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Juvenile hormone titers in the haemolymph of juglone treated *Dysdercus Cingulatus* (Heteroptera)

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Abstract
Juvenile hormone regulates metamorphosis and reproduction in insects. Compounds with anti-juvenile hormone activity are the new addition to the integrated pest management strategies to control pests. Among the eight known types of juvenile hormones in insects, the JH III is the most common one. Several compounds have been explored for their ability to disrupt the JH titers in insects and plumbagin, naphthoquinone is one of those. Hence it was thought worth exploring another naphthoquinone, Juglone for its effect on JH titers in *Dysdercus cingulatus*. Insects were collected, reared in the laboratory and then treated topically using a micro Hamilton syringe. Haemolymph of the treated and control group was subjected to liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) to identify and quantify the JH titers. In the present study, the dose-dependent decrease in the JH titers was observed before and after mating, which explains the chemosterilent effect of juglone. Hence, it can be concluded that anti Juvenile hormone research renders new opportunities for the development of integrated pest management strategies targeted at the disruption of juvenile hormone titer.

Keywords: Plant products, IGR, juglone, LC-Q-TOF-MS, juvenile hormone, *Dysdercus cingulatus*

Introduction
Juvenile hormone is an acyclic sesquiterpenoid that is very unique to insects. As the name suggests this hormone is responsible for sustaining the insect in juvenile state. The JH titer is high in the larval stage and is mantained till the final instar. Eventually it reduces to induce larval-pupal or larval adult transformation [1]. In female insects, it facilitates vitellogenin synthesis in the fat body and its uptake by maturing oocytes. Hence, the JH titer fluctuates as per different phases of the ovarian cycle [2]. In males, JH controls several crucial aspects like courtship behavior, migration, caste differentiation, and reproduction diapause [3]. This led Williams (1956) to predict the dawn of juvenile hormone (JH) based insecticides, which has boosted the research on this hormone. It took some time to realize that JH-analogues (juvenoids) insecticides were effective only against certain groups of insects. Then, came the compounds with anti-JH activity which are considered as new representatives of IGRs lacking some disadvantages of juvenoid-type chemicals [4]. Therefore, the current JH research is focused on developing strategies to disrupt the JH functions. Moreover, as JH is specific to insects, its signalling is also specific to insects and some other arthropods making vertebrates immune to this. This makes it an ideal strategy for pest management with low toxicity to non-target organisms [5].

The disruption of JH functions is based on a basic concept, of disrupting the JH titers, which means an induced increase of JH titers at developmental stages when titers are generally low, and the induced reduction of JH titers at developmental stagest when titers are generally high. At present, there are eight JH known [6]. however, the most commonly found Juvenile hormone in insects is JH III [7].

The compounds that can alter this titer can act as insect growth regulators. Compounds like indoxacarb alters the juvenile hormone titer of *Nilaparvata lugens* and *Chilo suppressalis* [8]. Similarly, a quinone, plumbagin is also known to disrupt the JH titers in *Dysdercus cingulatus* [9]. Juglone is an another natural quinone. It has been isolated from several plants of Juglandaceae members, including *Juglans nigra* L., *J. regia* L., *J. cinerea* L., *J. ailantifolia* Carr., *J. mandshurica* Maxim., *Carya tomentosa* Nutt., *C. ovata* (Mill.) K. Koch, *C. illinoensis* (Wangenh.) K. Koch (pecan), *Ptero-carya fraxinifolia* (Lam.) Spach, and *Platycarya strobilacea* Siebold & Zucc. [10, 11]. Hence in the current study, it was thought worthwhile to explore the insecticidal potential of this quinone, a juglone by analysing its effect on JH titers in *Dysdercus cingulatus.*

Materials And Methods
Test compound
Juglone, 5-hydroxy-1,4-napthoquinone, is a quinoid compound that functions as an allelochemical when released from trees in the walnut family (Juglandaceae) into the rhizosphere. It was purchased from Sigma.

Test organism
The red cotton bug, *Dysdercus cingulatus* Fabr, (Pyrrhocoridae: Heteroptera) has been used in the present study as a test insect. Sensitivity to compounds, easy handling, short life cycle, and easy rearing make the red cotton bug, the insect of choice for investigation.

Collection and Rearing
Dysdercus cingulatus were collected from the field of Rahuri, Ahmednagar Maharashtra. The adult male and female were hand picked and separated by observing their size. The rearing jars were prepared by layering an inch of moist soil. Three pairs of adult *Dysdercus cingulatus* per insect-rearing jar were introduced into these jars. They were

fed on soaked cotton seeds and maintained at 28º±2ºC and 85% humidity with 12 hours of photoperiod.

Treatment

Freshly moulted 24-36 hours old adults were selected and treated topically with the requisite amount of Juglone using a Hamilton microliter syringe.

Collection of Haemolymph

Haemolymph from control and treated males and females were collected separately after 24, 96, and 168 hours of treatment. Haemolymph was pooled, mixed with 70% methanol (1:30), and kept in the refrigerator till further use.

Sample preparation

15µl of haemolymph sample was mixed with an equal volume of 70% methanol and kept at room temperature for 30 min. And was later centrifuged at 8500 rpm for 30 minutes. The upper phase was transferred carefully in a new vial and the process was repeated twice. Later, 500 µl Methoprene was added as an internal standard. Further, the mixture was dried to a pellet under a stream of N_2 and dried pellets were dissolved in 10 µl of methanol and subjected to liquid chromatography quadrupole time-of-flight mass spectrometry (LC-Q-TOF-MS) as mentioned by Magdum *et al*. 2024 to identify and quantify the compound [9].

The concentration of Juvenile hormone present in the haemolymph samples was calculated from the chromatogram using the equation

$$\frac{Concentration\ of\ standard}{area\ of\ standard} = \frac{concentration\ of\ sample}{area\ of\ sample}$$

Statistical Analysis

Two-way ANOVA analysis was performed in Excel.

Observation And Results

Juvenile hormone titer was carried out in the present investigation in Juglone treated and control group of *Dysdercus cingulatus* and the results were compared with standard JH**.**

Quantitative analysis of Juvenile hormone using Electro spray ionization- Mass spectroscopy (ESI-MS): Juvenile hormone extract was prepared as described in the materials and methods and 10 µl of standard III with 1.25 µg concentration was injected into LC-Q-TOF-MS instrument. It was interesting to note that JH III was not very prominent in adult *Dysdercus cingulatus.*

The juvenile hormone was separated on C18 column (50 mm × 2.1 mm× 1.7 µm) by gradient elution in water and methanol in 15 min. MS analysis was carried out using electrospray ionization for Juvenile hormone analysis in haemolymph of Control and Juglone-treated *Dysdercus cingulatus* under the conditions mentioned in materials and methods.

Standard Juvenile Hormone III

JH III was identified based on retention time. The number of ions differing in the samples from the haemolymph of different treated groups was visually inspected and compared with the standard JH III and the control group. Due to the high abundance of hydrogen in haemolymph primarily $[M+H]^+$ was formed. The limit of detection and quantification was 15 and 2-4 ng/10µl. The visual inspection of Standard JH III at m/z 267 is shown in Fig 1. The visual inspects of standard JH showed prominent 6 ions 147,189, 217,235, 249 and 507.

Retention time on the X-axis and relative abundance of ions are shown in Fig. 2 which is collectively describing the total ion chromatogram (TIC). The retention time was found to be 11.58 min and the area was 268091. This was used to calculate the total concentration of the hormone in the samples.

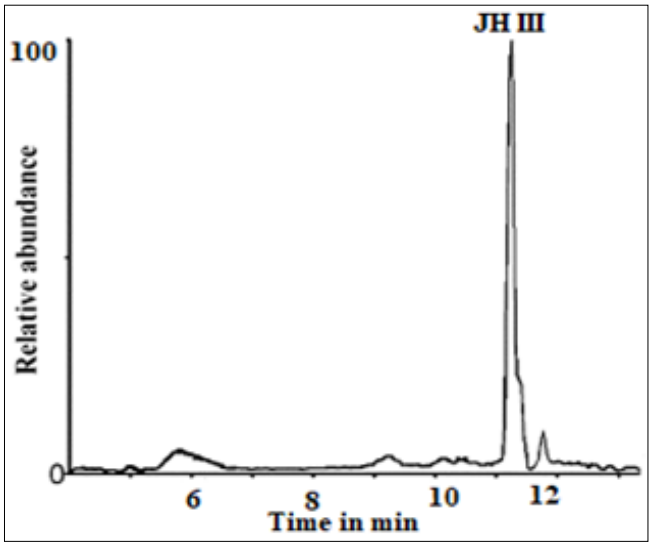

Fig 1: Standard JH III hydrogen adduct (*m*/z 267) chromatogram with the following gradient: 0–1 min 60% methanol,1–4 min 60%–80% methanol, 4–6 min 80% methanol, 6–9 min 80%–90% methanol, 9–18 min 90%–100% methanol

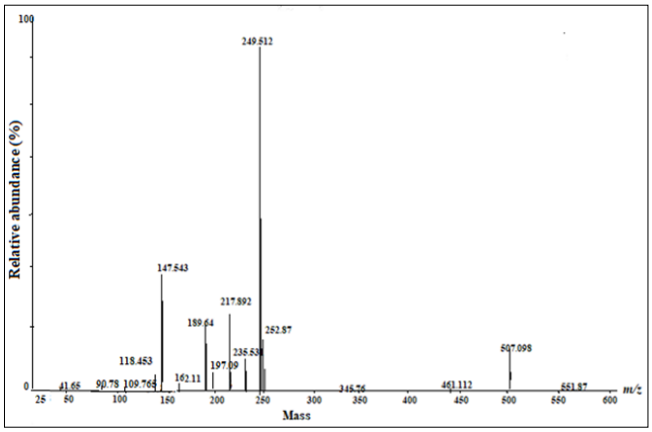

Fig 2x: ESI-MS spectra of the standard JH-III

Analysis of Juvenile hormone titer in Haemolymph Sample from Control group

Samples of haemolymph from both male and female *Dysdercus cingulatus* (24, 96, and 168 hours of post-treatment period) were analysed.

Analysis of Haemolymph of Juglone-treated Dysdercus cingulatus

Haemolymph samples from 3 and 5µg/ml Juglone-treated male and female individuals were analysed separately on 24, 96, and 168 hours of treatment. In all the treated male groups the ion chromatogram (TIC) showed the presence of 13 ions differing from the background which was similar to the Juglone treatment. The mass of ion with *m/*z92 was missing.

Male Dysdercus cingulatus (Fig. No. 3): After 24 hours of treatment in a male haemolymph sample in 3µg/ml dose, the total area of absorption was found 1.56% less than the control group and the JH concentration was 2.35ng/10µl. Similarly, 5µg/ml Juglone treatment in males caused a reduction by 2.77% and the JH concentration was 2.32ng/10µl in the haemolymph sample.

With a further increase in post-treatment period of 96 hours, 3µg/ml Juglone caused a reduction in total peak area by 11.34% and the JH concentration was 2.36ng/10µl. The higher dose i.e. 5µg/ml Juglone caused a reduction by 15.82%. There was a further reduction in the area of absorption noted with this dose. The JH concentration in 5µg/ml Juglone was found as 2.24 ng/10µl.

The last post-treatment period i.e., 168 hours caused a further reduction in the area of absorption of peak and JH concentration in both the doses of Juglone. It was found to be 5.09% less than the control group when treated with 3µg/ml Juglone. Treatment with 5µg/ml Juglone caused a reduction in peak absorption area by 10.66%. The JH concentration was 2.17 and 2.06 ng/10µl in 3 and 5 µg/ml Juglone doses respectively.

Female Dysdercus cingulatus (Fig. No. 3): For all three post-treatment periods in Juglone-treated female *Dysdercus cingulatus*, A total of 12 ions were noted differing from the background against 14 of the control group. The mass of ion m/z 63 and 93 was missing.

After 24 hours of treatment, 8.95 % of reduction in the total area of absorption was noted in a haemolymph sample of 3µg/ml dosage. The JH concentration was 2.54 ng/10µl which was less than the control group. Similarly, further reduction in the absorption area was found with a 5 µg/ml dosage (10.29%) and the JH concentration was 2.50 ng/10µl.

With the increase in the post-treatment period of 96 hours in 3µg/ml Juglone-treated females, the haemolymph sample showed the presence of 18.64% lesser absorption area and therefore the JH concentration was found as 2.65 ng/10µl when compared with the control group. With the same post-treatment period, in 5µg/ml dosage, a further reduction by 23.22% was noted. 2.50 ng/10µl JH concentration was found in haemolymph sample of 5µg/ml dosage of Juglone-treated female *Dysdercus cingulatus*.

3µg/ml of Juglone-treated females, further caused a reduction in the total area of absorption of maximum peak after 168 hours of treatment. It was found 14.97% less than the control and the total JH concentration was found as 2.17ng/10µl of haemolymph sample. With the same post-treatment period, but with a higher dose (5µg/ml) of Juglone, the reduction was found as 18.40% and JH concentration was 2.13ng/10µl

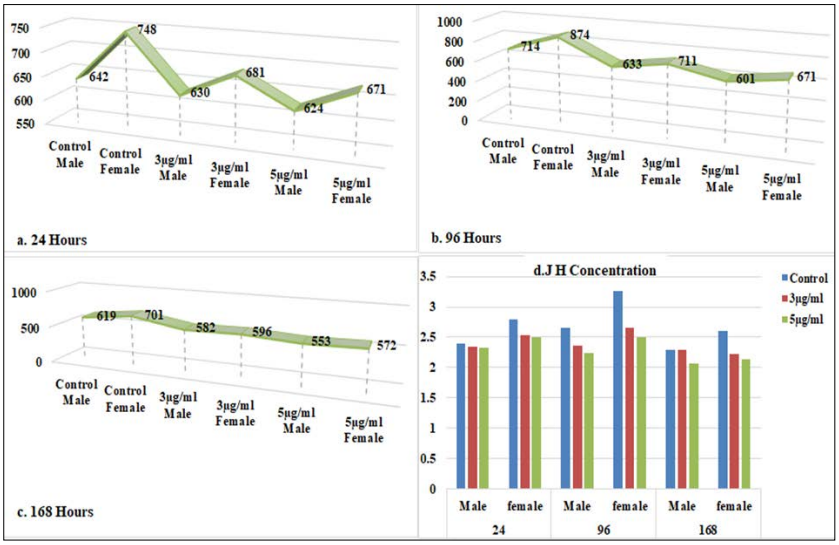

Fig 3: a-c Total area of absorption of a prominent peak in haemolymph sample of Juglone treated *Dysdercus cingulatus d.* Total Juvenile hormone concentration in the different post-treatment periods in Juglone-treated *Dysdercus cingulatus*

Hence the result is consolidated as an overall decline in the JH titers in males and females upon treatment with Juglone.

Statistical Analysis for Juglone-treated Dysdercus cingulatus

ANOVA Two factors without replication were carried out and the following are the details.

Table 1: Summary of Two factor ANOVA without replication for Juglone treated *Dydercus cingulatus*

Summary	Count	Sum	Average	Variance
Control	6	16.03	2.67167	0.11342
3µg/ml	6	14.28	2.38	0.03404
5µg/ml	6	13.76	2.29333	0.03339
Males	3	7.05	2.35	0.0013
Females	3	7.83	2.61	0.0247
Males	3	7.26	2.42	0.0468
Females	3	8.41	2.80333	0.16203
Males	3	6.55	2.18333	0.01703
Females	3	6.97	2.32333	0.06323

Table 2: Summary of Two factor ANOVA without replication for Juglone treated *Dydercus cingulatus*

ANOVA						
Source of Variation	**SS**	**df**	**MS**	**F**	**P-value**	**F crit**
Rows	0.47143	2	0.23572	14.8467	0.00101	4.10282
Columns	0.74545	5	0.14909	9.39051	0.00154	3.32583
Error	0.15877	10	0.01588			
Total	1.37565	17				

The table No. 1 and 2 shows that the p-value is 0.00101, which is less than 0.05. Hence, we consider that the effect of Juglone is statistically significant. So, there is a significant difference among the different doses of Juglone.

Discussion

JH titer can be correlated with the reproductive cycle in the life cycle of insects in both male and female *Dysdercus*. It's observed that these adults enter into the second and third cycles of the reproductive phase after 96 and 168 hours of treatment. This enhances the increase in JH concentration in the haemolymph. Hence JH titers increase before and after mating as reported in *Nilaparvata lugens* and *Chilo suppressalis* [8] as JH stimulated after mating increases egg production [12].

Similar correlation was observed in *Heliothes virescens* during normal reproduction and oviposition [13]. According to studies carried out on adult cabbage looper, *Trichophusia ni*, it has been observed that JH titer affect the regulation of egg development [14]. Hence, Any change in the titer disrupts the reproductive cycle, as observed in male boll weevil where corpora allata (CA) produced very less or no JH. This suggested the role of low JH titers in the failure of males to produce Vitellogenin [15].

Similarly, in the present study, with the increase in the post-treatment period, there is a relative decrease in the amount of JH when compared with the control group. Moreover, there is a dose-dependent effect observed in all the treated groups.

Hence, earlier studies of Plumbagin, Juglone, and Menadione revealed the fact that these compounds act as chemosterilants and also adversely affect fecundity and fertility in *Dysdercus cingulatus* [16] could now be co-related to the low level of JH in the haemolymph of insects. This could be owing to either the inactivation of hormones or the degradation of JH from certain nonspecific esterases [17, 18]. Moreover, it is stated that this concentration of JH could be due to low JHBP (juvenile hormone binding protein) and probably results in uneven distribution of JH in the body. The reverse is also crucial for the tissue clearance of JH when required and its degradation in the haemolymph [19].

For these further studies understanding the nature of binding proteins and their role in transportation is required.

Conclusion

Anti Juvenile hormone research renders new opportunities for the development of integrated pest management strategies targeted at the disruption of juvenile hormone titer.

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