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Lethal and Sublethal Effects of Juglone on the Life-History Traits of *Galleria mellonella* L. (Lepidoptera: Pyralidae)

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Abstract: The greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) is a model insect widely used for ecotoxicological studies and a serious pest of honeycomb in hives causing considerable losses in apiculture. Our aim was to explore the lethal and sublethal effects of juglone (5-hydroxy-1,4-naphthoquinone), a secondary metabolite naturally synthesised in leaves and fruits of the walnut species in the genus *Juglans*, on *G. mellonella*. According to probit analysis, the lethal (LC_{99}) and median lethal (LC_{50}) concentrations of dietary juglone given to *G. mellonella* larvae were determined as 5.49 and 2.38 mg / 2 g of diet, respectively. All tested juglone concentrations decreased the survivability of larvae to the pupal and subsequently to the adult stages. Selected juglone concentrations at LC_{30} (1.59 mg / 2 g), LC_{50} (2.38 mg / 2 g) and LC_{70} (3.18 mg / 2 g) incorporated into the diet of first instar larvae altered the life-history traits by prolonging the larval developmental time, decreasing pupal and adult weights, lowering total egg numbers and egg hatchability. Our results indicated that juglone has ecotoxic potential for insects and could be incorporated in novel studies on plant-based insecticides against storage insect pests.

Key Words: *Galleria mellonella*, juglone, ecotoxicology, naphthoquinone, storage insect pests.

Introduction

The uncontrolled and high-dosage application of pesticides used in pest control affects human and animal health and, in turn, causes deterioration of the ecological balance. Due to their rapid degradation from the environment and low toxicity against vertebrates, alternative plant-based insecticides are safer than non-environmental insecticides (Lindroth et al. 1990). Today, eco-friendly, plant-based chemical-related research has gained importance in pest management programs to reduce the negative effects caused by non-biodegradable chemicals (Raja 2014, Kwadha et al. 2017). Over the years, integrated pest management programs (IPM) including alternative eco-friendly strategies have been one of the major research areas to solve the economic and environmental health related problems caused by pest insects. Additionally, laboratory cultivation of pest insects provides significant information about the biological and insecticidal potential of various phytochemicals (Thiboldeaux et al. 1994, Piskorski & Dorn 2011, Altuntaş et al. 2016). In this way, it is possible to evaluate the ecotoxic effects of phytochemicals which could be applied against insect pests of stored products and considered as candidates for bio-insecticides.

In this study, the greater wax moth *Galleria mellonella* L. (Lepidoptera: Pyralidae) was studied because it poses a great threat on Apiary and its larvae feed on honeycombs, pollen, honey and beeswax in hives (Kwadha et al. 2017), thus causing considerable losses in apicultural products (Charriere & Imdorf 1997). Due to its rapid growth and inexpensive laboratory cultivation, the wax moth is also used as a

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host for rearing biological control agents (Kwadha et al. 2017) and a model insect for ecotoxicological and immunological studies. *Galleria mellonella* can also be used instead of mammalian species for studies related to in vivo toxicity of environmental pollutants as a model organism (Altuntaş et al. 2016, Maguire et al. 2016, Sugeçti et al. 2016). A variety of fumigants including paradichlorobenzene, such as naphthalene, are used in the chemical control of wax moth infestation but these fumigants are very toxic to bees and humans and besides, leave residues in honey (FAO report, Charriere & Imdorf 1997, Kwadha et al. 2017). Alternatively, eco-friendly insecticides, such as secondary metabolites from plants, could be used in pest control for the protection of stored apiculture and other animal products. Evidence from earlier studies has shown that juglone (5-hydroxy-1,4-naphthoquinone) and various other naphthoquinones have toxic effects on some phytophagous insects, leading to weight reduction, antifeedant effects, deterioration in morphology and sexual development and reduction in egg hatching (Lindroth et al. 1990, Thiboldeaux et al. 1994, Sun et al. 2007, Sorokin & Whitaker 2008, Hu et al. 2017, Lv et al. 2018). However, juglone-induced impacts on the biological fitness of *G. mellonella* have not been determined yet. In addition to this, some beekeepers in Turkey use green leaves and (or) walnut husks of *Juglans regia* L. (black walnut) to control greater wax moth *G. mellonella* infestation in beehives. This application is known anonymously by beekeepers but not scientifically researched and approved. Juglone is one of the secondary metabolites naturally synthesised in walnut husks of *J. regia.* The current study aimed at determining the lethal and sublethal effects of dietary juglone on survival and various life-history traits (e.g. developmental times, longevity, fecundity and egg hatchability) of the wax moth *G. mellonella.*

Materials and Methods

Insect rearing

A colony of *Galleria mellonella* was maintained by feeding the insects on an artificial diet including 340 g of wheat bran, 20 g of pollen, 75 ml of filtered flower honey, 150 ml of glycerol, 100 g of ground old dark honeycomb and 75 ml of distilled water. All stock and experimental laboratory cultures were maintained at Eskisehir Technical University, Eskişehir, Turkey at 27 ± 1°C, 60 ± 5 % RH in constant darkness**.**

Survivorship assay

The lethal concentrations (LCx) of the juglone was determined with probit analysis by incorporating pure juglone (Sigma, St. Louis, MO) at concentrations of 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4, 4.5 and 5 mg in 2 g artificial diets of the first instar of *G. mellonella*. Since each larva feeds on up to 2 g of diet until pupation, the desired juglone concentrations were directly mixed homogeneously into 2 g of artificial larval diet. For each analysis, an individual mating pair of 1–2 days old *G. mellonella* adults was placed in 1 L jars containing 1 g honeycomb for mating and oviposition. After oviposition, the eggs were observed until the emergence of the first instars. Under standard laboratory rearing conditions, each of the first instars was transferred to a sterile plastic box (10 ml) containing 2 g of diet treated with different concentrations of juglone (0.5 – 5 mg into 2 g diet, w/w). Larval diets without juglone were fed to the control groups. Larval mortality was monitored daily until the 7th instar emerged (170 ± 10 mg). The surviving last instar larvae were counted and transferred into another plastic box including filter paper for pupation. Thus, the percentages of survival to pupal and adult stages were recorded on survivorship assay at all tested juglone concentrations. For all experimental and control groups, experiments were replicated four times with 15 larvae per replicate (60 individuals per concentration).

Sublethal effects of juglone on life-history traits

According to probit analysis, juglone concentrations at LC_{30} (1.59 mg / 2 g), LC_{50} (2.38 mg / 2 g) and LC_{70} (3.18 mg / 2 g) in larval diets were used to determine the sublethal effects of juglone on the life-history traits of *G. mellonella* (Table 1). Larval and pupal developmental time, adult longevity, pupal and adult weights, fecundity and fertility were examined as biological fitness indicators. Bioassays were prepared as mentioned above using first instar larvae and kept under the same laboratory conditions. Both experimental and control group diets were inspected daily and the developmental stages of larvae were followed until the 7th instar emerged. The day the eggs hatched was considered as the first day of the larval developmental time. The time required for completion of the larval stage was recorded as "larval developmental time". Upon the emergence of the seventh instar, the larvae were removed from the plastic cups containing diets and individually transferred into another sterile plastic cup lined with a filter paper for pupation. So, these pupation cups were observed daily until the emergence of the adults and that time interval was recorded as "pupal developmental time". Then, newly emerged adults were transferred into new plastic cups covered with screened lids and observed daily until the adults had

died to record the "adult longevity". In addition, weights of pupae and adults were recorded for each specimen. All biological assays for each experimental and control group were repeated four times with 15 larvae (n = 60 larvae) at different times.

To explore the effects of juglone on the fecundity and fertility of *G. mellonella*, a pair of newly emerged adult male and female reared on the juglone containing (LC_{30}, LC_{50} and LC_{70}) diets were placed in sterile plastic cups covered with a plastic lid and allowed to oviposit for 2 days (Sugeçti et al. 2016). After oviposition, adults were removed from cups and eggs were transferred into petri dishes which were held on a black background for easy counting of the eggs under stereomicroscope (Leica, Zoom 2000). The female fecundity was assessed as the total numbers of eggs laid per female per day. The fertility ratio was expressed as the percentage of total number of hatched eggs per female per day. Each experiment was repeated four times using ten females per replicate (i.e. n = 40 for each of tested juglone concentrations).

Statistical analysis

According to the larval mortality data, lethal concentrations of pure juglone with associated 95% confidence levels ($P < 0.05$) were determined using probit analysis in Statistical Package for the Social Sciences (SPSS; version 18.0 for Windows, SPSS Science, Chicago, IL). Survivorship data were analysed using a chi-square test (χ^2) in SPSS. The differences were statistically significant when F and $x^2 > 0.05$. Further, percentage data on fecundity and fertility were normalised using arcsine transformation before analyses. One-way analysis of variance (ANOVA) was performed to compare the data of survivorship, biological assays and percentage of fecundity and fertility. To define the significant differences among means, the least significant difference (LSD) test in SPSS program was used. Values of $P < 0.05$ were accepted as significant.

Results

Survivorship

Using probit analysis, the cumulative percentage mortality of larvae of *G. mellonella* after exposure to different concentrations (in a range of 0.5 – 5.0 mg / 2 g of diet) of juglone are shown in Table 1. This assay showed that LC_{99} and LC_{50} concentrations of juglone mixed with the diet of first instar larvae, were 5.49 (5.10 – 5.99) and 2.38 (2.21 – 2.55) mg / 2 g of diet, respectively ($x^2 = 4.76$, df = 9, P = 0.86). Dietary juglone at the same concentrations decreased significantly the survival of *G. mellonella* during the pupal and adult stages. The survival percentages from the first instar larva to pupation were significantly decreased at all concentrations compared to the control group (Fig. 1). These results were also shown in a concentration-dependent manner (F = 223.93; df = 5, 12; P = 0.00) and a 100 % mortality was recorded at the highest concentration of juglone (5 mg / 2 g of diet). Survivorship of pupae to the adult stage also decreased in a concentration-dependent manner at all juglone concentrations with respect to the control group (F = 321.44; df = 5, 12; P = 0.00). However, all data on survivorship to adult stage were lower than results of survivorship for the pupal stage (Fig. 1).

Sublethal effects of juglone on life-history traits

The time from the first stage to the completion of the larval development of *G. mellonella* fed on juglone-free diet was determined to be 25.27 days and

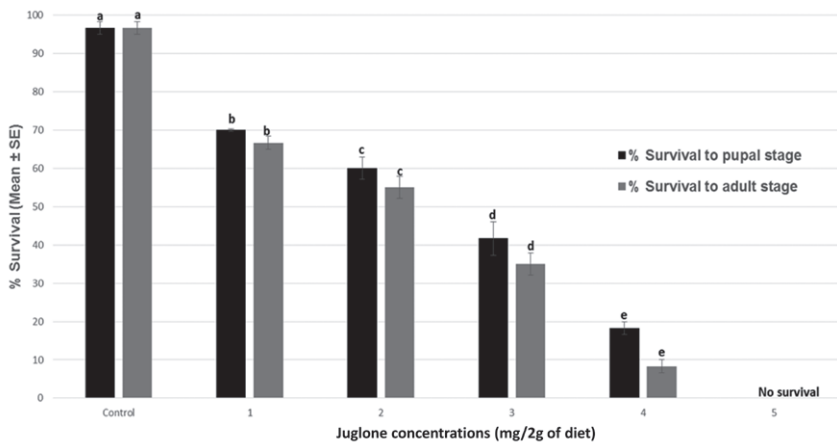

Fig. 1. The percentages of survival to pupal and adult stages of *G. mellonella* fed on different concentrations of juglone in larval diets. *Each bar represents the mean ± standard error (SE) of four assays with 15 larvae per replicate. Means within a bar followed by the different letter are significantly different ($x^2 > 0.05$).

increased in the juglone-treated groups (F = 22.15; df = 3, 236; P = 0.00). There was no statistically significant increase in the larval developmental time at LC_{30} and LC_{50} but at the highest concentration (LC_{70}), a statistically-significant increase was observed as compared to the lower concentrations and control group. Furthermore, it was observed that the larval stages could not be completed at concentrations above LC_{50} of juglone and the deaths occurred earlier as concentrations increased. The pupal developmental time and adult longevity did not change at all tested concentrations of juglone compared to the control (P > 0.05, Table 2). However, pupal weights showed a decrease at all juglone concentrations and a reduction by approximately 20 % was observed at LC_{70} when compared with the control (F = 44.695, df = 3, 236, P = 0.000). In addition, there was a significant decrease in adult weights at LC_{50} and $LC_{70,}$ when compared with control and low juglone concentration (F= 3.86; df= 3, 236; P= 0.01, Table 2).

Fecundity and fertility

Female fecundity and fertility ratio reduced significantly in the juglone-treated groups (F = 12.51, df = 3, 116, P = 0.00) as compared to the control group, even though adult longevity did not change in all groups (Tables 2 and 3). The percentage of hatched eggs was 73.8 ± 2.7 % at LC_{70} for juglone, and 90.8 ± 0.9 % for the control group (F = 8.964, df = 3, 116, P = 0.000, Table 3).

Discussion

This survivorship assay showed that dietary juglone had lethal and sublethal effects on the larval and pupal stages of *G. mellonella* and caused a concentration-dependent larvicidal effect. Thus, the LC_{50} of juglone incorporated in the larval diets of *G. mellonella* were determined as 2.38 mg / 2 g. It was also observed that juglone at different concentrations given with the diet had a lethal effect during larval development of the model insect *G. mellonella* even at lower concentrations than the value found in walnut husks of up to 5 mg / g at the end of the growing season (Stampar et al. 2006, Piskorski & Dorn 2011). Duke & Ayensu (1985) proposed for the first time that juglone may have herbicidal, pesticidal and repellent effects. Furthermore, various studies have reported that dietary juglone and other naphthoquinones caused toxic and antifeedant effects in a concentration dependent manner on some phytophagous lepidopteran species i.e., *Lymantria dispar* (Lepidoptera: Erebidae) and *Spodoptera frugiperda* (Lepidoptera: Noctuidae) (Yu 1987, Lindroth et al. 1990, Thiboldeaux et al. 1994, 1998, Meizhi et al. 2003, Sun et al. 2007, Piskorski et al. 2011, Lv et al. 2018). Similar to these previous studies, the current study showed that survival of the storage pest *G. mellonella* depends on the juglone concentrations in the larval diet. In contrast to our data, it is known that a lepidopteran insect, the polyphagous luna moth *Actias luna* (Lepidoptera: Saturniidae), is exposed to juglone in its natural diets and it has the ability to counteract the toxic effects of juglone even at high concentrations: juglone is converted to the non-toxic 1,4,5-trihydroxynaphthalene in the digestive tract of larvae (Piskorski & Dorn 2011). Further, studies on the larvae of the oligophage codling moth *Cydia pomonella* (Lepidoptera: Tortricidae), which uses walnut *J. regia* as a secondary host, revealed that when treated with high juglone concentration (50 mg / g) there was 100% mortality, while the low-concentration (5 mg / g) treatment had no effect on the survival rates of larvae (Piskorski & Dorn 2011). On the other hand, in the present study, we observed a 100% larval mortality at 5 mg / 2 g juglone concentrations added to the larval diet. It is quite evident from the earlier study and present findings that juglone is highly toxic even at lower concentrations to the storage pest *G. mellonella*. At the same time, this study showed a significant decrease in the survival during the pupal and adult stages depending on the amount of juglone incorporated in the larval diet. Our results indicate that juglone has an insecticidal efficiency depending on insect's physiological capacity. To the best of our knowledge this is the first study showing the lethal concentrations of juglone and its insecticidal potential against the storage pest *G. mellonella.*

In this study, juglone concentrations under LC_{50} were used to determine the alterations in life-history traits of *G. mellonella* and a concentration of 3 mg / 2 g diet was used to determine changes as a result of an overdose. These concentrations of dietary juglone reduced the larval developmental time, pupal weight, egg number and hatchability, thus influencing the biological fitness of *G. mellonella*. Similar to the present study, different researches evaluated that various naphthoquinones, including juglone, affect the biological parameters of some species of Lepidoptera. However the species evaluated in these studies are phytophagous insects, such as *L. dispar*, *S. frugiperda*, the cabbage looper *Trichoplusia ni* (Lepidoptera: Noctuidae) and the oriental fruit moth *Grapholita molesta* (Lepidoptera: Tortricidae: (Yu 1987, Lindroth et al. 1990, Thiboldeaux et al. 1998, Sun et al. 2007, Piskorski & Dorn 2011, Piskorski et al. 2011). Low doses of juglone administered to the diet of the gypsy moth L. *dispar* caused a significant decrease in vital and growth activities, nutrient consumption and pupal weight and

Table 1. Lethal concentrations (LC_{10}, LC_{20}, LC_{30}, LC_{40}, LC_{50}, $LC_{70,}$ LC_{95} and LC_{99}) of juglone (mg / 2 g of diet) incorporated in the diet of *G. mellonella* larvae.

Lethal concentrations[#] (mg/ 2g in diet)	Probit concentrations (mg / 2 g in diet)	95 % confidence interval *	
		Lower bound	Upper bound
LC_{10}	0.44	0.09	0.72
LC_{20}	1.11	0.84	1.33
LC_{30}	1.59	1.36	1.78
LC_{40}	1.99	1.81	2.17
LC_{50}	2.38	2.21	2.55
LC_{70}	3.18	2.99	3.39
LC_{95}	4.87	4.55	5.29
LC_{99}	5.49	5.10	5.99

* Values are displayed with lower and upper confidence limits, probit = 0.660 x concentrations (mg / 2 g) – 1.570.
[#]All assays were performed with 15 larvae for each concentration (n = 60 per treatment) and repeated four times.

Table 2. Effects of juglone on the biological parameters of *G. mellonella* (LDP: larval developmental time; PDP: pupal developmental time; AL: adult longevity; PW: pupal weight; AW: adult weight; SE: standard error).

Juglone (mg / 2 g in diet)[#]	Mean ± SE*				
	LDT (day)	PDT (day)	AL (day)	PW (mg)	AW (mg)
Control	25.27 ± 0.40 **a**	7.40 ± 0.11 **a**	14.78 ± 0.83 **a**	117.06 ± 3.68 **a**	22.39 ± 0.95 **a**
LC_{30}	27.53 ± 0.48 **b**	7.50 ± 0.11 **a**	14.35 ± 0.91 **a**	104.72 ± 2.34 **b**	22.54 ± 1.33 **a**
LC_{50}	28.47 ± 0.46 **b**	7.43 ± 0.12 **a**	13.47 ± 0.94 **a**	103.82 ± 2.48 **b**	18.95 ± 0.69 **b**
LC_{70}	31.25 ± 0.71 **c**	7.47 ± 0.13 **a**	13.18 ± 0.86 **a**	96.42 ± 2.28 **c**	19.19 ± 0.91 **b**

* Means ± standard error within a column followed by the same letter are not significantly different ($P \geq 0.05$, LSD test).
[#]All assays were performed with 15 larvae for each concentration (n = 60 per treatment) and repeated four times

Table 3. Effects of dietary juglone on the fecundity and fertility of *G. mellonella* female.

Juglone (mg / 2 g diet)[#]	Fecundity (Number of Eggs; per day / Female)	Fertility (% Eggs Hatched)
	Mean ± SE*	Mean ± SE*
Control	362.3 ± 21.1 **a**	90.8 ± 0.9 **a**
LC_{30}	239.5 ± 11.1 **b**	82.6 ± 2.6 **b**
LC_{50}	306.7 ± 15.9 **c**	78.8 ± 2.2 **bc**
LC_{70}	310.6 ± 17.5 **c**	73.8 ± 2.7 **c**

* Means ± standard error within a column followed by the same letter are not significantly different ($P \geq 0.05$, LSD test).
[#]All assays were performed with ten adults for each concentration (n = 40 per treatment) and repeated four times.

also prolonged developmental time (Lindroth et al. 1990). In the study on *S. frugiperda,* increased larval developmental time and mortality were reported (Yu 1987). The cause of these toxic effects was related to the decrease in food consumption and digestibility due to juglone. Previous studies also evinced that increases in doses led to decreases in larval-pupal body weights and prolongation of larval developmental time in some saturnid species and *G. molesta* fed with juglone (Thiboldeaux et al. 1998, Piskorski et al. 2011). Further, a study on species of Coleoptera revealed that methanol extracts of juglone negatively affect pupal development, nutrition and insect growth regulators in the bean weevil *Acanthoscelides obtectus* (Coleoptera: Chrysomelidae) and the Mexican bean beetle *Epilachna varivestis* (Coleoptera: Coccinellidae) species which are agricultural pests (Cespedes et al. 2016). Similarly, all concentrations of juglone given with larval diet caused prolongation of the larval period and a decrease in pupal and adult weights of *G. mellonella*. In addition to these effects, early stage deaths in larvae exposed to dietary juglone concentrations above LC_{50} (3, 4 and 5 mg / 2 g in diet) were observed. On the other hand, no changes occurred in juglone-treated groups of the non-feeding adult and pupal stages of *G. mellonella*. The reason for these effects may be due to the antifeedant effect of juglon or toxicity caused by the limitation of gut peristaltic movements and the mouth parts of *G. mellonella* larvae as mentioned in a previous study (Akhtar et al. 2012). For this reason, we suggest that the relationship between these data and the antifeedant effect be explored in histopathological studies

in the future. Further, the reduction in the total average number of eggs laid by a single female and egg hatchability showed that juglone had adverse effects on the reproductive potential of *G. mellonella*. These findings concur with the findings of the above-mentioned studies on phytophagous lepidopteran species (Lindroth et al. 1990, Thiboldeaux et al. 1998, Sun et al. 2007, Piskorski et al. 2011). Our results indicate that healthy egg production of *G. mellonella* exposed to sublethal concentrations of juglone in larval diets has a reduced ability to overcome the lethal effects. Therefore, the reduced adult fecundity of *G. mellonella* exposed to sublethal concentrations of juglone can lead to a decrease in pest population in the next generations.

Conclusion

Juglone is a naphthoquinone that has a larvicidal potential in a concentration dependent manner and could be used in IPM studies as a novel plant-based insecticide for *G. mellonella* or other storage pest insects instead of environmentally non-friendly chemicals, such as naphthalene, acetic acid, sulphur or other fumigants. Additionally, the results from our study indicate that the adverse effects of walnut leaves or fruits, used traditionally by beekeepers during storage of honeycombs or hives, on *G. mellonella* are due to juglone. Therefore, we suggest that further studies be performed to determine the ecotoxic effects of sublethal juglone concentrations detected in this study on non-target organisms.

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