Journal of Entomology and Zoology Studies
Available online at www.entomoljournal.com

E-ISSN: 2320-7078
P-ISSN: 2349-6800
JEZS 2016; 4(5): 956-959

Received: 09-07-2016
Accepted: 10-08-2016

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Assessment of antifeedant potential of azadirachtin, a plant product of *Azadirachta indica* against *Papilio demoleus* L. (Lepidoptera: Papilionidae) larvae

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Abstract
The lemon butterfly *Papilio demoleus* is one of the economically important pests whose larval forms cause serious damage by both wild and cultivated species of citrus during the later stages of their development. Nowadays increasing efforts are made to develop environmentally safer pest control methods, one such method can be the use of natural antifeedants. Azadirachtin a plant product and secondary metabolite isolated from *Azadirachta indica* was evaluated their antifeedant activity against fourth instar larvae of *Papilio demoleus,* following no-choice leaf bioassay method. The feeding deterrence of Azadirachtin was evaluated at 200, 150, 100, 50 ppm concentrations for 24 hrs. and 48 hrs. exposure. Results showed that the Azadirachtin treated larvae showed maximum antifeedant activity of 86.28% and 70.43% at 200ppm concentration The antifeedant activity were increased with increasing concentration. Azadirachtin could also be considered for use in the management of pests.

Keywords: Antifeedant activity, plant products, *Papilio demoleus*, Azadirachtin, *Azadirachta indica*

1. Introduction
Citrus is one of the important commercial fruit crops of the world and it is prominently cultivated in the tropical and subtropical regions. It is found in much of southern Asia from Saudi Arabia east to Australia, New Guinea and the Philippines. Recently, it has been introduced into the Caribbean [1].

Citrus industry is the third largest fruit industry in the world occupying six per cent of the total area under various fruits. Sweet orange grown in an area of 204.1 lakh hectares with an annual production of 30.61 lakh tonnes of fruits and productivity of 15 MT/Ha in Andhra Pradesh and Telangana states (Indian Horticulture Database - 2013). In Indian more than 250 insect pests are attacked on citrus at all stages of growth right from buildings and seedlings in nurseries. Out of these, 165 species are causing an estimated loss of 30 per cent in yields [2]. Among the various insect pests that attack citrus, the citrus butterfly, *P. demoleus* Linnaeus is a regular pest in nurseries, young seedlings, and on new flush of full grown up tress. The caterpillars feed voraciously and cause extensive damage to nurseries and young seedlings leaving behind midribs only. Severe infestation results in defoliation of the tree [3] and leads to retarded plant growth and decreases fruit yield [4]. Information on the morphometry and biology of citrus butterfly on Sweet orange will be useful to evolve effective management strategy, against citrus leaf miner [5].

Currently, synthetic pesticides are used in controlling crops insect pests and have usually provided strong defense against insect pests. Worldwide, it is estimated that approximately 1.8 billion people engage in agriculture and most of them use approximately 5.6 billion pounds of synthetic pesticides to protect the food and commercial products that they produce [6]. Synthetic pesticides and their metabolites have high persistence in soil, water and crops themselves and therefore affect environment and the health of human being during preparation, application and the consumption of crops. These constraints of synthetic pesticides have led to increased interest in the application of botanical pesticides for crop protection in the field and during storage [7].

Botanical pesticides have long been publicized as an attractive alternatives to synthetic insecticides for pest management since they pose either little or no threat to the environment, to ecosystems and to human health. Mankind has used plant extracts for thousands of years to the prevent diseases, treatment of disease, as insecticides to control microbial growth, weeds and many more functions [8]. Therefore, many plants which were used for medicinal purposes locally, also demonstrated potential as insect control agents. Normally, botanical pesticides comprise a mixture of bioactive compounds with many have advantages in terms of efficacy and short life span, no resistance is developed in pests and pathogens [9]. The botanical pesticides are generally pest-specific and are relatively harmless to non-target organisms including man and natural enemies of insect pests, and environmentally eco-friendly, degrade rapidly in sunlight, air, and moisture, so they are less persistence in the environment, and are rapid in action to the insect pests, no adverse effect on plant growth, seed viability and cooking quality of the grains and are less expensive and easily available in the farmers natural environment [10]. Now it becomes necessary to search for the alternative. Therefore the use of plant products has been recommended ever more as a suitable alternative of plant protection with low negative risks [11]. In the present study *P. demoleus* L. was selected as a test species to evaluate the antifeedant activity by using the plant product azadirachtin from *Azadirachta indica*

2. Materials and Methods

2.1 Test insect

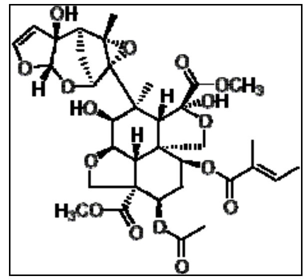
The lemon butterfly, *P. demoleus* is a key pest of citrus in India. Whose larval forms cause serious damage to citrus family by devouring the foliage heavily during the later stages of their development It feeds voraciously on vegetative growth of citrus plants throughout the year. It is most destructive to citrus seedlings as well as new flushes [12]. The *Papilio demoleus*, is also called as the citrus Swallowtail and it belongs to the family Papilionidae.

2.2 Collection of larvae and maintenance

Larvae of different stages of *P. demoleus* were collected from Citrus fields in Nalgonda (District) of Telangana State, India from 2007 – 2010 period, were reared in the laboratory at 27 ± 2 °C temperature and 70% ± 5% R.H., in the glass petri plates, kept in a wooden box (20cmx20cmx20cm), with wire-netted sides and top. The larvae of the species were daily fed on fresh leaves of lemon. The completely grown fourth instar larvae were sorted out and placed in a separate glass dish at room temperature for the experiment.

2.3 Plant material

Azadirachta indica tree is in Meliaceae family the active compound in the neem is azadirachtin which is found in the leaves, and also concentrated in the seeds. It is a bitter, complex chemical compound which belongs to the limonoid group, molecular formula is $C_{35}H_{44}O_{16}$ and it show strong biological activities among various insect pests, this compound is a feeding deterrent and growth regulator [13]. This compound can affect about 200 species of insects by acting as antifeedant and growth disruptor. Azadirachtin has a toxicity and fascinating effect on insects [14]. It has very low toxicity to mammals so practically non-toxic to mammals and also has been reported to be non-mutagenic [15]. Azadirachtin has been found to degrade rapidly under environmental factors such as UV radiation in sunlight, heat, air moisture, acidity and enzymes present in foliar surfaces. Therefore, there is a need to use azadirachtin as environmentally compatible insecticides, with selective toxicity to targeted pests, low toxic to plants and mammals and environmental friendly desired stability.



Chemical structure of Azadirachtin

2.4 Preparation of Test Compound

Acetone was used as the solvent in preparing the test solutions, since the solubility of the test compounds was very high in acetone. 1% stock solution was prepared using acetone and 200ppm, 150ppm, 100ppm and 50ppm concentrations of azadirachtin were prepared from the stock by dilution method.

2.5 Experimental Procedure: Antifeedant activity evaluation method

Antifeedant activity of the Azadirachtin a pure compound isolated from *Azadirachta indica* was assayed against fourth instar larvae of *P. demoleus* using a leaf disc in no-choice bioassay method. The experiment was done in Petri dishes. A moist paper towel was kept at the bottom of each container in order to maintain relative humidity and to keep the lemon leaves fresh. The observations were recorded after 24 hrs. and 48 hrs. after treatment. Fresh, tender leaf discs (36.5 sq.cm) of lemon were used as food. Concentrations of the pure compounds such as 50ppm, 100ppm, 150ppm and 200ppm were prepared. The leaf discs treated with acetone were used as a negative control, and those treated with azadirachtin were used as a positive control. Single *P. demoleus* fourth instar larva was introduced into the Petri dish. Parallel controls of *P. demoleus* maintained with similar leaf discs. Ten such Petri dishes were taken for each experiment. The experiment was conducted under laboratory conditions of 28 ± 1°C, 14:10 L:D photoperiod, and 75 ± 5% relative humidity. Progressive consumption of leaf area by the larvae was recorded after
24 hrs. 48 hrs. using a LI-COR-3100 leaf area meter.
The area of the leaf discs was measured by the leaf area meter before treatment as well as 24 hrs. and 48 hrs. after treatment, percentage of protection was able to be calculated and inserted into the formula. The percentage of antifeedant activity was calculated using the formula [16]

$$\text{Antifeedant activity} = \frac{\text{Leaf area consumed in control} - \text{Leaf area consumed in treated leaf}}{\text{Leaf area consumed in control} + \text{Leaf area consumed in treated leaf}} \times 100$$

2.6 Statistical analysis

Antifeedant activity was analyzed using one way ANOVA. Significant differences between treated and control groups were determined. The results are expressed as Mean ± SD and data was statistically analyzed with the level of significance set at $p<0.05$ using SPSS software.

3. Results and Discussion

Botanical insecticides are an excellent alternative to synthetic pesticides to reduce negative impacts on human health and environment. One possible way to reduce the high consumption of synthetic insecticides is the application of botanical insecticides, which are considered to be environmentally and medically safe [17].

Table 1: Undamaged leaf area (sq.cm) and antifeedant activity (%) with different concentration treatments of azadirachtin

Conc. in ppm	No of Insects	Mean ± SD After 24 hrs.	Mean ± SD After 48 hrs.	Antifeedant activity (%) after 24hrs.	Antifeedant activity (%) after 48hrs.
200	10	33.37 ± 1.04*	27.15 ± 0.74*	86.28	70.43
150	10	28.24 ± 0.78*	24.60 ± 0.80*	73.67	62.13
100	10	25.17± 0.76*	19.75± 0.93*	65.83	52.08
50	10	21.61 ± 0.91*	15.51± 0.99*	58.73	43.28
Control	10	14.05 ± 0.92*	09.73 ± 1.14*	---	---

Mean and SD values are significant at $p < 0.05$.

According to the results various concentrations that was used for an antifeedant experiment were below the mortality concentration, so the dose that were used was an efficient dose without killing the insect but had an antifeedant activity

Plant kingdom is a rich source of biologically active natural chemicals. More than 10,000 secondary metabolites have been chemically identified from the plant kingdom. Neem products such as neem seed kernal extract, neem leaf extract, neem oil and neem cake are widely used as insect repellents and insecticides against a vast number of pests [18]. Azadirachtin, is the most potent natural insect antifeedant. According to various studies higher antifeedant index normally indicates decreased rate of feeding. In the present study, the extract azadirachtin was applied on the leaf surface using different concentrations such as 200ppm, 150ppm, 100ppm and 50ppm for 24 hrs. And 48 hrs. Duration. The results of present study showed 86.28% and 70.43% antifeedant activity at 200ppm concentration after 24 hrs. and 48 hrs. Exposure, respectively. The protected leaf area at 200 ppm was 33.37 ± 1.04*sq.cm and 27.15 ± 0.74*sq.cm after 24 hrs. and 48 hrs, respectively. The antifeedant activity varied significantly based on the concentration and type of plant product used for the formulations. Antifeedant is a chemical that inhibits the feeding without killing the insect directly, while the insect remains near the treated foliage and dies through starvation [19]. Similar results were observed in *Spodoptera mauritia*, *Ephestia kuehniella Zell* and *Manduca sexta* when subjected to azadirachtin [20].

In our study, an additive effect in the antifeedant activity was recorded in the azadirachtin treatment, which was significantly high when treated with 150ppm concentration the antifeedant activity was 73.67% and 62.13% for 24 hrs. and 48 hrs. Exposure and the protected leaf area was 28.24 ± 0.78* sq.cm and 24.60 ± 0.80* sq.cm after 24 hrs. And 48 hrs., respectively. This result coincided with the findings of Kumar [21] who reported that methanolic extracts of neem and karanj oils showed increase in the activity against *Tetranychus* species they further stated that the neem and karanj combination showed synergistic activity against aphid *Macrosiphoniella sanborni.* Schoonhoven [22] were also in agreement with the present work on feeding deterrency and repellency of neem antifeedant property of neem leaf extract on various agricultural pests and found the highest antifeedant effect shown by neem therefore their work also supported present investigation.

This study indicated that the *P. demoleus* larvae is determined not only by feeding inhibition, as indicated by reduced food uptake at 100ppm concentration 65.83% and 52.08% antifeedant activity was observed for 24 hrs. And 48 hrs. Exposure and protected leaf area was 25.17± 0.76*sq.cm and 19.75± 0.93*sq.cm after 24 hrs. And 48 hrs. Respectively, digestibility as indicated by reduced efficiency of *P. demoleus* larvae in converting ingested food to growth. Similar results were found the Dancewicz [23] also was in agreement that *Allium sativum* and *Ocimum sanctum* suppress the feeding behavior of *Myzus persicae.* Feeding behavior is governed by both neural input from the insect's chemical senses and central nervous integration of this sensory input. The primary antifeedant effects might be attributable to a direct action of neem-based biopesticides on the centers that control feeding and metabolism. Azadirachtin stimulates specific deterrent cells in chemoreceptors on insect mouthparts and blocks the receptor cells that normally stimulate feeding. A secondary antifeedant effect of azadirachtin results from the disturbance of hormonal and other physiological systems.

Antifeedant activity was concentration dependent at lower dose of 50ppm concentration reduced the larval feeding up to a large extent that was 58.73% after 24 hrs. And 43.28% after 48 hrs. And the protected leaf area was 19.05 ± 0.92*sq.cm and 10.73 ± 1.14*sq.cm after 24 hrs. And 48 hrs, respectively. Similar observations were also noticed in *P. demoleus* treated with plant products such as andrographolide and Costunolide [24, 25]. Plant–based antifeedant can be of great value in protecting crops from insect attack and pest infestation and may replace synthetic insecticides in future. Antifeedant activity causing the pest to stop feeding or to move from the treated crop to another plant. Plant extracts often consist of complex mixtures of bioactive constituents plant metabolites may produce toxic effects if ingested leading to rejection of the host plant.

The plant products and active compounds may act as antifeedants, disturb insect growth, development and inhibit oviposition. Azadirachtin had higher activity of feeding deterrence at 200ppm and reduced the food consumption, at 150 and 100ppm were found to have a moderate activity of feeding deterrence. Similar findings were reported earlier by the main active compound in the *C. odorata* wood, gedunin, had previously shown moderate antifeedant activity against many insect species [26] so antifeedants have some physiological or toxic actions on insects, depending on the treatment concentrations.

4. Conclusion

The present findings suggest that plant extracts of Azadirachtin, contain biologically active compound which possesses a potentially vital antifeedant effect on *P. demoleus.* Thus, these plant extracts offers significant promise for combating the threat posed by Citrus farmers. The major thrust of this work is its adaptability for use by small scale farmers plagued by the challenge of not being able to afford conventional pesticides on the market.

5. Acknowledgement

The authors are very much thankful to Prof. Naidu Ashok, former Principal and Prof. V. Vanita Das, former Head, Department of Zoology, Nizam College (Autonomous) Osmania University for extending lab facilities and for their constant encouragement.

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