	30.00 ± 1.58h	36.00 ± 2.44 g	46.00 ± 3.31g
	3	17.00 ± 1.22fg	27.00 ± 1.22gh	38.00 ± 1.22g	48.00 ± 2.00f	61.00 ± 2.92ef
Neem oil	1	14.00 ± 1.00gh	23.00 ± 1.22hi	36.00 ± 1.87g	49.00 ± 1.87 f	56.00 ± 1.00f
	2	17.00 ± 1.22fg	28.00 ± 1.22g	44.00 ± 1.87f	63.00 ± 1.22d	74.00 ± 1.00c
	3	21.00 ± 1.00ef	33.00 ± 1.22f	58.00 ± 1.22d	76.00 ± 1.87c	88.00 ± 2.55b
Confidor	Recommended dose	94.00 ± 2.92a	100.00 ± 0.00a	--	--	--
Control	No treatment	8.00 ± 1.22i	12.00 ± 1.22j	20.00 ± 1.58i	25.00 ± 1.58h	29.00 ± 1.87h

[1]Means in each column followed by the same letters are non-significant at 5% level, using LSD test following ANOVA.

Table 2: Percent mortality of *Phenacoccus solenopsis* in comparison with control in different neem extracts during a week exposure time.

Treatments	Concentration (%)	% Mortality (after hrs/days)				
		24hrs	48hrs	72hrs	5days	7days
Neem seed (acetone extracts)	1	42.86	66.67	59.18	55.36	53.23
	2	65.21	76.00	68.75	66.67	65.48
	3	70.37	80.00	72.60	72.53	70.10
Neem seed (n-hexane extracts)	1	34.48	69.23	60.78	56.14	57.35
	2	79.49	76.47	70.15	67.11	66.67
	3	81.39	81.54	75.31	74.49	71.00
Neem seed (water extracts)	1	27.27	42.86	23.08	07.41	0.00
	2	38.46	47.83	33.33	30.56	36.96
	3	52.94	55.56	47.37	47.92	52.46
Neem oil	1	42.86	47.83	44.44	48.98	48.21
	2	52.94	57.14	54.55	60.32	60.81
	3	61.90	63.64	65.52	67.11	67.05
Confidor	Recommended dose	91.49	88.00	--	--	--

Mortality (%) = $(M_o - M_c)/M_o \times 100$

M_o = Observed mortality (%) in treated Petri dishes.

M_c = Controlled mortality (%) in untreated (i.e. control) Petri dishes.

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