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INSECTICIDAL AND BACTERICIDAL EFFECTS OF ETHANOLIC LEAF EXTRACT OF COMMON OLEANDER, *NERIUM OLEANDER*

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Abstract: Crude ethanolic extract of *Nerium oleander* leaves was evaluated for its insecticidal and antibacterial activities. Stored grain pest, *Trogoderma granarium* and fruit fly *Drosophila rufa* larvae were used as insect model. The leave extract was prepared in ethanol. The recovered dried extract was 9.4g/100gram of fresh leaves. Dried extract was suspended in ethanol for exposing *T. granarium* and *D. rufa.* To check the contact effect of the crude extract, 2nd and 4th instar larvae of *T. granarium* and 3rd instar larvae of *D. rufa* were used. Three methods viz.., filter paper, residual film and feeding were used. Concentration of *N. oleander* extract used were 10, 25, 50 and 100mg. *T. granarium* larvae showed 10% mortality after 72 hours at 100mg dose level. In D. *rufa* 10% mortality occurred after 48 hours and 15% after 72 hours at 20mg dose level. Antimicrobial activity of N. *oleander* crude extract was evaluated by disc diffusion method against both Gram-positive and –negative bacterial strains. Nine doses of crude extract *i.e.,* 0.4, 0.5, 0.7, 0.8, 0.9, 1.00, 1.2 and 1.4 mg/disc for each strain. Four strains *i.e*., *Bacillus cereus*, cellulose producing *Bacillus*, *B. alves* and *Acinetobacter anitratus* showed growth inhibition zones at all above concentrations**.**

Keywords: Toxicity, oleandrin, stored grain pests, *Trogoderma granarium, Drosophila rufa,* antibacterial activity, *Escherichia coli, Pseudomonas* sp., *Bacillus cereus, Acinetobacter anitratus*

INTRODUCTION

Chemicals play important role in the biological activities of animals, plants and microorganisms. Indiscriminate and unplanned use of chemicals has resulted in acute and chronic poisoning,

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environmental damage and appearance of insecticide resistant pest strains. Unplanned use of synthetic pesticides to control the pest of agriculture and veterinary importance has posed a serious threat to the environment as well as human health in the 3rd world countries (Zahida and Masud, 1988). Plants normally grow on soil which is rich in microorganisms. To protect them from these invaders, plants produce a wide range of antibacterial and antifungal compounds either in a constitutive or an inducible manner (Cammue *et al*., 1992). Among these compounds several low molecular weight proteins or peptides with antibacterial or antifungal activity have been isolated from various plants (Hejgaard *et al*., 1992) and are believed to be involved in a defense mechanism. There are number of toxic plants, which contain variety of natural products with the ability to kill the pest at very reasonable and moderate dose (Wagner and Bladt, 1995; Bai *et al.,* 2007). Plant insecticides and microbial pesticides are highly effective, safe and ecologically acceptable (Matthews, 1999; Nathan and Kalaivani, 2005). Various novel botanical extracts have been investigated and are being used for their insecticides properties; these include extracts from neem tree, thyme, avocado etc (Mansoor *et al*., 2000).

Nerium oleander Linn, Apocynaceae is known as white oleander in the Mediterranean region and subtropical Asia is indigenous to the Indo-Pak subcontinent. Al-Yahya *et al*. (2000) stated that *N. oleander* has shown insecticidal properties. It has also been shown that *N. oleander* leaves possess antibacterial properties (Farnaz, 1996; Siddiqui *et al*., 1997). The metabolism of oleandrin, a cytotoxic component of *N. oleander* has been studied by Madden *et al*. (2002). Polysaccharides isolated from the flowers of *N. indicum* exert partial protection (Yu *et al*., 2007).

The aim of present study is to evaluate the bactericidal and insecticidal effect of indigenous plant extract of *N. oleander*.

MATERIALS AND METHODS

Preparation of Plant Extract

Fresh leaves (100g) of *N. oleander* plant was crushed and soaked in 400ml ethanol for three days with occasional shaking. After three days the extract was centrifuged at 5000rpm for 10 minutes. Clear supernatants were allowed to dry at 45°C in incubator for few days. The total dried crude

extract obtained in this way was 9.42g from 100g fresh leaves of *N. oleander.*

Rearing of Insect Culture

The stock culture of *Trogoderma granarium* was obtained from the Biochemistry and Toxicology Lab., of the Dept. of Zoology, University of the Punjab, Lahore which was refreshed on partially broken wheat in sterilized jam jars covered with muslin cloth. Experiment was performed on the 2nd and 5th instar larvae. Another insect, the fruit fly *Drosophila rufa* were collected from fruit shop of New Campus area of the university. Its fresh culture was also prepared in glass petri plates, with semi-solid corn meal-molasses-agar medium in them, covered with muslin cloth. The 3rd instar larvae of *D. rufa* were used for further studies.

Concentrations of N. oleander crude extract used

Four concentrations of *N. oleander* extract were prepared in ethanol for both insects, separately following getting some clues about their tolerance. For *T. granarium* 10, 25, 50 and 100mg/ml concentrations were used. Similarly, for *D. rufa,* 5, 10, 15 and, 20 mg of *N. oleander* extract per gram of feed were prepared.

Determination of Insecticidal Activity of N. oleander Extract

For evaluation of LC_{50} of *N. oleander* extract against *T. granarium,* three methods, *i.e.,* filter paper method, residual film method and feeding method were used in sterilized glass Petri plates.

For exposing 3rd instar larvae to *N. oleander* leaf extract, corn meal-molasses-agar medium was prepared (Strickberger, 1962). Four concentrations of feed having 5, 10, 15 and 20mg of crude oleander extract per gram of feed were prepared in petri plates. Two controls were prepared, one without any extract and second with only solvent (ethanol). All plates were kept in incubator at 45°C overnight to evaporate the ethanol. After 24 hours 10, 3rd instar larvae were introduced into each plate. Mortality counts were recorded after 24, 48 and 72 hours of exposure to extract. Three replicates were used for each concentration.

Determination of Antibacterial Activity of N. oleander Extract

Eight bacterial species were used to test antibacterial activity of the extract namely; *Bacillus cereus*, *Bacillus* (cellulose producing sp), *B. alves, B. coagulans, Acinetobacter anitratus, Pseudomonas aeruginosa, Escherichia coli* and *Salmonella sp.* were obtained from the Lab stored culture of the department. The bacterial isolates were cultured in nutrient broth which were diluted 10 and 100 folds to spread on agar plates and incubated for 24 hours at 37°C. To evaluate the antibacterial activity of ethanolic crude extract of *N. oleander* disk, diffusion method was used (Murra *et al*., 1995). Two controls were also used, one control disk with ethanol and in second without any application *i.e.,* simple disk of filter paper were used. These disks after drying were applied on inoculated agar medium and it was incubated at 37°C for 24 hours. Antimicrobial activity was evaluated by measuring the zones of inhibition against each test organisms. Each assay in this experiment was repeated twice.

RESULTS

Insecticidal Activity of N. oleander Extract

Acute toxicity of crude extract of *N. oleander* was determined on the larvae of stored grain pest, *T. granarium* and fruit fly, *D. rufa* following exposing them to its various concentrations for 24, 48 and 72 hours in different experiments.

During this study, the contact action of crude extract was not found as in both (filter paper method, and residual film method) experiments indicative of contact action, no significant mortality occurred in all tested concentrations up to 72 hours in larvae of *T. granarium* and *D. rufa*. Similarly in feeding experiments, no significant mortality was found in 2nd and 5th instar larvae of *T. granarium* after 24 and 48 hours of exposure to the given concentrations of crude extract (Table I). However, after 72 hours exposure 10% mortality occurred in both larval instars at 100mg dose level. In 3rd instar larvae of *D. rufa,* 10% mortality was observed after 48 hours of exposure to crude oleander extract at highest given (20mg/g) concentration. The highest mortality was 15% in *D. rufa* at above concentration when exposed for 72 hours to crude oleander extract in feed (Table I).

Table I: Effect of crude leave extract of *Nerium oleander* on *Trogoderma granarium* (100 mg) and *Drosophila rufa* (20mg) larvae in feed.

S.#	Insect name & Stage	Mortality (%)		
		24 hours	48 Hours	72 hours
1	***Trogoderma granarium*** **2nd instar larvae**	--	--	10
2	***Trogoderma granarium*** **5th instar larvae**	--	--	10
3	***Drosophila rufa*** **3rd instar larvae**	--	10	15

Antibacterial Activity of N. oleander Extract

Antimicrobial potential of *N. oleander* crude extract was tested on both Gram-positive and Gram-negative bacterial strains as given in previous section. The antibacterial activity of the extracts was quantitatively assessed by measuring the inhibition zone around the discs containing crude extract of *N. oleander* applied in different concentrations. The results and screening of antimicrobial activity of *N. oleander* leaf extract against Gram positive bacteria indicated that *Bacillus cereus*, cellulose producing *Bacillus* sp., and *B. alves* showed growth inhibition zones at all tested concentrations of the above crude extract. On the other hand *B. coagulans* showed no inhibition zones at low doses but it gives inhibition zones at high concentrations *i.e.,* 1, 1.2 and 1.4mg/disc. The maximum zone of inhibition shown by cellulose producing *Bacillus* sp., *Bacillus cereus*, and *B. alves* was 19.3, 15 and 11.7mm, respectively at 1.4mg/disk concentration in Gram positive bacterial strains (Table II).

All tested Gram positive bacteria used in this study showed susceptibility against the ethanolic leaves extract of *N. oleander.* The results obtained showed that the extract exhibited moderate activity against *B. alves* and weak activity against *B. coagulans* (Table II).

Table II: Effect of crude extract of *Nerium oleandar* on Gram +ve bacterial growth in term of zone of inhibition (mm)

Bacterial isolates	Concentration of *Nerium oleander* extract (mg/disk)								
	0.4 (n=3)	**0.5 (n=3)**	**0.6 (n=3)**	**0.7 (n=3)**	**0.8 (n=3)**	**0.9 (n=3)**	**1.0 (n=3)**	**1.2 (n=3)**	**1.4 (n=3)**
Bacillus cereus	11.0^{a} ±0.00	11.7^{a} ±0.33	12.3^{a} ±0.33	12.7^{a} ±0.33	12.7^{a} ±0.33	13.0^{a} ±0.00	13.6^{b} ±0.33	14.7^{b} ±0.33	15.0^{b} ±0.00
Cellulase producing *Bacillus*	12.0^{b} ±0.00	13.3^{b} ±0.33	15.7^{b} ±0.33	15.0^{b} ±0.00	16.3^{b} ±0.33	16.3^{b} ±0.67	16.7^{c} ±0.33	17.7^{c} ±0.33	19.3^{c} ±0.33
Bacillus coagulans	R	R	R	R	R	R	11.0^{a} ±0.00	11.3^{a} ±0.33	11.7^{a} ±0.33
Bacillus alves	11.0^{a} ±0.00	11.3^{a} ±0.33	11.7^{a} ±0.33	12.3^{a} ±0.33	12.0^{a} ±0.33	12.7^{a} ±0.33	11.0^{a} ±0.00	11.0^{a} ±0.00	11.0^{a} ±0.00

Values not sharing common alphabets are significantly different from each other in respective column. Single factor of variance at $p \leq 0.05$

Table III: Effect of crude extract of *Nerium oleandar* leaves on Gram -ve bacterial growth in term of zone of inhibition (mm)

Bacterial isolates	Concentration of *Nerium oleander* extract (mg/disk)								
	0.4 (n=3)	**0.5 (n=3)**	**0.6 (n=3)**	**0.7 (n=3)**	**0.8 (n=3)**	**0.9 (n=3)**	**1.0 (n=3)**	**1.2 (n=3)**	**1.4 (n=3)**
E. coli	R	R	R	R	R	R	R	R	R
Acinetobacter anitratus	12.70^{ac} ±0.33	13.3^{a} ±0.33	12.3^{abc} ±0.33	11.7^{b} ±0.33	12.3^{abc} ±0.33	12.0^{abc} ±0.57	11.3^{cb} ±0.33	11.0^{b} ±0.33	13.0^{a} ±0.33
Pseudomonas aeruginosa	R	R	R	R	R	R	R	R	R
Salmonella	R	R	R	R	R	R	R	R	R

Values not sharing common alphabets are significantly different from each other in respective column. Single factor of variance at $p \leq 0.05$

In case of Gram negative bacteria *Pseudomonas aeruginosa, Escherechia coli* and *Salmonella* sp. showed resistance against the extract. However *Acinetobacter anitratus* showed zones of inhibition against all the concentrations *i.e.,* 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, 1.0, 1.2 and 1.4mg/disc concentrations of the extract. The maximum zone of inhibition was 13mm at 1.4mg/disc concentration in Gram-negative strains (Table III).

DISCUSSION

Extensive use of chemicals is posing severe threats to our environment including animal and plant life. Since many decades scientists are working for the development of relatively safe and non-persistent chemicals for the control of diseases and pests. To achieve this end, researchers are looking for plant metabolites, which have been showing any potential as a biocidal agents. In the present study *N. oleander* leaves crude ethanolic extract was evaluated for its insecticidal potential against 2^{nd} and 5^{th} instar larvae of *T. granarium* and 3^{rd} instar larvae of *D. rufa* after 24, 48 and 72 hours exposure. Antibacterial activity was tested against some laboratory preserved, Gram-positive and Gram-negative bacterial strains.

During this study, mortality was not observed in both types of larvae in contact experiments. The results showed that *N. oleander* ethanolic extract is totally ineffective as contact poison against the larvae of *T. granarium* and *D. rufa.* However, feeding experiments, with both insect larvae showed that with low concentrations of *N. oleander* extract no significant mortality was noticed after 24, 48 and 72 hours of exposure. At higher doses (100mg concentration), 10% mortality was observed in *T. granarium* larvae in both 2^{nd} and 5^{th} instar larvae. In feeding experiments with *D. rufa,* 10% and 15% mortality of larvae was found after 48 and 72 hours of exposure to *N. oleander* leaves extract at 20mg dose level under the present experimental conditions. This might be due to the effect that insects and larvae have thick cuticle in their body and extract might not penetrate through the cuticle. Moreover, the Insect larvae have the ability to store lot of fats in the body and utilize it during the unfavorable conditions. The results revealed that *T. granarium larvae* were more resistant to crude ethanolic extract of *N. oleander* as compared to *D. Rufa* larvae. Literature review reveals that *N. oleander* exhibits insecticidal activity against insects. Amr *et al*. (2001) reported insecticidal activity of *N. oleander* leaf extract

against the green lacewing, *Chrysopa carnea* steph. Ethanolic extracts of *N. oleander* showed insecticidal and feeding deterrent properties against rice weevil, *Sitophilus oryzae* infesting stored wheat and rice (Satpathi *et al*., 1992). Exposure of leaf moths (*Spodoptera littoralis*) with *N. oleander* extract induced remarkably increase mortality (Hossain *et al*., 1996).

Antimicrobial activity of different plant parts of the *N. oleander* is well documented (Sawhney, 1978; Srinivasan *et al*., 2001). To test antimicrobial activity, both Gram positive and Gram negative bacteria were used. Results in the present study indicated that extract showed antibacterial activity in all tested Gram positive bacterial strains used in this study. *Bacillus cereus* showed remarkable growth inhibition against the extract which was in accordance with previous study of Nimri *et al*. (1999), which showed 13mm zone of inhibition with 25mg/disk concentration of *N. oleander* extract.

In case of Gram negative bacterial strains used during this study only *Acinetobacter anitratus* showed inhibition both at high and low concentrations. All other tested bacterial strains (*E. coli, P. aeruginosa* and *Salmonella* sp.) showed no inhibition zones against *N. oleander* extract which was in accordance with other studies (Nimri *et al*., 1999). In present study *Pseudomonas aeruginosa* showed zone of inhibition against *N. oleander* extract which was in contrast to the findings of (Nimri *et al.,* 1999). This difference in tolerance against *N. oleander* extract may also be attributed to the differences in the bacterial physiology and cell wall structure in Gram- positive and -negative bacterial strains.

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