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Effect of Betulinic Acid on the Ovarian Development of *Papilio Demoleus* L. (Lepidoptera: Papilionidae) Larvae

[1]Srinivasa Rao Vattikonda, [2]Madhavi. M, [3]Sabita Raja. S

Department of Zoology, Nizam College (A), Osmania University, Hyderabad, Telengana State, India

Abstract: **Betulinic acid, a triterpenoid isolated from the bark of *Ziziphus Jujuba* exhibited insect growth regulating activity of the IV and V instar larvae and Zero hour pupae of *Papilio demoleus*. The larvae were treated tropical application with 2, 4, 6, 8 and 10 µg/µl doses concentrations of Betulinic acid, in solvent acetone and observe the effect on ovarian development and ovarian maturation. Ovarioles showed a considerable reduction in length and exhibited morphological and histological abnormalities. Comparative anatomical and histological studies of the treated and untreated ovarioles revealed that there was inhibition in the development of ovarioles. The untreated *Papilio demoleus* showed normal ovarioles with large amount of yolk in the oocytes localized in the vitellarium portion. However Betulinic acid affected ovarioles showed disrupted structures of the germarium and vitellarium with either complete or partial damage of few or all oocytes. Untreated ovarioles revealed accumulation of large amount of yolk in the oocytes whereas in case of Betulinic acid treated ovarioles, the yolk was markedly reduced and acquired several vacuoles indicating the resorption of the yolk or reduction in its synthesis. The results demonstrated that Betulinic acid causes rapid cessation of oviposition due to disruption of ovarian structure and inhibition of oocyte growth following topical treatment on of *Papilio demoleus.***

Keywords: **Betulinic acid, *Papilio demoleus,* Ovarioles, Sterility, Biological control.**

1. INTRODUCTION

Two-thirds of today's world population depends upon agriculture for livelihood, but nowadays, growth and production of agricultural crops are getting hampered day by day [1]. When farmers see their agricultural crops declining in yield and production, they often expect a dramatic, magical treatment to make them lush, green, and healthy again, so that the productivity increases. Humanity faces many problems that arise from its rapidly increasing population and one is the provision of the population with good quality food that is accessible for all [2]. There are various strategies that may be used to increase crop yield and improve food production but various problems remain, among them, the destruction of crops by pests is one of the most difficult, especially in developing countries, and although pests make up only a small percentage of insects, they cause significant losses to agricultural and forest crops, such as contributing to 20% annual loss of cereal crops [3]. The most voracious of the insect pests are Lepidoptera larvae, which have huge nutritional needs and are thus the most detrimental to food production [4]. Moreover, many insect species are vectors of diseases that lead to millions of human deaths each year. For example, malaria, which is transmitted by mosquitoes, kills over 600 thousand people annually around the world [5]. Therefore, the search for effective tools to control insect populations is one of the most intensively developing fields of research. Presently, the most common way to control insect pests is through the use of synthetic pesticides, but they negatively impact the natural environment. These compounds have a wide spectrum of activities against diverse groups of insects and can almost completely remove pests from agro ecosystems. However, although the immediate impacts and high efficiency of synthetic insecticides seem positive, there is no way to limit their action to only agricultural areas. Conventional methods of insect pest management with chemical pesticides have resulted in severe problems of environmental pollution such as contamination of water, air and soil [6]. Pollution adversely affects the ecosystem destroys biodiversity such as wild life, changes soil microbial diversity, disrupts the dynamics of food chains in the community and changes system processes. Furthermore, there is an increase in resistant pest populations [7], a decline in non target organisms such as earthworms, mites, spiders, fish, aquatic organisms, amphibians, birds, predators, and pollinators (wild bees, bumble bees, honey bees, fruit flies, humming birds, honey eater birds and sun birds). All of these factors also pose dangers to human beings. Acute and chronic human illnesses are rapidly increasing due to polluted water, air and food as a result of biomagnifications of the toxic compounds [8]. These problems have forced humankind to search for alternatives to these compounds, and the demands of agriculture include inexpensive insecticides that cause the least amount of damage to the environment.

Integrated pest management (IPM) involves various plant protection strategies with an emphasis on a variety of biological control agents such as predatory animals, plant-derived substances, crop rotation and mechanical damage to pests. An intense search for alternatives less harmful to the environment has been initiated in laboratories around the world. Agrochemical research on plant products over the last 30 years has resulted in the discovery of chemically novel insecticides that mimic the action of insect hormones. secondary plant metabolites, such as alkaloids, glycoalkaloids, terpenoids, organic acids or alcohols, are regarded as promising sources of plant-protecting substances [9]. These compounds are produced by a variety of plant species in practically all their organs, and they are one of the most important lines of plant defense against pests This concept of plant products interfering with the insect hormones as a selective mechanism to control pest insects was introduced by Carrol Williams as “third generation pesticides”, the insect growth regulator (IGR). An IGRs may be defined in terms of its mechanism of action, as a substance which acts within an insect to accelerate or inhibit a physiological regulatory process essential to the normal development of the insect. Insect growth regulators impair insect endocrine regulation of moulting and metamorphosis processes, such as juvenile hormone analogues (JHAs), moulting accelerating compounds (MACs), and chitin synthesis inhibitors. Insect hormones, especially ecdysteroids, are growth promoting at low concentrations and causes differentiation at higher (moulting inducing) levels [10]. The chitin synthesis inhibitory prevent the molting process by inhibiting chitin synthesis, thereby causing abnormal endocuticular deposition and abortive molting [11]. In addition, the chitin synthesis inhibitors (CSIs) affect reproduction in several insect orders, primarily by causing a reduction in egg hatch. From the review it is evident that IGRs based plant products to be effective tools for pest control though the problem of insects developing resistance to them is observed in a few cases.

Citrus is one of the important fruit crops and is grown in more than 52 countries around the world. Citrus industry is the third largest, in the world after mango and banana. Citrus is the largest cultivated group of fruits in the world, which includes mandarin, sweet orange, limes, lemons, tangerines and grape fruit. In the group of citrus plants, mandarin is the largest cultivated fruit in India and world, where as sweet orange is the second largest growing species among the group of citrus fruits.

All over in India, citrus plants are attacked by more than 250 insect pests alone at all stages of growth right from budding and seedlings in nurseries [12]. Out of these, 165 species are important in India causing an estimated loss of 30 per cent in yield [13]. Among the various insect pests that attack citrus, the citrus butterfly, *Papilio demoleus* (Linnaeus) is a regular pest of nurseries, young seedlings and flush of full grown up trees. Keeping in view its economic importance, the study has been carried out citrus butterfly, *Papilio demoleus*. A number of plant products in various forms have been screened against major agricultural pests. Considering the importance of phytochemicals in pest management the present work is designed to investigate the sterilant activity effect of the phytochemical, Betulinic acid, a terpenoid isolated from the bark of *Ziziphus jujube* (Rhamnaceae) against the fourth and fifth instars and pupae of *Papilio demoleus,* in laboratory assays.

2. MATERIALS AND METHODS

Test Insect:

The lime butterfly *Papilio demoleus* L. (Lepidoptera:Papilionidae) is tropical or subtropical butterflies it is widely distributed in Southern Asia, Australia, Taiwan, Hainan, New Guinea, and Southeast Asia. *P. demoleus* are found throughout the Malaysian Peninsula, including Kedah, Langkawi, and Tioman Island. *Papilio demoleus* (Linnaeus) is a regular pest of nurseries, young seedlings and flush of full grown up trees. The caterpillars feed voraciously and cause extensive damage to nurseries and young seedlings leaving behind midribs only [14]. Severe infestation results in defoliation of tree and retarding plant growth and decreasing fruit yield. The caterpillars feed voraciously and cause extensive damage to nurseries and young seedlings leaving behind midribs only. Severe infestation results in defoliation of tree and retarding plant growth and decreasing fruit yield [15].

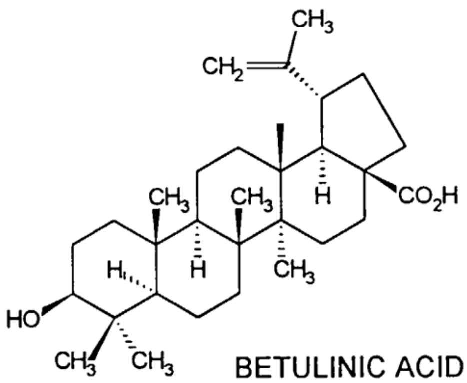
Collection of larvae and maintenance:

Collected from infested citrus plants in Nalgonda (District) of Telangana State, India from 2007 – 2010 period, and reared in a laboratory at 28±2°C, 70±5% relative humidity and 10L:14D photoperiod. Larvae were provided with fresh lemon (*Citrus aurantifolia*) and bael (*Aegle marmelos*) leaves for feeding in 6″×4″ glass troughs. After feeding the larvae were shifted to tall glass jars for pupation. The larvae clung onto the walls of these jars and transformed into pupae. Adults emerging from the pupae were kept in wooden box (20cmx20cmx20cm), provided with netting and a cloth sleeve on one side for handling. Adults were fed 10% honey solutions, and fresh twigs of lemon were provided for oviposition. The eggs were reared at 28±2°C to obtain fresh batches of larvae. The completely grown fourth instar and fifth instar larvae were sorted out and placed in a separate glass dish at room temperature for the experiment for each experiment 3-5 replicates were done and each experiment was repeated at least 3 times.

Test Product: Betulinic acid:

Betulinic Acid ***($C_{30}H_{48}O_3$)*** Betulinic acid was an important compound isolated from bark of *Ziziphus Jujuba*. It's medicinal benefits are mentioned in Ayurveda and Chinese Medicine. Betulinic acid, a triterpenoid found in many plant species, has attracted attention due to its important pharmacological properties, such as anti-cancer [16] and anti-HIV activities[17], anthelmintic activity [18], antifeedant activity [19]. It also exhibits antibacterial [20], anti- inflammatory [21] and anti-malarial [22] properties.

Chemical Formula: **$C_{30}H_{48}O_3$**



BETULINIC ACID

Preparation of test solution:

Different concentrations of Betulinic acid doses were prepared by dissolving a known amount of Betulinic acid in 1 µl of acetone to obtain 2, 4, 6, 8 and 10 µg / µl doses.

Treatment with Betulinic acid:

Thirty Freshly moulted fourth instar, fifth instar larvae and thirty zero-hour pupae were treated topically on the abdominal region with 2, 4, 6, 8 and 10 µg/ µl of Betulinic acid with acetone as the carrier solvent with the help of Hamilton micro syringe. Thirty larvae and pupae were treated each time with Betulinic acid and the experiments were performed in triplicate. Controls were treated each time with an equivalent volume of carrier solvent acetone. After total absorption of Betulinic acid The larvae and pupae were transferred into the diet. The treated resultant females were observed for ovarian deformities and the results were compared with controls ovaries (Figure-1).

3. RESULTS & DISCUSSION

The present study reveals morphological and morphometric variations of ovarian components as a result of the topical application of the Betulinic acid. All treated ovarioles were of abnormal shape and size. Fusion and improper orientation of follicles are conspicuous.

From the present study it was seen that there is reduction in body weight and ovary weight in treated resultant. Hence it is suggested that normal metabolism may be interrupted. Length of the ovariole, length and breadth of the oocytes, length of the oviducts (lateral and median) and the number of follicles exhibit variations in the treated resultant (Figure-2). Similar results have been recorded in other insects. Length of proximal oocytes was reduced in azadirachtin induced overage nymphs of *Locusta migratoria* [23]. Ovariole size, length, and oocyte number were reduced in *Dysdercus cingulatus* treated with extracts of *Vitex negundo* and *Eupatorium odoratum*[24]. Reduced size of ovary was reported in *Bactrocera cucurbitae* reared on food treated with methanolic extract of *Acorus calamus* [25]. The present results are in agreement with these reports.

The present study shows that in Betulinic acid treated ovary of *Papilio demoleus* the terminal filament cell cluster (Figure-3) are either of reduced length floating free in the body cavity or absent. Similar condition of free and short terminal ends of ovarioles and reduced ovariole length were reported in *Corcyra cephalonica* emerged from neem fed larvae [26]. According to Buning [27] terminal filament is important in maintaining the integrity of the ovariole. Direct contact with the somatic cells at the apical tip of the ovariole controls the asymmetric division of the oogonial stem cells in the germarium. In *Drosophila*, ovariole morphogenesis starts with the formation of terminal filament. For ovariole morphogenesis terminal filament cell cluster is essential [28].

Betulinic acid treated resultants, germarium is degenerated, a major part of it including the prefollicular region is diffused, empty and vacuolated. A few trophic nuclei are embedded in the diffused mass. Presence of empty spaces, vacuolated cytoplasm and fragmented nuclei in the germarium might be due to the cellular degeneration leading to reduction in the number of cells(Figure-4). Prameela (1997) reports degeneration of trophocytes in the ovary of *Dysdercus cingulatus* treated with, *Eupatorium odoratum, Vitex negundo.*

Betulinic acid treated resultants, some follicles are fused and some are of varying shape and size. Fusion of follicles observed in the study is due to the absence of inter follicular tissue possibly due to the failure of multiplication, differentiation and orientation of the interfollicular cells. Abnormal shape and size of the follicles is apparently due to the defective orientation of the follicle cells. The follicle cells fail to surround the oocyte as an intact, continuous layer, which is indicated by discontinuous follicular epithelium in many follicles. Abnormalities were seen in the follicular epithelium in all treatments but its intensity varies. In many follicles, the follicular epithelium is very thin formed of cells of diminished size. Number of follicles was reduced in the treated resultants treated with Betulinic acid. Ghazawi [29] reported that topical treatment of azadirachtin in *Heteracris littoralis* resulted in the shrinkage of ovaries with abolished oocyte growth, decrease in the number of depositesd eggs, disintegration and destruction of follicular cells in females

Yolk deposition in the basal oocytes of Betulinic acid treated ovariole is defective since the yolk is not organized into globules. Instead many yolk bodies of abnormal shape and size occur. This indicates altered integration and processing of yolk directing to defective and asynchronous vitellogenesis. Excessive vacuolation is correlated with degenerative changes and cytoplsasmic shrinkage as reported in *Callosobruchus maculates* and *Apis mellifera* [30] [31]. Shrinkage of ooplasm, irregular shaped eggs, loosely arranged follicular cells, arrested vitellogenesis or partial accumulation of yolk, compound egg chambers and vacuolated follicular epithelium were observed [32] (Figure-5).

Strategies based on botanical pesticides have evoked a renaissance in insecticide research seeking sustainable and eco friendly pest control devices. It is considered that the plant based insecticides have a margin of safety for both user and consumer. Botanical pesticides may also have a useful role to play in resistance management.

4. CONCLUSIONS

The study illustrates that the Betulinic acid extracts of bark of *Ziziphus Jujuba have* some anti-reproductive compound, which results the ovarian inhibitory effects. The present results demonstrate that Betulinic acid affects morphology of ovarioles during ovarian development of *Papilio demoleus*. The precise biochemical mechanisms of the effects of Betulinic acid on reproduction and ovarian development can reveal the nature of the effects of the sub lethal doses. Thus the present study clearly indicates that Betulinic acid acts as insect growth regulator and it influences the ovarian growth. Ovaries also exhibited the deformities like large oocytes, chorionated oocytes which blocked the ovarioles and reduced the fecundity. Future research should explore the biochemical nature of the action of sublethal doses of Betulinic acid in *Papilio demoleus*. Hence these plants can be considered potential candidates for regulating the pest population.

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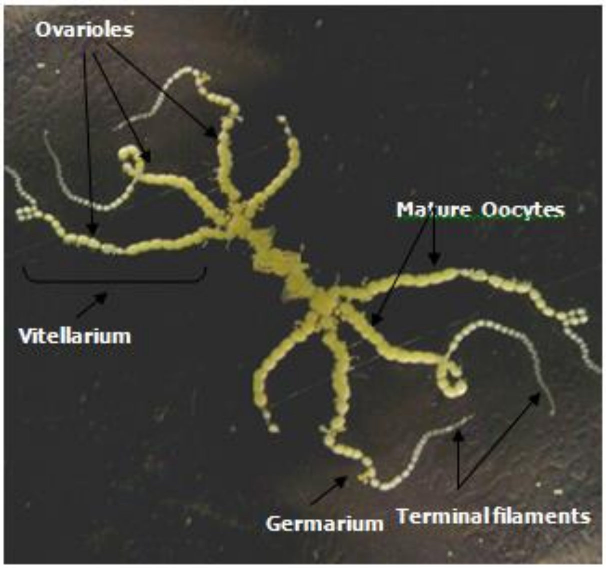

Figure 1: Ovary of *Papilio demoleu*

Figure 2: Ovarioles in defferent lengh & sizes Fused Oocytes of abnormal ovariole

Figure 3: Treated-Terminal oocytes abnormally large and unovulated

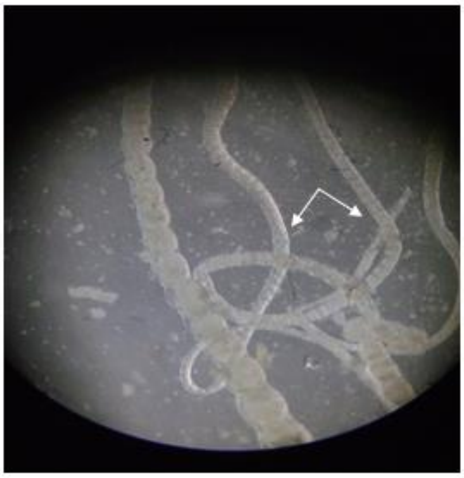

Figure 4: Empty and vacuolated ovarioles

Figure 5: The ovarioles and oocytes varing in size fused and matured unovulated oocytes blocking the Common oviduct

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