International Journal of Entomology Research
ISSN: 2455-4758
Impact Factor: RJIF 5.24
www.entomologyjournals.com
Volume 3; Issue 2; March 2018; Page No. 23-27

Effect of Andrographolide on ovarian development of *Papilio demoleus* L. (Lepidoptera: Papilionidae) larvae

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Abstract
Andrographolide is the main bioactive component of *Andrographis paniculata* it is diterpenoid and bitter substance which is extracted from the stem and leaves of the *Andrographis paniculata.* andrographolide exhibits a broad range of biological activitie. It is an insect growth regulator, we observed the effect of different concentrations (2, 4, 6, 8 and 10 µg / µl doses) of Andrographolide on the reproductive system of the *Papilio demoleus*, in our observations various morphological abnormalities like deformation and degeneration were observed in the ovaries and accessory glands of sterile insects. Histological investigation of the ovaries in infertile insects indicated a degeneration of ovarian follicle cells, defective vitellogenesis. 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 Andrographolide 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 Andrographolide 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 Andrographolide causes rapid cessation of oviposition due to disruption of ovarian structure and inhibition of oocyte growth following topical treatment on 4^{th} 5^{th} instar and pupae of *Papilio demoleus.*

Keywords: andrographolide, *Papilio demoleus*, ovarioles, sterility, biological control

Introduction
The modern insecticide researches started almost 65 years ago with the chlorinated hydrocarbons, organophosphates, methyl carbamates and botanicals, the use of these conventional organic insecticides to control insect pests [9]. In the recent years, the use of synthetic pesticides for pest management has become highly controversial. These pesticides are known to cause extensive environmental hazards as these pesticides accumulated at various concentrations in different levels of ecosystem. Also, the development of pesticide resistance in the pest is another reason of controversy. Even though the insects are exposed to an insecticide for long duration they manifest slowly, the insect not only develops resistance against the specific insecticides to which they are exposed to, but also a group of insecticides develop resistance by way of cross-resistance. To overcome these problems, attempts were made to develop alternate methods of pest control including the use of cultural practices, biological control, use of antifeedants, hormonal insecticides (IGRs) plant extracts [2, 31].
In the search for safer insecticide approaches, interest on plant products acting as an insecticides has grown as more and more pesticides are eliminated from use due to environmental and food safety problems[14] The plant kingdom affords a rich storehouse of chemicals of diverse biological effects on insects. In recent years several plants with insecticidal properties have been identified. Environmentally safer pesticides are selectively toxic, do not bio-accumulate, and exhibit relatively short persistence in the environment. More selective modes of action and reduced risks for non-target organisms and the environment they are most desirable in the modern integrated pest management programs, in the last two decades with the development of natural and synthetic compounds capable of interfering with the processes of growth, development and metamorphosis of the target insects. Plant products (insecticides) appear to be eco-friendly and mixtures of biologically active substances, because they have been found to be selective [24] and pose less negative impacts to ecosystems than conventional insecticides [27].
Use of plant products to insect pest control that adversely affect insect growth and development without causing environmental hazards. These substances were classified as "Insect hormone mimics" or "insect growth regulators" (IGRs). They were considered to have reduced risk as reported by the [29] to being soft to beneficial insects and target specific for juvenile stages. There are three types of insect growth regulators- hormonal, enzymatic and chitin synthesis inhibitors. Both juvenile hormone analogues and ecdysone inhibitors disrupt the ratio of hormones in the young insect. For an insect to be moult in the next stage, the correct ratio of juvenile hormone and ecdysone must be present. Ecdysone is a primary moulting hormone which is necessary for insects to change from the larval to pupal stage. If the ratio of one hormone to other is not proper, the insect fail to become adult, reducing reproduction and subsequently population increase. With some IGRs, adults even fail to produce viable eggs.
Plants are potential producer of novel chemical compounds which cannot yet be synthesized. Estimations suggest that over 2000 plant species have the potential to identify and

develop new chemistries to reduce bacteria, fungal, and insect/arthropod pests [13]. The complexity in chemical compounds in biorational products can also make development of resistance by insect pests is more difficult [21]. These environmental issues have driven agricultural researchers to search for better ecofriendly based pesticides [30]. Fruits and vegetables play a vital role in providing essential protective nutrients like vitamins and minerals and are used as selective diets by everybody. Citrus is one of the most important crops grown all over India. Citrus is heavily infested by a number of insects/pests among that *Papilio demoleus* L. (Lepidoptera: Papilionidae) which is one of the most destructive pests extensively found all over India and in other countries. It is a polyphagous pest which shows its presence on *Murraya koenigi, Psoralea corylifoli* and other economically important crops.

In the present study Andrographolide which was isolated from *Andrographis paniculata* we investigated the sterilant activity effects of Andrographolide on the fourth and fifth instars and pupae of *Papilio demoleus,* in laboratory assays.

Materials and Methods

Test Insect

Papilio demoleus Linnaeus, the Citrus Butterfly (also commonly known as the Lime Swallowtail), is a major pest of Citrus sp. (Citrus) and *M. koenigii* (Curry Leaf) plantations [1]. It is one of the economically important pests whose larval forms cause serious damage to citrus family by devouring large quantity of foliage during the later stages of their development. The genus *Papilio* is widely distributed all over the world. Among the various insect pests that attack lemon, curry leaf, orange and other plants of rutaceae. The caterpillars feed voraciously and cause extensive damage to nurseries and young seedlings. They are foliage feeders, prefer blossoms and young nurseries of citrus and curry leaf. Severe pest attack resulted in entire defoliation of the tree and leads to retardation of plant growth [5]. In India it is mostly found in the plains but can be found on the hills of peninsular India and up to 7000 feet in the Himalayas. Several insecticides offer quick and effective control of the pest. Keeping in view their adverse effects on the environment and other non-targeted organisms here an attempt is made to review the historical distribution, biology and biological management methods which are best suited to integrated pest management programme. *Papilio demoleus* incidence was noticed to be high during the rainy and winter periods. Pest activity was severe during October to December and first fortnight of January on sweet orange and during November to January in acid lime in Rayalaseema region in AP and high relative humidity, low temperatures favoured its larval activity [16].

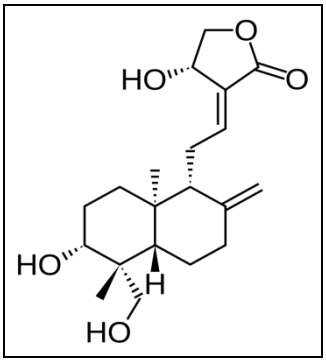
Collection of larvae and maintenance

Eggs and larvae of various stages of *Papilio demoleus* were 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: Andrographolide

Andrographis paniculata is a herb endogenous in southeast Asia, China and India. It is widely used in traditional remedies and folkloric medicine to cure a variety of human illness. The dried herb is a remedy for a number of ailments related to digestion, vermicidal, analgesic, antibacterial, antityphoid, antibiotic activities, hypoglycemic, besides immune enhancement [25]. Andrographolide the main bioactive component of *Andrographis paniculata* it is diterpenoid and bitter substance it is extracted from the stem and leaves of the *Andrographis paniculata.*. Extracts of this plant and andrographolide exhibit pharmacological activities such as those that are immunostimulatory[15], antiviral. As major active constituent, andrographolide exhibits a broad range of biological activities, such as antitumor, antidiabetic, antimalarial, hepatoprotective, anti-inflammatory, antiallergic, antiplatelet aggregation [18], hepatoprotective, and anti-HIV [20]. Many previous studies reported that extracts from *A. paniculata* have antifeedant and antioviposition properties against insects [8]. Andrographolide inhibits ovarian development, affecting the fertility and the reproductive potentiality of various insects suggesting its use for the development of safe and specific anti-fertility agent for the control of the pest [17]. Evaluation of Andrographolide for its larvicidal efficacy, Early reports of oral administration of powdered of stem of *Andrographis paniculata* indicated an antifertility effect in male Wistar mice, but no impact on fertility in female mice [26].

Chemical formula ------ $C_{20}H_{30}O_5$



Structure of Andrographolide

Preparation of test solution

Different concentrations of Andrographolide doses were prepared by dissolving a known amount of Andrographolide

in 1 µl of acetone to obtain 2, 4, 6, 8 and 10 µg / µl doses.

Treatment with Andrographolide

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 Andrographolide with acetone as the carrier solvent with the help of Hamilton micro syringe. Thirty larvae and pupae were treated each time with Andrographolide and the experiments were performed in triplicate. Controls were treated each time with an equivalent volume of carrier solvent acetone. After total absorption of Andrographolide.

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 (Fig- A).

Results & Discussion

One of the relatively new method to control the *Papilio demoleus* is apply a plant product causing physiologic and morphogenetic abnormalities that result in reproductive failure in insects [11]. At this stage, ecdysone is released to initiate the development and differentiation of the reproductive organs, leading to metamorphosis, the morphological abnormality of the internal reproductive organs and histological disruption of testis and ovaries were similar to the findings reported by other researchers [4, 19]. Different concentrations of the plant product Andrographolide applied on fourth instar, fifth instar larvae and pupae. The phytochemical exhibits the ecdysis inhibition the inhibition rate is increasing as per concentration increased. The treated resultants developed abnormalities in larvae, pupae and the adults. The adult survival rate is decreased as per the increase of concentration. The treatment with Andrographolide some of the treated larvae pupated normally but ovaries of these resultant adult were drastically affected.

The development of the ovary in insects depends upon the deposition of yolk i.e., vitellogenesis. vitellogenin is a name given to a unique group of proteins that are synthesized extraovarially, specifically by the fat body, released and transported through the haemolymph and deposited in the oocytes as vitellins, the major yolk proteins[6]. The vitellogenins have proved to be exceptionally useful for studying the control of reproduction in insects vitellogenesis in *Papilio demoleus* was influenced by Andrographolide. The ovaries of treated resultant adults which could not extricate from the pupal case, were small and reduced in number of ovarioles similar finding observed in [12] on *Callosobruchus maculatus*, [7] on *Tenebrio molitor*. The ovarioles remained underdeveloped and one or two chorionated oocytes blocks the common oviduct and some of the terminal oocytes were abnormally large and blocks the oviduct few are fused oocytes of abnormal ovariole (Figure - 2). In abnormal adults, the ovaries were in advanced stages of degeneration. The yolk was reabsorbed and the oocytes lost their shape and these were varied in sizes. In some ovarioles the dislodgement of linear arrangement of oocytes were observed.

The presence of Andrographolide has led to the incomplete development of ovaries and different abnormalities such as defective and deteriorated ovariols and partial developed eggs and ovarioles with fused abnormal oocytes are varies sizes and shapes (Figure - 3). In some cases, the external part of the oviduct was extensively wrinkled and ovariole with fused abnormal oocytes (Figure - 4) Also, the ovariols were degenerated in some parts at 10 ppm. Growth and development of the ovaries were completely inhibited [28]. This concentration caused the complete degeneration of the accessory glands, while lower doses were less effective. Application of Andrographolide prevented the normal development of follicle epithelium and caused abnormality, hypertrophy and disruption of ovarian layer, in some other cases small and deformed ovarioles and a large chorionated oocyte blocking the common oviduct formed the compound egg chamber (Figure - 5) in un-extricated adult Similar results were observed with different phytochemicals, in *Locusta migratoria* Azadirachtin inhibited both oogenesis and ovarian ecdysteroid synthesis hence preventing oviposition [23]. This compound also caused the lysis of the nucleus of the follicle cells. In addition, the absorption of yolk in the eggs of the treated insects was incomplete due to the disruption of the epithelium layer formation [10]. In some cases, the formation of autophagic vacuoles in the vitellus caused the reabsorption and digestion of proteins. Moreover, these vacuoles led to the degeneration of the nucleus of oocytes causing their sterility.

According to the results of this study and other reports, the application of Andrographolide on the *Papilio demoleus* not only induces morphological deformities, but also causes the sterility of adults. This may be due to the destructive effects of these compound on reproductive organ development. We found that application of Andrographolide had a direct role on fecundity and fertility of treated insects, therefore it may be concluded that this compound had an effect on the growth of ovaries and oogenesis. Reduction in fecundity similar observations has been reported by [22] on *Aphis glycines* and [4] on *Rhyzopertha dominica*.

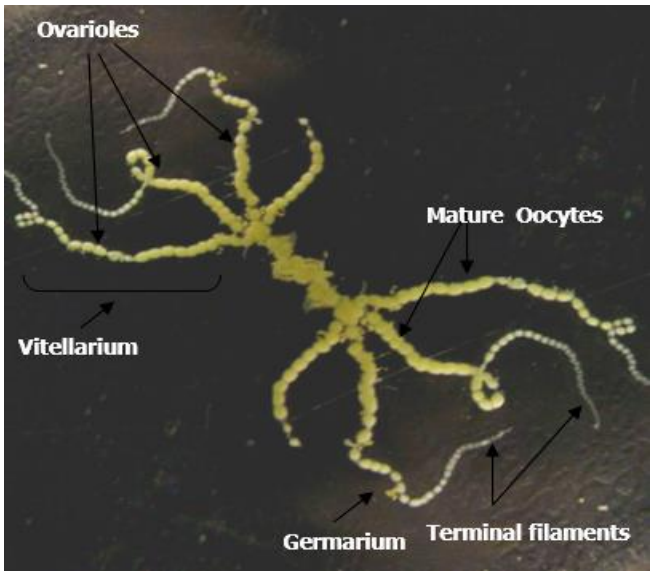


Fig 1: Ovary of *Papilio demoleus*

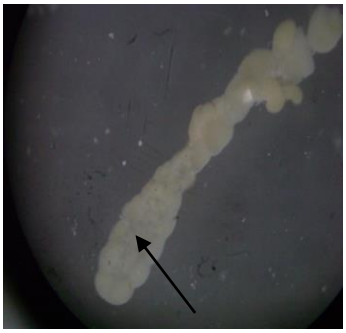


Fig 2: Fused Oocytes of abnormal ovariole

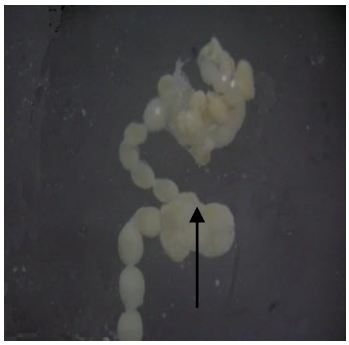


Fig 3: Abnormal and varied sizes and shape of oocytes

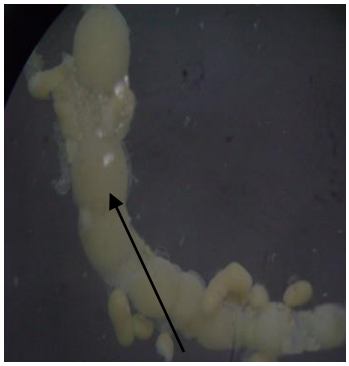


Fig 4: Ovariole with fused abnormal oocytes

Fig 5: The Ovarioles and oocytes varing in size fused and matured unovulated oocytes blocking the common oviduct

Conclusions

On the basis of overall findings, it can be concluded that Andrographolide is toxic to *Papilio demoleus*, as it mimics the action of JH and maintains the insect in an immature state. Andrographolide caused mortality in larvae and produced abnormal adults and it also affected the sterility of adults. This may be due to the destructive effects of these compounds on reproductive organ development. The plant extract inhibited the growth and development and fecundity of the *Papilio demoleus.* Thus Andrographolide may be considered as a leading target compound having the potential to control *Papilio demoleus* and can therefore form an important component of various Integrated Pest Management (IPM) programs for other such insects.

Acknowledgments

The authors would like to thank the Prof. Naidu Ashok, Principal, Nizam College, Osmania University for the facilities to perform the experiments.

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