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In vitro acaricidal activity of *Melia azedarach* ripe fruit extract against *Hyalomma excavatum* (Acari: Ixodidae)

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ABSTRACT

The current study aimed to evaluate the effect of dichloromethane extract of *Melia azedarach* ripe fruit on larvae and adult females of *Hyalomma excavatum* at concentrations of 0.25, 0.5, 1, 2, and 4%, using the larval immersion test (LIT) and adult immersion test (AIT). The results showed that in LIT, the percentage mortality of larvae was significantly higher at concentrations 1, 2, and 4% than that in the control group after 24 h. While the mortality rates varied from 8.66% to 72.66% after 24 h post-treatment, complete mortality of the examined larvae was achieved at a concentration of 4% after 48 h post-exposure whereas, it was13.33% in the negative control group. In AIT, the percentage inhibition of oviposition in the treatment groups was significantly greater than that in the control group ($p < 0.01$). The maximum inhibition of oviposition was 17.72%, which was achieved at a concentration of 4% and it was 0% in the control group. The difference between reproductive index in treatment and control groups was not statistically significant ($p > 0.01$). This study showed that the ripe fruit extract of *M. azedarach* was toxic to *H. excavatum* under laboratory conditions.

Keywords

Melia azedarach, Hyalomma excavatum, Azadirachtin, plant-based acaricide

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Abbreviations

DCM: Dichloromethane
LIT: Larval immersion test
AIT: Adult immersion test
AZA: Azadirachtin
IO: Inhibition of oviposition
RI: Reproductive index

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Introduction

Hard ticks (*Arachnida: Acarina: Ixodidae*) are obligate blood-feeding ectoparasites that affect both human and animal health via sucking blood and transmission of some pathogenic agents such as *Babesia spp., Theileria spp., Anaplasma spp.,* and *Nairovirus* [1, 2]. Anxiety, irritation, stress, skin damage, weight loss, tick paralysis, decrease in milk production, loss of production, and anemia are direct adverse consequences of infestation with the hard ticks [2].

Nowadays, tick control relies on using synthetic pesticides. Although these compounds are more available and possess fast-killing effects, intensive and repeated use of them to control tick infestations has resulted in developing resistance to an array of acaricides. *Rhipicephalus (Boophilus) microplus* resistance to permethrin was recorded in the USA and Mexico [3]. Resistance to cypermethrin and deltamethrin in *Hyalomma anatolicum* has been reported from India [1, 4]. Tick resistance to conventional pesticides and increased demand for organic products has accelerated the research on plant-based acaricides [5].

Melia azedarach, belonging to the *Meliaceae* family, is a well-known source of various bioactive components with insecticidal properties. This deciduous tree species native to Indomalaya and Australasia is now cultivated in most subtropical and tropical regions of the world [6, 7]. Acaricidal, insecticidal and larvicidal efficacy of *M. azedarach* extract against agriculture pests, mosquitoes, important veterinary ticks, and mites have already been reported [8-11]. *M. azedarach* has been reported to have a complex mixture of compounds including saponins, terpenoids, flavonoids, tannins, alkaloids, and limonoids [6]. Limonoids particularly azadirachtin (AZA) constitute the biologically active components of *M. azedarach* fruits. However, other limonoids such as nimbin, nimbolinin, and salannin have also been reported from the *M. azedarach* fruits [6, 12, 13] AZA is the most important limonoid and biopesticide of this plant which its toxicity and adverse effects on feeding, growth, fecundity, and oviposition of arthropods, especially for phytophagous insects have been proven [14-16]. This compound is found in different parts of the *M. azedarach* tree and the highest level of AZA is generally found in seeds [17]. Although several methods have been reported for the identification and quantitative determination of azadirachtin, most studies have used high-performance liquid chromatography (HPLC) for this purpose worldwide [17]. The AZA content in various parts of trees is influenced by several factors such as genetic, climatic conditions, harvesting time, geographical area, and time of collection/storage of plant materials [17].

Adult *Hyalomma excavatum* ticks (known as large Anatolian *Hyalomma*) infesting cattle, sheep, horses, goats, camels, and donkeys are found almost all over Iran except the Caspian Sea area [18-21]. This *Hyalomma* species serves as a vector for some pathogens particularly *Theilera annulata* and Razmi et al. (2003) reported a high rate of *T. annulata* infection in examined *H. excavatum* collected from cattle in Mashhad area, Iran [21, 22].

Due to the high prevalence of *H. excavatum* in most parts of Iran and the necessity of searching for less hazardous and eco-friendly alternatives for synthetic acaricides, the present study aimed to evaluate acaricidal effects of *M. azedarach* fruit extract on *H. excavatum* under laboratory conditions.

Results

Larval immersion test

In larval immersion test (LIT), the percentage mortality of larvae was significantly higher at concentrations 1, 2, and 4% than the control group after 24 h. The mortality rates varied from 8.66% to 72.66%, 24 h post-treatment, and 100% mortality of examined larvae was achieved at concentrations 4%; 48 h post-exposure. The extract killed 100% of the tick larvae at all concentrations after 72 h, while the mortality rate was 17.30% in the control group (Figure 1, Table 1).

The LC50 and 99 (lethal concentrations of 50 and 99%) values of this extract against examined tick larvae were calculated at 24 and 48 h post-exposure and is presented in Table 2.

Adult immersion test

In adult immersion test (AIT), the percentage inhibition of oviposition in treatment groups was sig-

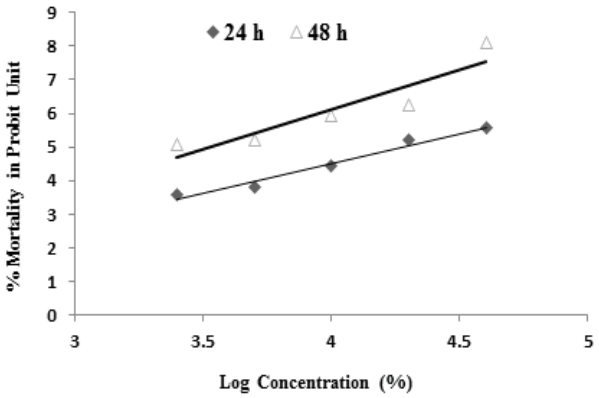

Figure 1.
Linear regression curve of percentage mortality of *H. excavatum* larvae in Probit unit versus logarithm concentration of *M. azedarach* ripe fruit extract.

nificantly greater than the control group ($p < 0.01$). The maximum inhibition of oviposition was 17.72%, which was achieved at a concentration of 4% and this parameter was concentration-dependent. The difference between reproductive indexes in the treatment and control groups was not statistically significant ($p > 0.01$). The detailed data about the inhibition of oviposition and reproductive index is presented in Table 3.

Discussion

The LIT results demonstrated a different level of larval mortality. Besides, the percentage of mortality of exposed larvae was concentration and time-dependent. These findings confirm several similar studies that investigated the effectiveness of *M. azedarach* crude extract against ticks, mites, and mosquitoes [8, 10, 11]. Borges et al. (2003) showed that the hexane extract of *M. azedarach* ripe fruit was effective against *Rhipicephalus* (*Boophilus*) *microplus* larvae in a concentration and time-dependent manner [8]. The acaricidal effects of this extract against *Demanyssus gallinae* and *Tetranychus urticae* have been reported [10, 23]. Furthermore, Selvaraj and Mosses (2011) observed that leaf and seed extract of this tree produced significant larval mortality in all larval stages of *Anopheles stephensi*, *Culex quinquefasciatus*, and *Aedes aegypti* [11]. Azadirachtin is recognized as the main active ingredient and the most important component of *Azadirachta indica* and *M. azedarach*. Feed deterrency, growth reduction, increase in mortality, abnormal/delayed molts and down-regulation of insects' reproductive organs have been observed after applying AZA [14]. The other known bioactive components of *M. azedarach* include meliartenin, meliacaprin, meliacin, meliantrol, melianol, salannin, nimbin, and pinoresinol bis-epi-pinoresinol with anti-feeding/growth activities, repellency, inhibition of oviposition, and embryogenesis properties [24, 25]. In the present study, a high mortality rate in examined tick larvae was observed after a short time post-exposure. This fast-killing effect may be related to inhibition of cell division and protein synthesis in cells of ectoparasites [14].

In this study, egg production in exposed ticks was significantly lower than that in the control group, and this extract at the concentration of 4% produced 17.72% inhibition of egg production. In a similar study, the ripe fruit extract of *M. azedarach* at the concentration of 0.25% caused complete egg production inhibition in *Boophilus microplus* [8]. Variation in efficacy with different concentrations may be associated with genetic characteristics of the plant, edaphoclimatic conditions, and even duration of storage affecting the chemical composition of a plant extract [26, 27]. Reproductive disruption in examined ticks can be attributed to azadirachtin that affects reproductive tissues at molecular/cellular levels and disrupts endocrine processes by altering ecdysteroids and juvenile hormone titers [14]. Sousa et al. (2013) observed a

Table 1.
Means ± SD of mortality rates of *Hyalomma excavatum* larvae in treatment and control groups exposed to different concentrations of *M. azedarach* ripe fruit extract 24, 48, and 72 h post-exposure.

Time (h)	Control group (water + tween)	Case groups (% *M. azedarach* extract)				
		0.25	0.5	1	2	4
24	3.33 ± 5.77 ^{Aa}	8.66 ± 3.21 ^{Aa}	15.33 ± 4.50 ^{ABa}	29.00 ± 9.64 ^{Ba}	58.33 ± 17.55 ^{Ca}	72.66 ± 16.16 ^{Ca}
48	13.33 ± 7.63 ^{Aa}	53.66 ± 9.81 ^{Bb}	59.33 ± 7.37 ^{Bb}	82.00 ± 23.06 ^{Db}	89.66 ± 17.89 ^{Db}	100.00 ± 0.00 ^{Db}
72	17.30 ± 8.32 ^{Aa}	100.00 ± 0.00 ^{Bc}	100.00 ± 0.00 ^{Bc}	100.00 ± 0.00 ^{Bb}	100.00 ± 0.00 ^{Bb}	100.00 ± 0.00 ^{Bb}

Different capital letters within rows and small letters within columns indicate significant differences ($p < 0.05$).

Table 2.
The LC50 and LC99 values of *M. azedarach* ripe fruit extracts against *H. excavatum* Larvae.

Time (h)	Slope (95% CL)	R^2	LC50 (%) (95% CL)	LC99 (%) (95% CL)
24	1.77 ± 0.17	0.97	1.86 (1.77-1.96)	38.40 (36.48-40.32)
48	2.33 ± 0.56	0.85	0.33 (0.31-0.35)	3.32 (3.15-3.48)

CL: Confidence Limit

Table 3.
Means ± SD of mortality rates of *Hyalomma excavatum* larvae in treatment and control groups exposed to different concentrations of *M. azedarach* ripe fruit extract 24, 48, and 72 h post-exposure.

Tick reproduction	Control group (water + tween)	Case group (% *M. azedarach* extract)				
		0.25	0.5	1	2	4
Inhibition of oviposition (%)	0.00 ± 0.00	4.14 ± 2.19	6.66 ± 2.89	8.74 ± 3.97[a]	14.26 ± 6.07[a]	17.72 ± 6.15[a]
Reproductive index	0.63 ± 0.06	0.60 ± 0.05	0.59 ± 0.03	0.57 ± 0.03	0.54 ± 0.06	0.52 ± 0.08

[a] Significant differences compared with the control group (p <0.05).

reduction in ovary weight, morphological changes in oocysts, vacuolization, chorion deformity, and disorganization of the yolk granules of engorged females *Rhipicephalus (Boophilus) microplus* treated with *M. azedarach* hexanoic extract [28]. To fully understand the various acaricidal effects of *M. azedarach*, further studies are required to identify all its bioactive acaricide components with their special effects at cellular and molecular levels.

Presently, there is scant published data on LC50 values for *Hyalomma spp.* exposed to *M. azedarach* extract. The current study recorded an LC50 value of 1.86% for *H. excavatum* treated with dichloromethane extract of *M. azedarach* 24h post-exposure. LC50 values of 0.26 and 4.17% were reported for *Hyalomma dromedarii* nymphal stage exposed to petroleum ether and ethyl alcohol extract of *M. azedarach*. Also, these extracts showed a significant effect on *H. dromedarii* eggs with LC50 values of 3.14 and 1.77%, respectively [29]. LC50 value of 1.78% was recorded for *Dermanyssus gallinae* tereated with Hexan extract of *M. azedarach* [10]. These variations in LC50 values can be attributed to the type of solvent used for plant extraction, susceptibility of exposed ectoparasite species, and its developmental stage.

Besides the laboratory studies, Borges et al. (2005) and Sousa et al. (2011) evaluated the efficacy of hexane extract of ripe fruits of *M. azedarach* against all developmental stages of *R. microplus* [30, 31]. Standardized laboratory and farm tests need to be developed to add along with this incremental process for finding natural pesticides.

In conclusion, the findings of this study showed that *M. azedarach* ripe fruit extract was effective against larvae and engorged females of *H. excavatum* ticks, and more studies are required to investigate its efficacy in field trials.

Materials & Methods

Collection of ticks

Adult males and females of *H. excavatum* were taken from an active tick colony rearing in the Faculty of Veterinary Medicine's parasitology laboratory, Ferdowsi University of Mashhad, Iran. The identification of ticks was made using a stereomicroscope based on morphological criteria [21].

The developmental stages of ticks

Unfed mixed sex adult ticks were experimentally fed on healthy pathogen-free rabbits (male) at room temperature. 180 adult engorged female ticks were used for adult immersion test (AIT), but a group was placed into tubes individually and kept at 28 ºC, 80% RH to oviposit. Eggs were harvested and divided into groups of 300 eggs and transferred into perforated tubes. Newly hatched larvae were maintained at 28 ºC, 80% RH in an incubator and used for larval immersion test [32].

Preparation of the crude extract

The collected *M. azedarach* ripe fruits from the campus of the Ferdowsi University of Mashhad, Mashhad, Iran, were dried in the shade at room temperature and powdered using a grinding machine. The powder was extracted with dichloromethane (DCM) in the Soxhlet extraction apparatus and the solvent was removed by a rotatory evaporator. The residue was serially diluted with distilled water to obtain desired concentrations of 0.25, 0.5, 1, 2, and 4% and Tween 20 was used as emulsifier to ensure complete solubility of the materials in water [8, 10].

Larval immersion test

Larval immersion test (LIT) was performed based on the methodology described by Singh et al. (2017) [33]. Approximately 300 14-21 day old larvae were immersed in 0.5 ml of each desired concentration for 10 min. Then, the larvae were transferred on a filter paper to dry and 100 larvae were taken and placed in a folded filter paper packet (7.0 cm by 7.0 cm) using an aspirator. The pockets were sealed with adhesive tape and incubated at 28 °C and 80% RH. The packets were opened after 24, 48, and 72 h for counting live and dead larvae [33]. The mortality rate was corrected using the Abbott formula if mortality in the control group was between 0 and 5%:
Corrected mortality % = % test mortality - % control mortality/100 - % control mortality × 100 [34].
Distilled water + Tween 20 was used as a negative control and the larval immersion test for each concentration was repeated three times.

Adult immersion test

The AIT was performed as described by Godara et al. (2015) and the FAO (2004) [35, 36]. The engorged female ticks were weighed and allocated to groups with 10 ticks and each group was immersed in 30 ml of the prepared concentrations, namely 0.25, 0.5, 1, 2, and 4% for 5 min. The control group was treated with distilled water + Tween20. After sieving, the retained ticks were placed onto a clean tissue paper towel for drying and kept separately in a Petri dish. The ticks were stored in an incubator at 28 °C and relative humidity of 80% to oviposit. Eggs laid by every tick were weighted and incubated to hatch

[35, 36]. There were three replications for each concentration and control.
The percentage Inhibition of Oviposition (IO) and Reproductive Index (RI) was calculated using the following formula:

Reproductive Index (RI) = Average weight of eggs laid / Average weight of live tick

Percentage inhibition of oviposition (IO) = RI of control ticks – RI of treated ticks / RI of control ticks × 100

Statistical analysis

The data were subjected to SPSS software ver. 25 (IBM corporation, USA). Statistical analysis of the data was performed using the variance analysis (ANOVA and General Linear Model) followed by Duncan's multiple range test with a probability level < 0.01.
The lethal concentrations for 50% (LC50) and 99% (LC99) with their respective 95% confidence limits (CL) were determined using regression analysis equation to the probit transformed data of mortality.

Authors' Contributions

AM and AMJ created the original idea. SG carried out the experiments, and AM, AMJ, SY, and MA directed the project. All authors analyzed and interpreted the data. AM and SG contributed to the writing of the manuscript.

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Conflict of interest

The authors declare that there is no conflict of interest.

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