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Evaluation of the chinaberry *Melia azedarach* extract against the tomato leafminer, *Tuta absoluta* (Lepidoptera: Gelechiidae) in vitro

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Evaluación del extracto de paraíso *Melia azedarach* contra el minador de las hojas del tomate, *Tuta absoluta* (Lepidoptera: Gelechiidae) *in vitro*

RESUMEN. El minador sudamericano de la hoja del tomate (TLM), Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae), es una plaga importante del tomate, la papa y otras especies solanáceas de relevancia económica. El manejo de plagas de TLM basado en el uso de insecticidas convencionales puede ser problemático debido a diversas causas, entre ellas, la reducción de los beneficios económicos por los altos costos de los insecticidas, la destrucción de las poblaciones de enemigos naturales, la presencia de residuos de insecticidas en los frutos del tomate y el rápido desarrollo de resistencia a los insecticidas, especialmente cuando se alcanzan niveles elevados de infestación. La búsqueda de compuestos activos menos persistentes beneficiaría a la producción hortícola, así como al medio ambiente y a los consumidores. En el presente estudio, evaluamos la toxicidad de un extracto de hexano en diferentes concentraciones obtenido de frutos frescos del árbol del paraíso Melia azedarach L. (MA), sobre huevos de TLM, larvas de segundo estadio y pupas mediante pruebas de contacto en el laboratorio. Los resultados indicaron que, al aumentar las concentraciones, también aumentaba la mortalidad de huevos, larvas y pupas. El análisis de los datos probit mostró que la CL50 para el extracto de MA en huevos de TLM, larvas del segundo estadio y pupas fueron 948,93, 346,72 y 1,75 µl/ml, respectivamente. Concluimos que este extracto tiene potencial para controlar *T*. absoluta, pero se deben realizar más estudios ecotoxicológicos para concluir sobre su seguridad en otros organismos y el medio ambiente.

PALABRAS CLAVE. Ensayos de toxicidad. Insecticida botánico. Minador sudamericano de hojas del tomate.

ABSTRACT. The South American tomato leaf miner (TLM), *Tuta absoluta* (Meyrick) (Lepidoptera: Gelechiidae), is an important pest of tomato, potato and other solanaceous species of economic relevance. Pest management of TLM based on the use of conventional insecticides can be problematic due to various causes, among them, the reduced economical profits from high insecticide costs, the destruction of natural enemies' populations, the presence of insecticide residues on tomato fruits and the rapid development of insecticide resistance, especially when high levels of infestation are reached. The search for less-persistent active compounds would benefit horticultural production, as well as the environment and consumers. In the current study, we evaluated the toxicity of a hexane extract at different concentrations obtained from the chinaberry tree *Melia azedarach* L. (MA) fresh fruits, on TLM eggs, 2nd instar larvae and pupae by means of contact tests in the laboratory. Results indicated that with increasing concentrations, mortality of eggs, larvae and pupae also increased. Analysis of probit data showed that LC₅₀ for MA extract in TLM eggs, 2nd instar larvae and pupae were 948.93, 346.72 and 1.75 µl/ml, respectively. We conclude that this extract has potential for controlling *T. absoluta* but further ecotoxicological studies should be carried out to conclude on its safety in other organisms and the environment.

KEYWORDS. Botanical pesticide. South American tomato leaf miner. Toxicity trial.

INTRODUCTION

The South American tomato leaf miner (TLM), Tuta absoluta (Meyrick) (Lepidoptera: Gelechiidae) is a key pest of tomato native to the western part of South America. It invaded Brazil around 1980 (Souza & Reis, 1992) and since then it became a devastating pest of tomato crops in South America, Europe, Africa, and Asia (Tropea Garzia et al., 2012; Zappalà et al., 2013). This pest continues spreading and affecting tomato production worldwide (European and Mediterranean Plant Protection Organization (EPPO), 2008, 2009; Urbaneja et al., 2012). In Iran, T. absoluta was first discovered in November 2010 infesting open-field tomato crops of Uromiyeh, Azerbaijan province. Northwestern Iranian region (Baniameri & Cheraghian, 2012). In this country, T. absoluta is currently considered a key insect pest on both greenhouse and open-field tomato crops. Damage is caused by larval tunneling and, ultimately, they can penetrate young stems and fruits resulting in the loss of over 80% of fruits (Desneux et al., 2011).

During the last decades, TLM control was directed to the use of chemical insecticides. Environmental safety of insecticides is the first and foremost criterion for TLM control programs (Tropea Garzia et al., 2012). In most countries where T. absoluta is present, the main control strategy includes frequent spraying with chemical insecticides (Gilardón et al., 2001; Tropea Garzia et al., 2012). Nevertheless, because of the development of resistance, chemical control has demonstrated limited efficacy even after the use of pesticides with different modes of action and increasing the application frequencies (Sigueira et al., 2000; Lietti et al., 2005). This has led to the use of alternative, nonchemical control strategies, such as conservation biocontrol provided by indigenous natural enemies (Zappalà et al., 2013) and the use of plant resistance among other techniques of less environmental impact (Gilardón et al., 2001). The utilization of eco-friendly and easily biodegradable plant products with natural insecticidal activity has increased in recent years. To control pests without disturbing the environment, natural products have been screened for potential sources of insecticides. These materials are considered alternative to conventional pesticides owing to their low toxicity to warm-blooded mammals, in addition to their high volatility. Botanical insecticides may be safer for the environment than synthetic insecticides, and they are usually easily processed and utilized by farmers and small industries (Belmain et al., 2001).

Interest on plant essential oils and extracts was renewed with emerging demonstrations of their insecticidal properties. They act in several modes of action on various types of pests and can be applied to plants in the same way as other conventional insecticides (Isman, 2000; Benelli et al., 2012). Most of plant extracts are known to possess ovicidal, repellent, larvicidal activities and

antifeedant activity against various insect species (Isman, 2000; Abbasipour et al., 2010, 2011; Rosa et al., 2010; Guedes et al., 2012; Sousa et al., 2013). The implementation of the Plantwise program resulted in reduction of control costs and environmental effects via the use of broad-spectrum pesticides against *T. absoluta* (Centre for Agricultural Bioscience International (CABI), 2019).

The "chinaberry tree" Melia azedarach L. (MA) is an ornamental species of the Meliaceae family considered to be native from Asia and exported to American, African, and European continents as well as to Northern Australia (Phua et al., 2008). It is a deciduous 3-10 m tree with sweet-scented lilac flowers during autumn and spring, dark green leaves, and a round-shaped fruit initially green and yellowish when mature (Botha & Penrith, 2009). Trees are cultivated in countries with template to warm climates where they are easily found in houses and parks as ornamental trees for protection against sunlight and winds. Previous studies demonstrated that this plant species has pharmacological and toxicological properties. Phytochemical composition of fruits includes melianoninol, melianol, melianone, meliandiol, vanillin, and vanillic acid (Han et al., 1991). Toxic compounds tetranortriterpens, known as meliatoxins, present in all the parts of the plant but specially concentrated in ripe fruits (Botha & Penrith, 2009). Aqueous and alcohol extracts prepared from different parts of MA have antibacterial (Sen & Batra, 2012), antiparasitic (Szewczuk et al., 2006), antifungal (Carpinella et al., 2003), antiviral (Alche et al., 2003), antioxidant properties (Marimuthu et al., 2013), and insecticide activity (Vergara et al., 1997; Carpinella et al., 2003). Studies demonstrated that extracts could have an anti-feeding effect due to tri-terpenoids that inhibit food intake capacity for some phytophagous insects, leading to lethal and sublethal impacts in next generations. Plant extracts prepared from leaves and fruits of MA have been tested on the bean weevil, Acanthoscelides obtectus (Say) (Coleoptera: Bruchidae) (Mazzonetto & Vendramim. 2003), mosquitoes (Pérez-Pacheco et al., 2004; Parra Henao et al., 2007) and moths (Rossetti et al., 2008). In Argentina, information exists regarding repellent and insecticidal properties of MA extracts on several insect species of economical and sanitary importance, such as Triatoma infestans (Klug) (Hemiptera: Reduviidae), Spodoptera eridania (Cramer) (Lepidoptera: Noctuidae), Cotesia ayerza (Brèthes) (Hymenoptera: Braconidae), and Epilachna paenulata (Germ) (Coleoptera, Coccinellidae) (Valladares et al., 2003, 2011; Rossetti et al., 2008). Considering the potential of this plant species to be applied as botanical insecticide, we performed tests using the hexane extract prepared from fruits of MA to assess the insecticidal properties on different developmental stages of T. absoluta. We aimed to include this less toxic and economic technique to complement the use of traditional synthetic pesticides for integrated management of this pest.

MATERIAL AND METHODS

Plant material and extract preparation

Chinaberry green fruits were collected from > 15-yr-old trees located in Shahed University Campus, College of Agriculture, Tehran, Iran as described by Chiffelle et al. (2019). A total of 3 kg of fruits were processed using the dry shade method, and afterwards grinded in a mechanical grain mill to obtain powdered material. The powder was dissolved in 100 ml hexane (Merck, Darmstadt, Germany) and then, the solution was homogenized with a magnetic stirrer (Heidolph, MR 3001K, Schwabach, Germany), at 37 °C for the first hour, and at room temperature to complete 24 h. The mixture was then covered with aluminum foil and parafilm and placed in the refrigerator. The homogenized mixture was filtered using Whatman No 1 filter paper and centrifuged for 15 min at 1500 rpm to obtain the supernatant (extract). After one hour, the remaining solution was filtered and placed in a glass Petri dish for 3-7 hours to completely removal of hexane, and finally a pure extract was obtained.

Insect rearing

To rear a colony of T. absoluta, tomato leaves with larvae were collected from infested tomato crops grown in greenhouses located in Karaj, Pakdasht and Varamin. Iran. Larvae were maintained in rearing cages 70 x 50 x 50 cm (height x width x depth) covered with voile containing tomato plants, Solanum lycopersicum L. var. Prances, in a climate-controlled room (27±2 °C, 65±5% RH and 16:8 h (L:D)) photoperiod. Second instar larvae used in the bioassays were obtained from rearing of the insect on tomato plants under greenhouse in the controlled climatic conditions. To obtain pupae of T. absoluta, leaves with larvae were collected from plants of greenhouse and placed in a plastic tray (40 x 30 x 6 cm) over a layer of sand (0.5 cm) arranged on a sheet of filter paper. The tray was kept in a thermo-conditioned room in the controlled climatic conditions. Daily, the sand was sieved, and the underlying sheet of paper was observed until insect reached the pupal stage. Pupae were transferred to a Plexiglas cage (50 x 30 x 30 cm) and observed periodically until adult emergence. Moths were transferred to new cages and were fed with honey droplets placed on the walls of the cage allowing oviposition for 24 h. Eggs were collected daily on individual tomato leaflets kept in a container with water and fastened at the top of the cage. Subsequently, the eggs were removed from the leaflets with a brush inside a glass recipient with distilled water, and then collected by filtration on polyester fabric.

Contact toxicity of extract on developmental stages of *Tuta absoluta*

Tuta absoluta eggs, 2nd- instar larvae and pupae were utilized to determine *M. azedarach* extract toxicity by means of a contact test (Hussein et al., 2015;

Ndereyimana et al., 2019). To determine the LC₅₀ for the egg stage, five concentrations (60, 144, 524, 1000, 2000 μl/ml) were selected. Two-day old eggs deposited by adult females on the leaves and trichomes were directly immersed in extract solutions for 5 seconds and were then kept under laboratory conditions to dry for 3 h. All the treated leaves and the eggs were moved to 8-cm Petri dishes. The treated Petri dishes were placed inside the growth chamber at 27±2 °C, 65±5% RH and 16:8 h (L:D) photoperiod and maintained for 6 days. To avoid the leaves getting dry, their petioles were put in a microtube containing water. For each concentration, 20 eggs of T. absoluta were considered along with an untreated control. The test was replicated three times. To determine the ovicidal effect of extracts, eggs with no growth or with dead embryos were recorded using a light stereo microscope (Zeiss Stemi 508) at the end of the experiment. To determine plant extract LC50 for the 2nd larval stage, six concentrations (60, 120, 250, 500, 1000, 2000 µl/ml) as treatments were selected. The leaf dip method was used for bioassay of larvae. The leaves were immersed for 5 seconds in the extract solutions and after drying, they were placed into 8 cm Petri dishes offered 10 TLM 2nd instar larvae (one-day old). The test was replicated three times. Control treatment was performed similarly but leaves were immersed into distilled water. The treated Petri dishes were maintained in a growth chamber at 27±2 °C, 65±5% RH and 16:8 h (L:D) photoperiod during 48 h. The larval mortality rate was recorded at the end of the experiment. Also, the effect of M. azedarach extract during 24, 48 and 72 h exposition on the mean mortality rate of 2nd instar larvae of *T. absoluta* was evaluated (Kim et al., 2002). Then, according to the number of insects killed, the percentage of losses for each concentration was obtained.

To determine LC50 at pupal stage, firstly individuals of similar age were reared from a cohort from the colony. To obtain the pupae, T. absoluta infested leaves were removed from plants and dissected to recover larvae, 14 days after oviposition. Third to fourth instar larvae were removed and placed in plastic, ventilated containers (15 x 10 x 7 cm) and provided with tomato shoots with leaves, to allow larvae to complete their development until pupation. Pupae for the bioassay were collected daily from the rearing containers. Ten 2 to 3-day old pupae were immersed for 20 seconds in solutions of same concentrations as used for larval bioassays. For each concentration, 10 pupae were considered per replicate. After drying, the pupae were transferred to a Petri dish and kept until the insects hatched (Park et al., 2002). This experiment was replicated three times for each concentration. The treated Petri dishes were placed inside the growth chamber at 27±2 °C, 65±5% RH and 16:8 h (L:D) photoperiod. Then, according to the number of lost pupae, the percentage of mortality for each concentration was obtained.

Statistical analysis

All experiments were assigned using completely randomized design. The data obtained were analyzed by two-way ANOVA analysis of variance using SAS software. If variance of means was significant, Tukey's test was used to compare them. Values of LC_{10} , LC_{25} , LC_{50} and LC_{90} with 95% fiducial limits (FL) were calculated, using probit analysis (SAS Institute, 2004).

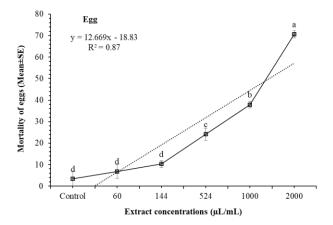
RESULTS

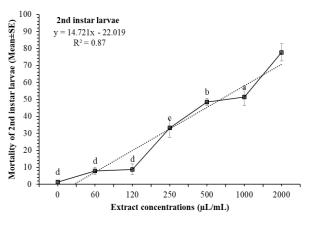
Melia azedarach extract significantly (P< 0.01) led to an increase in the number of killed eggs of T. absoluta (F= 97.69; df= 5; P< 0.01) compared with control treatment (concentration at 3.3%; α = 0.05) (Fig. 1). However, mortalities at the two initial concentrations (60 and 144 µl/ml) yielded 6.8 and 10.3% of dead eggs, respectively, and these did not show a significant difference with the control (α = 0.05). Greater concentrations (524 and 1000 µl/ml) increased mortalities significantly and reached 24.12% and 37.89%, respectively. Exposure of eggs to the maximum concentration (2000 µl/ml) produced the highest mortality (70.61%) (Fig. 1). A regression analysis between M. azedarach extract concentrations tested against mean egg mortality showed a significant linear correlation (P< 0.001) (Y= 12.67X - 18.83). The coefficient of explanation was equal to 0.87 (Fig. 1).

In relation to the TLM 2nd larval instar experiment, the application of *M. azedarach* extracts significantly (P< 0.01) led to an increase in the number of killed larvae (F= 113.23; df= 5; P< 0.01). Also, results showed that mean mortality of larvae increased significantly compared to the control treatment (Fig. 1). The three initial levels of concentration including control, 60 and 120 µl/ml with 1.23%, 7.78% and 8.80%, respectively, showed the lowest larval mortality (α = 0.05), and no significant differences were observed among means. Mortality of larvae exposed to other concentrations tested (250 and 500 µl/ml) reached 33.18% and 48.40%, respectively. The highest larval mortality (77.65%) was obtained at the maximum concentration (2000 µl/ml) (Fig 1). A linear regression analysis between M. azedarach extract concentrations and mean mortality of 2nd instar larvae showed a significant linear correlation (P< 0.001) (Y= 14.721X -22.019). The coefficient of explanation was equal to 0.87 (Fig. 1). Larval mortality recorded at 24, 48 and 72 h reached 21.67%, 29.44%, and 37.41%, respectively and differences among means were significant (F= 15.98; df= 2; P< 0.01). However, interactions of two factors, concentration and time were not significant (F= 1.09; df= 10; P> 0.05).

The exposure of *T. absoluta* pupae to the different *M. azedarach* extract concentrations also significantly (P<

0.01) led to an increase in the killed number (F= 39.32; df= 5; P< 0.01) (Fig. 1). Concentration at 120 μ l/ml increased mortality and reached 57.5%. Higher concentration levels (250, 500, 1000 and 2000 μ l/ml) led to pupal mortalities of 95.5%, 100% and 100%, respectively with no significant difference among these three levels (Fig. 1). Analysis of the relationship between applied *M. azedarach* extract concentrations and mean mortality of pupae showed a significant linear correlation (P< 0.001) (Y= 19.81X - 4.61). The coefficient of explanation was equal to 0.87 (Fig. 1).





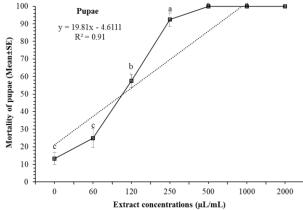


Fig. 1. Mortality of different developmental stages of the tomato leaf miner, *Tuta absoluta* exposed to *Melia azedarach* hexane extract in different concentrations. Different letters on the bar show statistically significant differences.

Bioassay results of LC₁₀, LC₂₀, LC₅₀ and LC₉₀ of $\it M.$ azedarach extract on TLM stages

The hexane extracts had a remarkable insecticidal activity against *T. absoluta*. The ovicidal activities were LC₅₀= 948.93 μ I/ml and LC₉₀= 10353.60 μ I/ml, the larvicidal activities were LC₅₀= 346.72 μ I/ml and LC₉₀=

3581.91 μ I/ml, and the pupicidial activities were LC₅₀= 1.75 μ I/ml and LC₉₀= 8.8 μ I/ml after 7 days, 72 hours and 7 days of exposure period, respectively. Also, the value of chi-square in the probit Table shows the appropriate fit of the data. This difference in LC₅₀ of different stages shows the difference in susceptibility of different stages (Table I).

Table I. LC_{10} , LC_{20} , LC_{50} and LC_{90} values (μ L/mL) of *Melia azedarach* hexane extract against different stages of the tomato leaf miner, *Tuta absoluta*.

Stage	$\mathrm{LC_{10}}^*$	$\mathrm{LC_{20}}^*$	LC_{50}^{*}	LC90*	slope ± SE	χ^2
Egg	86.97	197.54	948.93	10353.6	0.54±0.07	3.664 ^{n.s.}
(n=360)	(44.71-133.92)	(127.02-273.34)	(690.02-1452.09)	(5156.7-32514.5)		
2 nd larval instar	6.62	6.616	7.876	10.27	2.891±11.114	0.221 n.s.
(n=210)	(5.29-7.27)	(5.295-7.27)	(7.14-8.64)	(9.19-13.74)		
Pupa	0.151	0.151	1.865	2.84	1.684±7.028	0.412 n.s.
(n= 210)	(0.02-0.22)	(0.02-0.22)	(1.57-2.17)	(2.39-4.16)		

n= number of insects; χ^2 = chi-square; degree of freedom = 3 for all stages; *Mean and (fiducial limits); n.s.= non-significant

DISCUSSION

The result of this study showed that LC₅₀ for MA extract in TLM eggs, 2nd instar larvae and pupae were 948.93, 346.72 and 1.75 µl/ml, respectively. The bioactivity of MA botanical extract against T. absoluta is explained by the secondary metabolites produced by plant extracts, which have various modes of action (Gurjar et al., 2012; Ndereyimana et al., 2019). The viability of T. absoluta larvae was significantly altered by fruit extracts of M. azedarach. All the extract concentrations investigated exhibited significant differences between the means in relation to the control treatment, with a reduction in the viability proportional to the increasing concentration of the extract. These results confirm the insecticidal activity of M. azedarach, as reported for other lepidopterans, including medinalis (Guenée) Cnaphalocrocis (Lepidoptera: Crambidae) (Nathan, 2006), S. eridania (Rossetti et al., 2008), Plutella xylostella L. (Lepidoptera: Plutellidae) (Dequech et al., 2009) and T. absoluta (Brunherotto et al., 2010).

The results showed that the effect of M. azedarach extract on T. absoluta pupae was greater than on eggs and 2nd instar larvae. The LC₅₀ value of pupal stage with 1.75 µl/ml was much lower than egg stage with 948.93 µl/ml and 2nd instar larval stage with 346.72 µl/ml which indicated that this stage was more susceptible than the other two stages. In insects, the pupa is an inactive, transformative stage, covered with a cocoon around it, so that different developmental stages of insects display different sensitivity to the same insecticide, which also provided a physiological explanation of the relevant mechanism of the difference of sensitivity of insect at its larval and pupal stages to insecticide (Pedersen et al., 2020). In the egg stage, the presence of chorion caused much less permeability than the other two stages. Experiments carried out using a similar extract showed higher percentages of mortality on the cabbage white

butterfly, Pieris brassicae L. (Lepidoptera: Pieiridae) larvae compared to Azadirachta indica A. Juss, Lantana camara L. Moldenke., Cannabis sativa Linn, Nerium indicum Mill., Eucalyptus sp., Ricinus communis Linn. and Solanum nigrum Linn. extracts. The effect of 2.5% concentration of aqueous extract showed that this extract can cause up to 28.6% larval mortality after 24 hours (Sharma & Gupta, 2009). In previous studies, the effect of M. azedarach aqueous extract on T. absoluta pupae extended the growth period and reduced the survival rate in compared to control. Also, it had a decreasing effect on the pupal weight of males compared to the control but had no effect on the weight of the female pupae. A comparison between larval and pupal data showed very similar values, which is a rare result. According to Rodríguez and Vendramim (1998), the effect of insecticides on insect residues in the larval stage was more severe than in the pupal stage, because it is at this stage that the insect swallows chemicals (Brunherotto & Vendramin, 2001).

Consistent with the results of this study, Abou-Fakhr Hammad et al. (2019), also pointed to the significant lethal effects of *M. azedarach* extract on *T. absoluta*. It has been suggested that the mortality is due to the systemic action of this extract, which leads to penetration into the eggand subsequent lethality. Also, in Abou-Fakhr Hammad et al. (2019) study, the lethal effects of *M. azedarach* extract on *T. absoluta* were evaluated like the effects of neem extract.

The results of the present study suggest that the *M. azedarch* extract can be used to protect crops against the extensive damage caused by *T. absoluta* larval feeding. Our study demonstrates that the hexane extract of *M. azedarach* fruits was the most active toxicant to eggs, second instar larvae and pupae of *T. absoluta*. This organic extract has potential for development as botanical insecticide. Since the plant is easily grown and widespread, resource availability should not be an issue and the crude extract of *M. azedarch* can be used as a

crop protectant for local use in Iran and many other regions of the world, however further ecotoxicological studies should be carried out to conclude on its safety in other organisms and the environment.

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