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Acaricidal Effect of Propolis against *Hyalomma* Spp. Ticks and Assessment of Its Toxicity with the MTT Assay

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ABSTRACT

Background: The *Hyalomma* species is one of the most important vectors of pathogens responsible for human and animal diseases. The use of substances of natural source has been proposed as a safe way to control ticks. We aimed to evaluate the acaricidal activity of hydroalcoholic extract of honey bee propolis against the *Hyalomma* spp. *in vitro* and to determine its toxicity by MTT assay.

Methods: The acaricidal activity of propolis in concentrations of 50, 100, 150, and 200 mg/ml was investigated after 15, 30 and 60 minutes by two spray and contact methods. The main compounds of propolis were carried out with Gas Chromatography-Mass Spectrometry (GC–MS). Then the toxicity of each concentration was evaluated by MTT assay. Data were analyzed by GraphPad Prism 6 software.

Results: The concentration of 200 mg/ml of propolis had the highest acaricidal effect (90%) in the exposure time of 60 minutes and spray method was more effective than the contact method. The GC-MS analysis identify that Hexane (CAS); n-Hexane (17.32%) is the main ingredient of propolis. The results of the MTT toxicity test showed that toxicity increases with increasing concentration, and low concentrations of propolis have very little toxicity.

Conclusion: The hydroalcoholic extract of propolis contains potent acaricidal compounds and it might be used as a natural acaricide compound to against *Hyalomma* spp. However, further studies are needed to evaluate the effectiveness of the propolis.

Keywords: Acaricide, Propolis, *Hyalomma* spp., GC-MS, Toxicity

Introduction

Ticks are blood-sucking ectoparasites of vertebrates and humans that transmit various pathogens including protozoa, bacteria, rickettsials, and viruses during blood feeding (1). Ticks are the cause of weight loss, anorexia, anemia, hemoglobinuria, poisoning and general stress of animals and decrease livestock production (2). *Hyalomma* spp. is one of the most important tick species in Eurasia and Africa. It is the most important vector of protozoa such as *Theilaria*, *Babesia*, *Anaplasma*, as well as Crimean-Congo hemorrhagic fever virus (CCHF) (3). The use of chemical acaricides significantly reduces tick populations but causes resistance in ticks and poses environmental risks (4). Recently, the use of green pesticides has been proposed as an alternative to chemical pesticides. This issue is particularly supported by organic food producers and nature lovers (5).

Propolis is a resinous substance made by bees. Propolis is composed of resin, wax, essential and aromatic oils, pollen, and other organic materials, and its color and flavor vary depending on the plant origin of each region (6). Bees use propolis to seal their cavities, and it can protect the colony due to its antiviral, antimicrobial, and repellent properties (7). Recently, anti-parasitic, anti-viral, immune-stimulating, healing, anti-tumor, anti-inflammatory, antioxidant, anti-tumors and analgesic activities of propolis have been evaluated worldwide (8).

We aimed to evaluate acaricidal activity of hydroalcoholic extract of propolis against the *Hyalomma* spp. *in vitro* and to determine its toxicity by MTT assay.

Methods

Ethics approval

This study was approved by the Ethics Committee of University of Tabriz, Tabriz, Iran. The reference number for the ethical approval is IR.TBZMED.VCR.REC.1401.042.

Preparation of propolis

The collection of propolis was done with the help of beekeepers from the hives of villages around Tabriz/Iran. One hundred g of propolis was mixed with 400 ml of 70% ethanol and the tubes were sonicated for 2 h. The solutions were filtered using Whatman cellulose filters. The obtained solution was placed in a 28 °C incubator to dry completely. The powder was weighed on the residue and working concentrations (50, 100, 150, and 200 mg/ml) of propolis were prepared by dissolving the required amount of propolis in distilled water. The rest of the source was kept in a refrigerator at 4 °C for GC-MS analysis and MTT assay (10).

Tick collection

Female ticks were collected from the bodies of infected sheep and cattle. The collected ticks were placed in wide-mouthed rubber containers and transported to the Parasitology Laboratory of the Faculty of Veterinary Medicine, University of Tabriz, Tabriz, Iran for species determination. Members of the genus Hyalomma are all characterized as being large ticks with an elongated palps, fairly long hypostome, distinct eyes, and banded legs (9).

The criteria for including ticks in the study were that they were alive and healthy. Female ticks were used, and only the *Hyalomma* species was included in the study. The remaining unhealthy and discolored ticks, males, and other species were excluded from the study. The sample size was 80 ticks.

Evaluation of acaricidal activity of propolis *by spraying methods*

In an *in vitro* condition, the acaricidal activities of the propolis were evaluated at concentrations of 50, 100, 150, and 200 mg/ml at exposure times of 15, 30 and 60 min, were performed.

Each was evaluated using two methods: spray and contact. For the spraying method, different concentrations of propolis were sprayed directly on ten adult female ticks. The cypermethrin 10% (Hekar, Iran) was administered as a positive control. Since cypermethrin is used in livestock farms to control ticks, it was used as a positive control in this study. Cypermethrin concentrations of 50, 100, 150, and 200 mg/ml were prepared and ticks were treated for 15, 30 and 60 min. To monitor the acaricidal activity, all groups were exposed to propolis preparation for 15, 30 and 60 minutes. After the end of the time, the legs of the ticks were touched with an entomological pin under the stereo microscope (Olympus, Okayama-shi Okayama, Japan), if the legs did not move, the tick was considered dead. Two repetitions were considered for each dilution (10).

Evaluation of the acaricidal effect of propolis by contact methods

For contact method, under optimal conditions, the circular filter papers of 4.8 cm in diameter were treated with the provided concentrations of propolis (50, 100, 150, and 200 mg/ml). After drying at room temperature, ten live adult ticks were transferred to dry filter paper. Water-soaked cotton was placed in the Petri dishes to provide humidity. Finally, the Petri dishes were capped and sealed with parafilms. After 15, 30 and 60 min, the legs of the ticks were touched with an entomological pin under the stereo microscope (Olympus, Okayama-shi Okayama, Japan) (10).

Gas-Chromatography/Mass Spectrometry (GC-MS) analysis

Chromatography was performed using (Agilent GC/MS19091S-433, USA). The propolis was mixed with hexane (Merck KGaA**,** Darmstadt, Germany) (1:1). The solution was placed on the shaker until it was homogeneously mixed. Then the mixture was put it a separator, kept for 15 minutes to form double phase and the hexane phase was isolated and injected in the GC/MS (11).

Toxicological effects of propolis *on* Hyalomma *spp.*

The cytotoxicity of the propolis was determined by the MTT assay. To measure cytotoxicity, 1×10^4 *Vero* cells were cultured at a 96-well plate for 24 hours. The cells were cultured in four replicates at 50, 100, 150, 200 and 250 mg/ml, respectively, and incubated again for 48 hours. Then, 50 µL of serum-free media and 50 µL of MTT solution (Thiazolyl Blue Tetrazolium Bromide 98%, Sigma-Aldrich Co., USA) into each well and the plate incubate at 37 °C for 3 hours .The supernatant was then removed and 150 µl of DMSO was added to dissolve the Formosan crystals. At each step, centrifugation was performed to remove the liquid. The absorbance was recorded at 570 nm using a plate reading spectrophotometer (AquaLabo, UVILINE 9600, France). Cell viability percentage was calculated by the following formula:
Percentage of cell viability = (OD negative control / OD of tested sample)100%

Statistical analysis

The data were analyzed using excel software version 2023 and expressed as a mean ± SD. Data were analyzed by a two-way ANOVA for the comparison between the test and control.

Results

The results of spraying method showed that the concentration of 200 mg/ml hydroalcoholic extract of propolis had the highest acaricidal effect (90%) during the exposure time of 60 minutes and propolis at a concentration of 50 mg/ml in 15 minutes exposure time had the lowest acaricidal effect (0%). The spraying method was more effective than the contact method. So that the highest acaricidal activity was observed in the contact method at a concentration of 200 mg/ml in 60 minutes (50%).

The mortality rate of ticks after exposure to different concentrations of the propolis at various exposure times are presented in Table 1, Figures 1 and 2. Different concentrations of all treatments (propolis and Cypermethrin) had a significant difference in the two methods of evaluating ($P < 0.0001$). The amount of IC 50 in the spraying method is at a concentration of 100 mg/ml in 15 minutes, and its amount in the contact method is at a concentration of 200 mg/ml in 60 minutes.

Gas chromatography-mass spectrometry (GC-MS) showed that hexane (CAS); n-hexane (17.32%), cyclopentane methyl (16.62%), hexadecanoic acid (6.03%), cyclohexane (3.27%), pyrrolidine (2.16%), pentane, 2-methyl- (CAS) (2.16%), 2-phenyl-3-ethyl-6-methoxyindeno (1.74%), Di-(2-ethylhexyl) phthalate (1.4%), 18-methyl-19-oxoicosanoic acid (0.7%), benzene ethan amine (0.6%), pentane (0.4%), benzene (0.3%), and acetic acid (0.3%), respectively as the ingredient of propolis (Figure 3). The results of MTT toxicity test showed that propolis have very little toxicity in all concentrations Figure 2.

Table 1: The results of evaluating the acaricidal effect (%) of propolis against *Hyalomma* spp. *in vitro*

Concentration	*Time of exposure*	*Spraying method*	*Contact method*	*Positive control*	*Negative control*
50 mg/ml	15 min	0.0 ± 0.0	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0
	30 min	10 ± 0.0	0.0 ± 0.0	100 ± 0.0	0.0 ± 0.0
	60 min	40 ± 0.0	10 ± 0.0	100 ± 0.0	0.0 ± 0.0
100 mg/ml	15 min	45 ± 4.62	25 ± 4.62	100 ± 0.0	0.0 ± 0.0
	30 min	60 ± 0.0	25 ± 4.62	100 ± 0.0	0.0 ± 0.0
	60 min	70 ± 0.0	35 ± 4.62	100 ± 0.0	0.0 ± 0.0
150 mg/ml	15 min	70 ± 0.0	35 ± 4.62	100 ± 0.0	0.0 ± 0.0
	30 min	75 ± 4.62	40 ± 0.0	100 ± 0.0	0.0 ± 0.0
	60 min	85 ± 4.62	40 ± 0.0	100 ± 0.0	0.0 ± 0.0
200 mg/ml	15 min	80 ± 0.0	45 ± 4.62	100 ± 0.0	0.0 ± 0.0
	30 min	85 ± 4.62	45 ± 4.62	100 ± 0.0	0.0 ± 0.0
	60 min	90 ± 0.0	50 ± 0.0	100 ± 0.0	0.0 ± 0.0

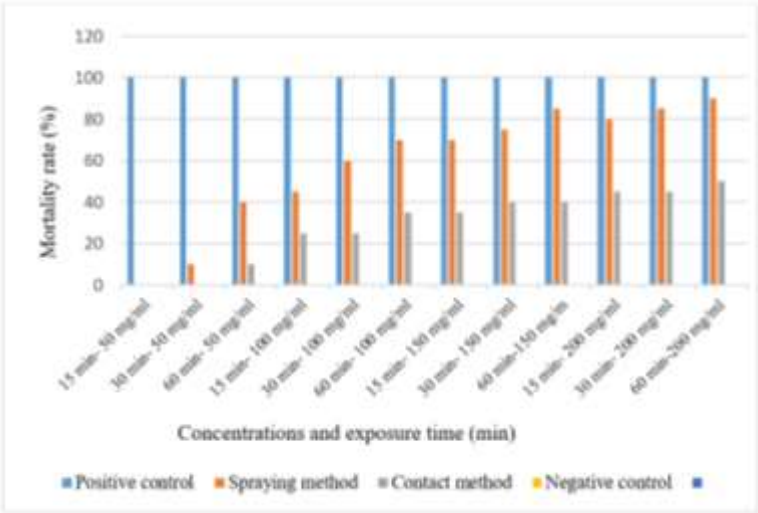

Figure 1: Acaricidal effects propolis against *Hyalomma* spp. by spraying and contact method

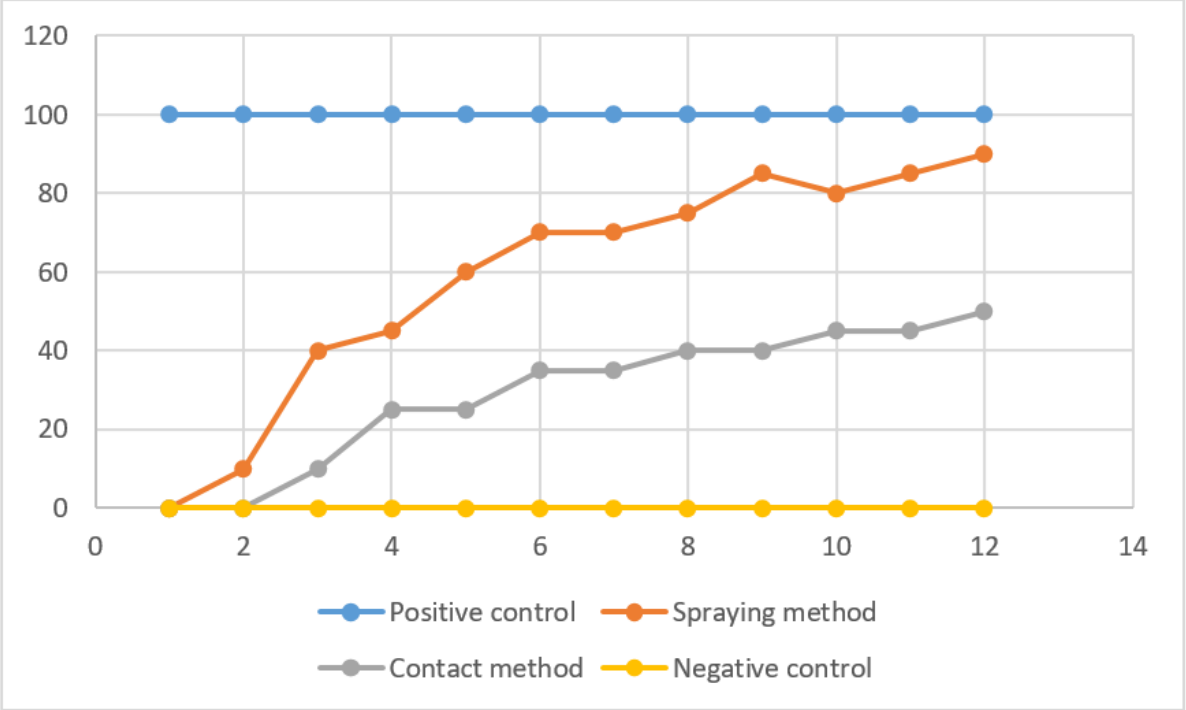

Figure 2: Linear regression diagram of the effect of propolis on *Hyalomma* spp.

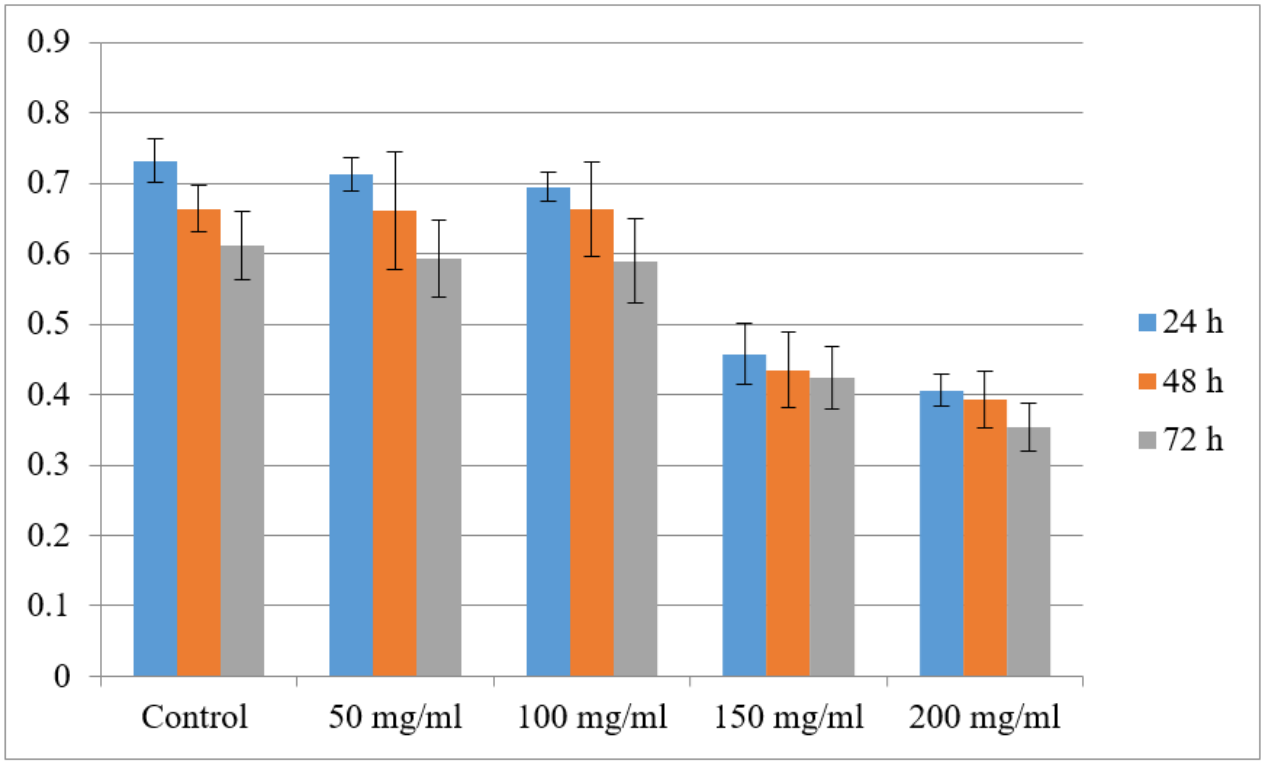

Figure 3: Result of MTT assay on *Vero* cells

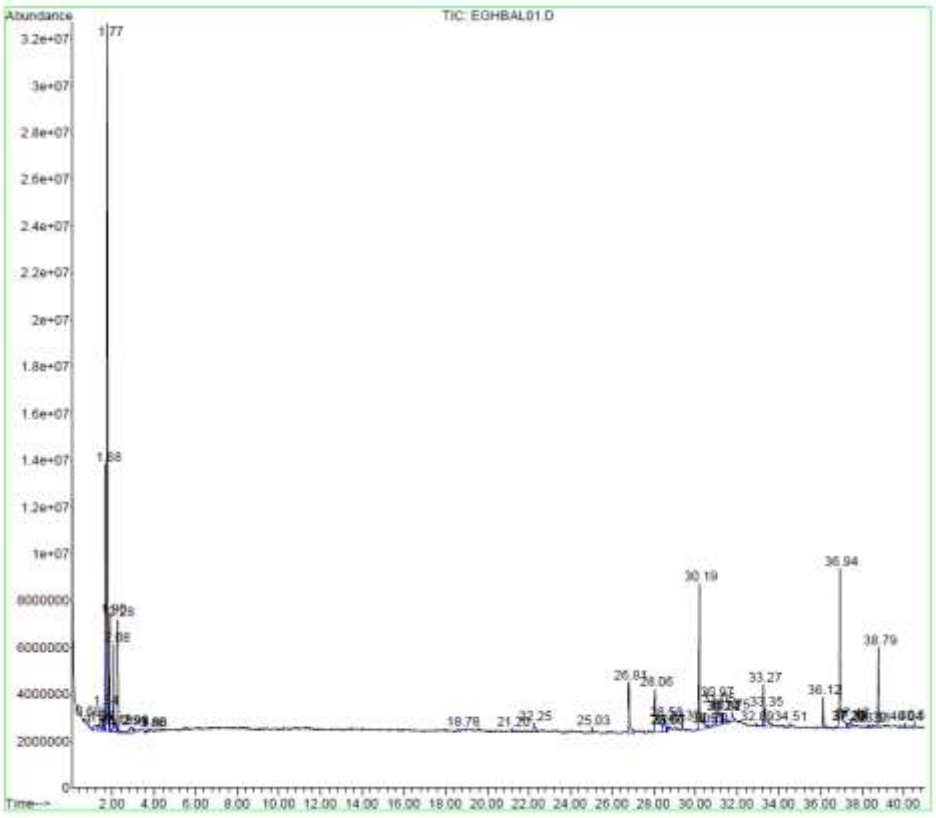

Figure 4: Gas chromatography/mass spectrometry (GC-MS) analysis of propolis

Discussion

We aimed to evaluate the acaricidal activity of hydroalcoholic extract of honey bee propolis against the *Hyalomma* spp. *in vitro* and to determine its toxicity by MTT assay.

The ticks as vectors transmit a large number of pathogens that cause diseases in animals and humans, commonly known as tick-borne diseases. The incidence of these human diseases in Iran has been underestimated due to poor surveillance and the small number of available studies (12).

Chemical acaricides have long been the most effective means of fighting ticks, but the emergence of resistance has called the effectiveness of chemicals into question. Nowadays, the development of alternative methods using natural products is of great interest (13).

Bees have been able to adapt to live in any habitat due to the production of special products such as: honey, wax, poison, propolis, flower pollen and royal jelly. Propolis is one of the bees' chemical weapons to protect the colony against pathogenic microorganisms. In traditional medicine, propolis is used to treat wounds and burns, sore throat, stomach ulcers, etc (14, 15). Because the plant fauna of each region from which bees prepare propolis is different, so the propolis of each region has a diverse chemical composition (16). Many studies have discussed the chemical composition of propolis using gas chromatography-mass spectrometry (GC-MS) (17-22). All of them contained mainly flavonoids and ferulic acids. The present study showed that hexane (CAS); n-Hexane (17.32%), cyclopentane methyl (16.62%), and hexadecanoic acid (6.03%) were respectively the main component of propolis. Due to the

difference in the type of propolis of each region and the difference in the device used for propolis analysis, the results of this research are different from other studies. In a number of studies, the anti-protozoal effect of propolis has been investigated, such as *Giardia intestinalis* (23), *Leishmania tropica* (24), *L. amazonensis* (25), *L. donovani* (26), *Naegleria* and *Balamuthia* (15), *Trypanosoma cruzi* (27, 28), *Plasmodium falciparum* (29), *Trypanosoma brucei brucei* (30), *Blastocystis* spp.,(31), *Toxoplasma gondii* (32), *Nosema ceranae* (33), and *Acanthamoeba castellanii* (14, 34).

Few studies have been carried out on the effect of propolis on ectoparasites. Propolis was used on the *Varroa destructor* mite and the results showed that there was no significant effect of propolis addition or removal on mite survival and infection level (35). Due to differences in the type of ectoparasite and differences in the type of propolis, concentration, and exposure time, it is not possible to compare the study with other studies. The effect of alcoholic extract of propolis on *Rhipicephalus microplus (Boophilus)* showed that the viability of propolis as an alternative for the control of cattle ticks, with the 70% extract concentration being most efficient and most effective for controlling *R. microplus* under laboratory conditions (36).

In our study, the highest acaricidal effect was associated with a concentration of 200 mg/ml at an exposure time of 60 minutes, and the difference with this study is the difference in the type of tick, type of propolis and type of unit, which is expressed in percentage in this study, but in our study it is mg/ml. The hydroalcoholic extract of propolis against *Haemaphysalis* species showed that propolis had 100% mortality at a concentration of 100 mg/ml after 60 minutes (4). In this study, 100% mortality was associated with a concentration of 100 mg/ml, but in our study, the highest mortality was associated with a concentration of 200 mg/ml, which is probably due to differences in the type of tick and differences in the components of propolis.

In the present study, there were limitations such as laboratory design, limited replication, and GC-MS limitations (volatile compounds only).

Conclusion

The natural substances have great potential to control ticks, so the constant search for environmentally friendly pesticides is necessary. Propolis had acaricidal effects against *Hyalomma* spp, and the concentration of 200 mg/ml of propolis had the highest acaricidal effect (90%) in the exposure time of 60 minutes and have very little toxicity in all concentrations, but more studies should be done *in vivo*. Therefore, in case of possible use of propolis as an acaricidal agent, skin cells will not suffer from tissue toxicity.

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Conflicts of Interest

The author declares no conflicts of interest.

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