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RESEARCH ARTICLE

In-vitro acaricidal activity of *Peganum harmala* and *Glaucium flavum* alkaloid against *Rhipicephalus* sp. of dog

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Köpeklerde *Rhipicephalus*'a (sp) karşı *Peganum harmala* ve *Glaucium flavum* alkaloidinin in vitro akarisit aktivitesi

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Öz

Amaç: Kene kontrolü için amaçlanan temel ilaç sorunu konakçılar üzerinde uygulama dozuna gelişen dirençtir. *Glaucium flavum* ve *Peganum harmala*'dan alkaloitlerin akarisit etkileri doğal ortamda enfekte olan köpeklerden toplanan invitro olarak yetişkin dişi *Rhipicephalus* sp türünde değerlendirildi.

Gereç ve Yöntem: Her iki ekstraktın akarisit faaliyetleri (3.12, 6.2, 12.5, 25 ve 50 mg / ml), erişkin daldırma testi, üreme indeksi ve inhibisyon yumurtlama kullanılarak değerlendirildi.

Bulgular: Metanolik ekstraktların neden olduğu mortalite yüzdesi her iki bitkinin % 3.66 ila 50 mg / ml arasındaki konsantrasyonlarda test edildiğinde % 41.66 ila 75 arasında değişmekteydi ve kontrole kıyasla önemli ölçüde farklıydı ($p<0.05$). Görsel olarak yumurtaların yumurtadan çıkması tamamen 50 mg / ml ile *G. flavum* tarafından bloke edilmiştir, ancak diğer her iki bitkinin ekstraktları da yumurtadan çıkmayı kısmen engelleyebilmiştir. Her iki bitkinin yumurtlama ve üreme oranı konsantrasyona bağımlıydı ve negatif kontrole kıyasla önemli ölçüde farklıydı ($p<0.05$). Ayrıca, *P. harmala* ekstraktları *G. flavum* ekstraklarından Rhipicephalus sp türüne karşı daha etkili olduğu görünmüştür. Her iki bitkinin de alkaloid ekstrakları, *G. flavum* için 6.25 ve 50 mg/ml ve *P. harmala* için 25mg/ml ve 50mg / ml düzeyi ile gözlemlenmiştir.

Öneri: *G. flavum* kökünden ve *P. harmala* tohumundan ekstrakte edilen toplam alkaloidler potansiyel olarak in vitro *Rhipicephalus* sp. yumurtlayan dişilerin azaltılmasında iyi akarisit aktivitelerinin olduğu gözlemlenmiştir.

Anahtar kelimeler: Alkaloid, akarisit, in vitro, *Rhipicephalus* sp., köpekler

Abstract

Aim: The major problem of drug intended for tick control is resistance developed to application doses on the hosts. The acaricidal effects of alkaloids from *Glaucium flavum* and *Peganum harmala* were evaluated in vitro on adult female of *Rhipicephalus* sp. collected in naturally infected dogs.

Materials and Methods: The acaricide activities of the both extracts (3.12, 6.2, 12.5, 25 and 50 mg/ml) were evaluated using the adult immersion test, reproductive index and inhibition oviposition. DMSO (1%) and SEB (1µl/ml) were used as a negative and positive control.

Results: The percent mortality caused by the methanolic extracts of both plants varied from 41.66 to 75 %, when tested at concentrations ranging from 3.12 to 50 mg/ml, and significantly different compared to control ($p<0.05$). Visually hatching of the eggs was completely blocked only by *G. flavum* with 50 mg/ml; however, other extracts of both plants were partially able to block the hatching. The oviposition and reproductive rate of both plants were concentration dependent in both plants and significantly different ($p<0.05$) compared to negative control. Also, the extracts of *P. harmala* seem to be more efficient against *Rhipicephalus sp.* adults at different concentrations than the extracts *G. flavum.* The alkaloid extracts of both plants high effects were observed with 6.25 and 50 mg/ml for *G. flavum* and with 25mg/ml and 50mg/ml for *P. harmala.*

Conclusion: The total alkaloids extracted from the *G. flavum* root and *P. harmala* seed have good acaricidal activities in vitro reducing potentially the egg laying of *Rhipicephalus* sp. females.

Keywords: Alkaloid, acaricide, in vitro, *Rhipicephalus* sp., dogs

Introduction

Ticks (Acari: Ixodidae) are obligatory hematophagous arthropods, with all stages of their development. Ticks are the focus of important studies in different countries, they are attack several animal species, including humans and they cause huge economic loss. The genus *Rhipicephalus* includes 70 species of small to medium-sized ticks and are mainly found in mammals of the Africa (Walker et al 2000). Currently, *Rhipicephalus sanguineus* is most worldwide spread tick, due to its wide distribution and reproductive habits, especially in Algeria (Kebbi et al 2019, Matallah et al 2013). It is a hard tick that feeds on warm-blooded animals, whose main host is a dog, but which can also be found on bison, camels, horses, goats, sheep, reptiles, and various birds (De Oliveira et al 2009). Ticks transmit to animals especially dog, a wide variety of viruses, protozoa and bacteria such as *Babesia*, *Theileria* and *Anaplasma* spp. (Gray et al 2013).

The biological control strategy of ticks is essentially by acaricides containing chemical components such as hydrocarbons and organophosphates. However, the major problem of drug intended for tick control is resistance developed to application doses on the hosts much higher than those recommended for the elimination of these ectoparasites (Klafke et al 2010). Surveys was reported that treatment failures due the development of parasite resistance to commercial acaricides such as ivermectin (Perez-Cogollo et al 2010) and fipronil (Miller et al 2013). Besides this, the residues chemical products have a considerable genotoxic and cytotoxic effect on human cells and the harmful effects on the environment are very concerning (Abduz-Zahir and Abdul-Rahuman 2012). The application of chemical acaricides generates serious damages accompanied by the environmental contamination and presence of residues in animal. This situation force to take into consideration with an aim to reduce the utilization of chemical acaricides often used in treatment of animals against les ticks. Due to these reasons, several researches have been undertaken to find adaptable alternative methods that are safer, reasonably effective and economically feasible to control of ticks. Plants and their bioactive products have been recognized as important natural resource are reported to have medicinal applications in control of ticks (Olivo et al 2009).

Glaucium flavum and *Peganum harmala* belongs to the family of *Papaveraceae* and *Zygophyllaceae*, respectively. They widely distributed in North Africa, it grows naturally along the entire Mediterranean coast, semi-arid and pre-desert area. These plants are used in folk medicine for the treatment of a variety of diseases. The important biological activities of *G. flavum* and *P. harmala* are attributed to its bioactive compounds rich in alkaloids. Plants and their bioactive products are reported to have used in different remedies or control of many health problems and infections, including its using as antimicrobial (Azizi et al 2017; Arafa et al 2016), antioxidant (Boulaaba et al 2019; Ait Abderrahim et al 2019), anti-inflammatory (Boulaaba et al 2019; Bensalem et al 2014), antitumoral (Bournine et al 2013), hypoglycemic (Komeili et al 2016) and phytotoxic effects (Sodaeizadeh et al 2010).

Because of problems cited above, it is important to highlight the need to establish of new strategies for development alternative, safer, and environmentally friendly acaricidal agents. The biological activities of *G. flavum* and *P. harmala* reported in the literature prompted to undertake the searches of acaricidal potential effects as biocontrol agents in ticks. To our knowledge, the acaricidal activity of *G. flavum* and *P. harmala* alkaloids has never been reported, especially these growing in Algeria. In the present study, the acaricidal effects of alkaloids prepared from two plants, i.e. *G. flavum* and *P. harmala* were evaluated in vitro on adult female of *Rhipicephalus* sp. (Acari: Ixodidae) collected in naturally infected dogs.

Material and Methods

Ethics committee approval was received for this study from the scientific committee of Faculty of Nature and Life Sciences, University of Bejaia (Report of Faculty Scientific Council #09 dated October 28, 2015).

Plant materials

P. harmala seeds and *G. flavum* roots were collected in June 2016 from areas far from any pollution in the fields of Batna (35°32'N, 6°10'E) and Bejaia (36°45'N, 5°3'E) provinces respectively. The scientific authentication of plants was carried out at the Ecology Laboratory (University of Bejaia, Algeria). After cut in small pieces of *G. flavum*, both plants were put to drying at room temperature (25-30 °C) for four weeks. Thereafter, plant material was pounded manually or using coffee grinder resulting in a fine powder and kept in dark.

Preparation of plant extracts

The extraction of alkaloids was carried out according to the method described by Suau et al. (2004). Briefly, dried plant materials (10g) were crushed and then extract with 100 ml methanol in a Soxhlet apparatus for 8 hours. The methanol extract was filtered and concentrated under rotavapor, acidified with 50 ml of HCl (2%, v/v) and extracted with petroleum ether (50ml) to remove fatty materials. The aqueous layer was brought to pH 8 with ammoniac and extracted three times with dichloromethane (25 ml). The organic layer was dried in the open air to obtain a total alkaloid extract.

Ticks

288 engorged adult Rhipicephalus sp. females were carefully collected in universal bottles using forceps from naturally in-

fested dogs in Bejaia Province (Northern Algeria) and then transported to the Animal Biology Laboratory (University of Bejaia). Adult ticks collected were selected morphologically and identified using stereomicroscope (MOTIC, ST-37C-2LOO) following the standard identification procedures described by Walker et al. (2014). These females washed with water and dried by paper toweling to evaluate the in vitro acaricidal activity of the both plant extracts.

Adult immersion test

Adult immersion test (AIT) was performed based on the previously described Drummond et al. (1973). The specimens were selected according to their integrity, motility and maximum engorgement. The ticks were then weighed, separated into groups of 8 with homogeneous weights, with three repetitions for each concentration. In order to improve solubility in water, the extract was dissolved in dimethyl sulfoxide solution (DMSO, 1%). Sebacil (SEB, at 50% Phoxim) was used as a positive control concentration at (1µl/ml). A total of 24 ticks were used for each dilution with three replicates of eight ticks for each treatment and control. A total volume of alkaloid extracts (100 ml) was obtained which produced a stock solution at a concentration of 50 mg/ml from which a series of dilutions were made to obtain solutions at different concentrations 3.12, 6.2, 12.5, 25 and 50 mg/ml. The females were immersed in 15 ml of the respective treatment solutions for 5 min in sterile Petri dishes with a slight agitation. Female ticks were maintained in the incubator at 27-28 °C and 70-80% relatively humidity to complete the life cycle until the laying eggs. The criteria for death of ticks were determined by observing any minor signs of life such as minimal legs movement with stimulation by forceps, categorize the parasites as alive. The ticks were discarded after collection of eggs. The estimated mortality rate (% MR) was calculated using the formula 1.

Table 1. Mean adult mortality at 15 days, mean of laid eggs mass and visual hatching rate of the alkaloid extracts of *G. flavum* and *P. harmala* at different concentrations against *Rhipicephalus* sp.

Concentration (mg/ml)	Ticks weight (TW±SE, g)	Adult mortality at 15 days (AM-15±SE, %)	Laid eggs mass (LEM±SE, g)	Visual hatching rate (HR%)
Glaucium flavum				
50	0.870±0.003	66.66±2.4^{a}	0.173±0.4^{a}	3
25	0.854±0.003	41.66±2.4^{a}	0.211±0.02^{a}	15
12.5	0.857±0.002	54.16±2.4^{a}	0.239±0.006^{a}	25
6.25	0.843±0.008	58.33±6.36^{a}	0.035±0.04	5
3.12	0.866±0.008	50.00±00.00^{a}	0.429±0.028^{a}	40
Peganum harmala				
50	0.886±5.09	75.00±00.00^{b}	0.057±8.38^{c}	20
25	0.872±2.50	62.50±00.00^{b}	0.061±4.38^{c}	10
12.5	0.884±2.52	58.33±2.40^{b}	0.113±4.19^{b}	12
6.25	0.886±0.88	62.50±00.00^{b}	0.163±15.56^{b}	20
3.12	0.880±1.52	70.83±2.40^{b}	0.183±19.61^{b}	20
DMSO (1%)	0.894±0.005	00.00±00.00^{a,b}	0.741±0.007^{a,b,c}	100
SEB (1 µl/ml)	0.886±1.17	100±00.00^{a,b}	00.00±00.00^{a,b}	0

^{a,b,c} Values by different letters superscripts in negative and positive control (DMSO and SEB, respectively) compared with each extract treatment are statistically different in the same column ($P < 0.001$). Number of replicates = 3. DMSO: dimethyl sulfoxide solution; SEB: Sebacil.

MR=(dead ticks)/(total ticks) x 100 (1)

Reproductive index and inhibition of oviposition

At the inhibition of oviposition, the eggs were weighted and transferred into tubes at different dilutions of each extract (3.12, 6.2, 12.5, 25 and 50 mg/ml). Each group consisted of eight ticks divided into three replicates. The eggs were incubated under the same conditions for larval hatching and the percentage of hatching was estimated visually. Sebacil concentration of 1µl/ml was used as positive control and DMSO (1%) as negative control. The hatching rate was read after ~25 days and the data obtained were used to determine the reproductive index (RI) and the percent inhibition of oviposition (% IO) using formulae 2 and 3, respectively (Goncalves et al 2007).

RI=Weightofeggslaid(g)/(Weightofadultfemales (g)) (2)

IO=(RI (control group)-RI (treated group))/(RI (contro group)) x 100 (3)

Statistical analysis

Data were expressed as the mean±SE. Groups were compared using ANOVA for repeated measurements using the R software version 3.4.4 (http://www.R-project.org/).The differences between means were determined by Tukey test at 5% significance level.

Results

Table 1 summarize the results of adult immersion test using the methanolic extracts of G. flavum and P. harmala, respectively. The efficacy of alkaloid extract of both plants against Rhipicephalus sp. was assessed by estimating the percent adult mortality (cut off was 15 days post-treatment) and eggs mass laid and visual hatching rate.

The percent mortality caused by the methanolic extracts of both plants varied from 41.66 to 75 %, when tested at concentrations ranging from 3.12 to 50mg/ml, and significantly different compared to control ticks (P<0.05). The laid egg mass effect was proportional to the extract concentration of G. flavum and P. harmala. It is also noteworthy that laid egg masses weight of the live ticks, treated with different concentrations of the various extracts were significantly (P<0.05) lower than ticks treated with DMSO.Visually hatching of the eggs was completely blocked only by G. flavum with 50 mg/ml; however, other extracts of both plants were partially able to block the hatching.

The effect of methanolic extracts of *G. flavum* and *P. harmala* extracts against engorged females was evaluated by calculating the oviposition inhibition and reproductive index. The variation of the mean reproductive rate and oviposition rate of both plants according to the concentration ranging from 3.12 to 50 mg/ml are illustrated in Figure 1 and 2, respectively. Generally, the oviposition rate and reproductive rate of both plants were concentration dependent in both plants and significantly different (P<0.05) compared to ticks treated by DMSO. The results indicated that the extracts of *P. harmala* seems to be more efficient against *Rhipicephalus sp.* adults at different concentrations than the extracts *G. flavum*. The alkaloid extracts of both plants high effects were observed with 6.25 and 50 mg/ml for *G. flavum* and with 25mg/ml and 50mg/ml for *P. harmala.*

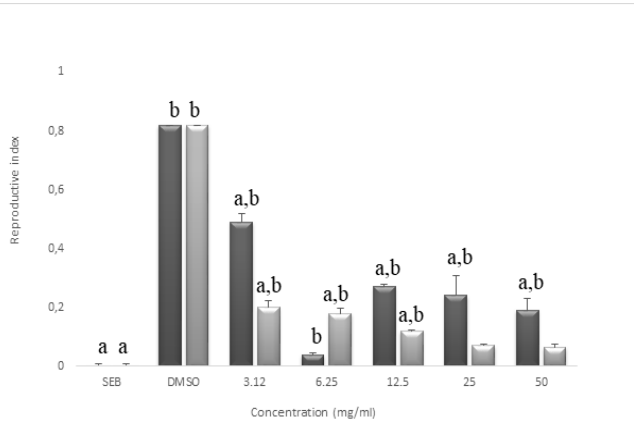

Figure 1. Reproductive index of the alkaloid extracts of *G. flavum* and *P. harmala* at different concentrations against *Rhipicephalus* sp.

a,b Values by letters superscripts in negative and positive control (DMSO and SEB, respectively) compared with each extract treatment are statistically different (p < 0.05). Number of replicates = 3. DMSO: dimethyl sulfoxide solution; SEB: Sebacil.

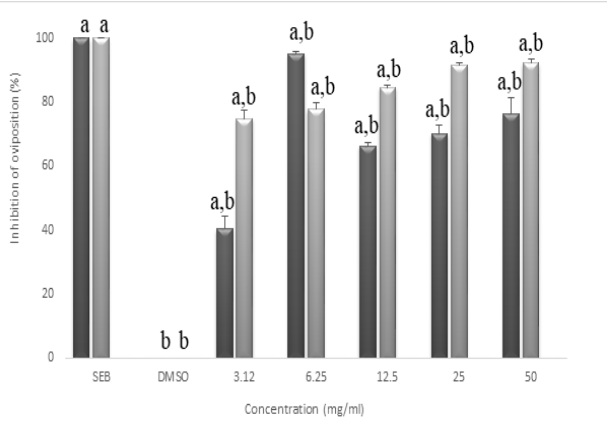

Figure 2. Percentage inhibition of oviposition of the alkaloid extracts of *G. flavum* and *P. harmala* at different concentrations against *Rhipicephalus* sp.

a,b Values by letters superscripts in negative and positive control (DMSO and SEB, respectively) compared with each extract treatment are statistically different (p< 0.05). Number of replicates = 3. DMSO: dimethyl sulfoxide solution; SEB: Sebacil.

Discussion

Since the last century, the plants as drugs known to be a rich source of beneficial compounds have been used by practitioners of traditional medicine. For decades, the acaricidal properties of the plant have been widely used in alternative veterinary medicine against ectoparasites and they became today a part of therapy traditional particularly in the rural area. In the literature, several studies showed significant biological activity of the *G. flavum* and *P. harmala* extract (Petropoulos et al 2018). The present study was conducted in order to provide an alternative therapy using of plant extracts are known residue less, flora and fauna friendly, biodegradable, which can intervene in all biological processes of the ticks, thus interrupting their life cycle. These tests measured the in vitro acaricidal effect on the processes of hatching, oviposition and reproductive index of *Rhipicephalus sp.*

The results of our study have demonstrated that extracts of *G. flavum* and *P. harmala* have an in vitro inhibitory effect on the eggs hatching and reproduction of adult female of *Rhipicephalus* sp. collected in naturally infected dogs. Previous studies reported that both plants studied extracts are rich in bioactive alkaloid compounds (Bensalem et al 2014, Bournine et al 2013). As described before, *G. flavum* contained protopine as major alkaloid compound magnoflorine, chelidonine, sanguinarine and chelerythrine. The HPLC profiling of the active total alkaloids of *P. harmala* indicates that possesses five β-carboline alkaloids e.g. harmine, harmaline, harmane, harmol and harmalol are major components. Recently, Shang et al (2016) reported that vasicine, harmalin and harmine, as the active compounds of *Peganum harmala* L., presented the marked acaricidal activities against *Psoroptes cuniculi*, and could be widely applied for the treatments of acariasis in animals. One study conducted by Misra et al (2008) reported that harmine and vasicine, two compounds found in *P. harmala*, are effective against *Leishmania donovani.* According to those authors, this acaricidal activity may be attributed to an individual or a combined effect of the alkaloid compounds. Also, Godara et al (2015) explained that the secondary metabolites can also act synergistically when used with or in combination with another plant with active ingredient. In agreement with our observations, many investigations demonstrated that bioactive compounds extracted from plants could be a source of alternative tick control with ecological and health benefits (Castilho et al 2017). The results of this study correspond also with those published previously, which demonstrated that the acaricidal activity resulted with increasing the plant extracts concentration (Gomes et al 2014). The results obtained for the inhibition of egg hatching and oviposition as well as for the adult ticks' mortality could be explained by the penetration of bioactive molecules of the plant extracts into the tick skins causing a significant decrease in the percentage of egg laying. Noted that the negative and positive control are taken into account in investigation in order to compare with both plant extracts this gives a strong support for the tested extract. Interesting, the data of this study shown that there had no inhibitory effect of DMSO on the egg hatching and oviposition; while SEB, a commercial organophosphate, totally blocked it. The results of present study correspond entirely with those published previously, which demonstrated that 1% DMSO did not presented any effect on the mortality of *Rhipicephalus microplus* (Goncalves et al 2007).

Previous investigations have demonstrated activity acaricidal of some plant extracts such as *Melia azedarach* (Sariosseiri et al 2018; Abdel-Ghany et al 2019) and *Azadirachta indica* (Avinash et al 2017). The alkaloid fraction of *Leucas indica* showed significant mortality of concentration-dependent adult ticks compared to the non-alkaloid fraction (Divya et al 2014). Further, the effectiveness of the acaricide depends also on the quality, quantity and degree of dispersal of the active ingredient. Our results are corroborated with those reported by Daemon et al (2012) which it presented a remarkable mortality rates (92-98 %) of thymol on *Amblyomma cajennense* and *R. sanguineus* larvae. A similar observation was reported by Ravindran et al (2011) concerning the inhibition effect on the hatching of eggs laid by *Rhipecephalus (Boophilus) annulatus* and the mortality of adult ticks treated with the ethanolic extract of *Leucas aspera* (Lamiaceae). Likewise, Muhammed et al (2012) showed that extracts of *Leucas martinicensis* are rich in alkaloids, flavonoids and volatile oils repelling adult mosquitoes culex. In 2009, Landau et al reported that a decrease of ticks' weight in lambs artificially infested by adult *Dermacentor vairiabilis* receiving high doses of *Azadirachta indica* Juss in feed additive. In the previous studies, the researchers have reported that eugenol extract has a total larvicidal effect and egg laying inhibition effect in engorged females of *R. microplus* (Monteiro et al 2012), which suggests its potential usefulness for the environmental control of ticks (Valente et al 2013). Friesen and Kaufman (2003) reported that inhibition of vitellogenesis and egg cell development in *Amblyomma hebraeum* by cypermethrin used as an insecticide in large-scale commercial agricultural applications. Alkaloid in the plant extracts was reported to cause mortality and inhibition of fecundity due to its neurotoxic properties (Valduga et al 2018). This effect attributed to the release of 20 hydroxy-20-ecdysone by the insecticide which may play a role in controlling the secretions of sex glands from the genetic organs of ticks. It is known that gamma aminobutyric acid (GABA) is found in most invertebrates and avermectins inhibit the GABA neurotransmission in nematode. It is noted also that active compounds effect may be explained by a block nerve signals by interfering with the glutamate-gated chlorid receptors causes a greater potentiation of GABA action on this receptor (Lumaret et al 2012). Indeed, one study reported that chemical compounds of plant extracts could penetrate into inside the egg and prevent the segmentation of blastomeres blocking the cuticle post-

synaptic receptors consequently, paralyzing larval formation of *Haemonchus contortus* (Engstrom et al 2016). Harmaline (7 methoxy 3,4-dihydro-b-carboline), alkaloid derived from the seeds of the plant *P. harmala,* is a monoamine oxidase inhibitor (Frostholm et al 2000) which may explain the inhibitory effect of this plant extract against ticks. Therefore, the acaricidal activity of the both plant extract used in this study could be attributed to its total alkaloids. Though most secondary metabolites are not fully identified in this study, the presence of more than one secondary metabolite was demonstrated usually for antitick activity.

Conclusion

The biological control of ticks presents many major challenges and opens up enormous opportunities for research to identify new and more important acaricides in reducing the use of synthetic chemicals that are harmful to humans and the environment. Total alkaloids extracted from the *G. flavum* root and *P. harmala* seed have good acaricidal activities in vitro reducing potentially the egg laying of *Rhipicephalus* sp. females. In perspective, it should be carried out in order to test the *in vivo* efficiency separately of the *G. flavum* and *P. harmala* bioactive compounds in domestic dogs. Also, it would be notable to investigate the toxicity level of these extracts *in vivo.*

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Conflict of Interest

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During this study, any pharmaceutical company which has a direct connection with the research subject, a company that provides and / or manufactures medical instruments, equipment and materials or any commercial company may have a negative impact on the decision to be made during the evaluation process of the study. or no moral support.

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