In vitro and *in vivo* Nematocidal Activity of *Allium sativum* and *Tagetes erecta* Extracts Against *Haemonchus contortus*

Allium sativum (Sarımsak) ve *Tagetes erecta* (Kadife Çiçeği) Özlerinin *Haemonchus contortus'a* Karşı *in-vitro* ve *in-vivo* Nematisidal Etkisi

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ABSTRACT

Objective: In the Mexican ethno-medicine, a number of plants have shown a successful anthelmintic activity. This fact could be crucial to identify possible green anti-parasitic strategies against nematodes affecting animal production. This research evaluated the *in vitro* and *in vivo* nematocidal effects of two single and combined plant extracts: bulbs of *Allium sativum* (n-hexane) and flowers of *Tagetes erecta* (acetone). The *in vivo* assay evaluated the administration of extracts either individually or combined against Haemonchus contortus in experimentally infected gerbils.
Methods: The *in vitro* larvicidal activity percentage (LAP) of *A. sativum* and *T. erecta* extracts against *H. contortus* (L3) was determined by means of individual and combined usage of the extracts. Similarly, the extracts were evaluated in terms of reduction in the parasitic population in gerbils infected with *H. contortus* by individual and combined usage.
Results: The LAP at 40 mg/mL was 68% with *A. sativum* and 36.6% with *T. erecta*. The combination caused 83.3% mortality of parasites. The oral administration of *A. sativum* and *T. erecta* extracts at 40 mg/mL, caused 68.7% and 53.9% reduction of the parasitic burden, respectively. Meanwhile, the combined effect of both extracts shown 87.5% reduction.
Conclusion: This study showed evidence about the effect of *A. sativum* and *T. erecta* plant extracts by means of individual and combined usage against *H. contortus* in *in vitro* and *in vivo* bioassays in artificially *H. contortus*-infected gerbils as a model. *(Turkiye Parazitol Derg 2015; 39: 260-4)*

Keywords: *Allium sativum*, *Haemonchus contortus, Meriones unguiculatus* (gerbil), *Nematode mortality, Tagetes erecta*

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ÖZ

Amaç: Meksika etnik-tıpta, bazı bitkiler başarılı bir anthelmintik etki göstermektedirler. Bu gerçek, hayvan üretimini etkileyen nematodlara karşı, olası yeşil anti-parazitik stratejilerin belirlenmesi için önemli olabilir. Bu araştırma iki tek ve kombine bitki özlerinin, *Allium sativum* soğanları (n-hekzan) ve *Tagetes erecta* (aseton) çiçeklerinin, nematosidal etkisini *in vitro* ve *in vivo* olarak değerlendirmiştir. *In vivo* çalışmada, deneysel olarak etkilenen gerbillerde *Haemonchus contortusa* karşı, bitki özleri tek olarak ya da birlikte uygulanmıştır.
Yöntem: *A. sativum* ve *T. erecta* özlerinin *H. contortusa* (L_3) karşı *in vitro* larvisidal etki yüzdesi (LEY), bitki özlerinin tek başına ve kombine olarak kullanılması ile belirlendi. Aynı şekilde bitki özleri, *H. contortus* ile infekte olmuş gerbillerdeki parazit popülasyonunu azaltması açısından, tek ve beraber kullanılarak değerlendirilmiştir.
Bulgular: 40 mg/mL'deki LEY, A. sativum için %68 ve *T. erecta* için %36,6 olarak bulunmuştur. Kombinasyon %83,3 oranında mortaliteye neden olmuştur. 40 mg/mL dozdaki bitki özlerinin oral olarak uygulanması ile parazit popülasyonu *A. sativum* için %68,7 ve *T. erecta* için %53,9 azalmıştır. Aynı zamanda, kombine etkisi % 87,5 olarak tespit edilmiştir.
Sonuç: Bu çalışma, bir model olarak, yapay olarak *H. contortusdan* infekte olan gerbillerle yapılan *in vitro* ve *in vivo* biyoanalizlerde, *A. sativum* ve *T. erecta* bitki özlerinin tek ve kombine olarak kullanılmasının *H. contortusa* karşı etkisini kanıtlamaktadır. *(Turkiye Parazitol Derg 2015; 39: 260-4)*

Anahtar Kelimeler: *Allium sativum*, *Haemonchus contortus, Meriones unguiculatus* (gerbil), *Nematod mortalitesi, Tagetes erecta*

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INTRODUCTION

Gastrointestinal parasitic nematodes (*gin*) are responsible for an important economic impact in ruminants (1). Particularly, *Haemonchus contortus* causes a severe damage on animal health because of its hematophagia habits. The immune system of young parasitized animals is dramatically diminished and they even can eventually die (2). Systematic deworming of animals using chemical anthelmintic drugs (CADs) is the most common method of *gin* control worldwide. Such a strategy reduces the parasitic burden in animals; however, there are a number of inconvenient factors that diminish the beneficial use of such drugs. One example is the anthelmintic resistance in the parasites that causes an inefficiency of the anthelmintic drugs. Also, once animals have been dewormed, some CADs are expelled into the environment in their bioactive form through feces or urine, creating a risk to non-target microorganisms (3).

Green technology offers an environmental friendly alternative. During the last decades, the use of natural bio-compounds obtained from plants with medicinal properties is gaining importance in human health. There are currently, a number plants extracts that have been commercialized worldwide (4). Similarly, plants and their products are being deeply studied searching for tools of control against animal parasitic nematodes. Many plants are currently being explored to identify possible candidates for control in animals of veterinary importance (5-10). Recently, two outstanding plants, *Allium sativum* and *Tagetes erecta,* have been identified and selected for investigating their green nematocidal activity.

A. sativum (garlic) is a member of the *Liliaceae* family and one of the most popular bulbs used worldwide to reduce various risk factors associated with several diseases, including activity against *gin* (11). Some bio-compounds obtained from garlic are allicin, allinin, ajoene, and diallylsulfide that are sulphured compounds.

T. erecta L (marigold) is known as death flower and is a medicinal and ornamental plant; it is also used for its nematocide, cosmetic, and medicinal properties (12, 13). The plant *T. erecta* has been shown to contain quercetagetin, a glucoside of quercetagetin, phenolics, syringic acid, methyl-3, 5-dihydroxy-4-methoxybenzoate, quercetin, and thienyl and ethyl gallate (13, 14). The leaves are reported to be effective against bleeding piles, kidney troubles, muscular pain, ulcers, and wounds. Phytochemical studies of its different parts have resulted in the isolation of various chemical constituents such as flavonoids, carotenoids, and triterpenoids.

Their importance is being evaluated in the control of animal parasitic nematodes (15). The present research aimed to evaluate the *in vitro* and *in vivo* nematocidal effects of the plant extracts obtained from *A. sativum* and *T. erecta* (administered either individually or combined) against *H. contortus* as a green alternative of parasitic control.

METHODS

2.1 Plant material

The selection of plants was based on the ethno-botanical background (either scientific or empiric) and its usage in traditional medicine as de-wormer products. *A. sativum* vegetative material was obtained from the local market in the City of Cuernavaca, and *T. erecta* flowers were obtained from a rural field in the Municipality of Tixtla; both places are situated in the State of Morelos, Mexico. The *A. sativum* bulbs were peeled, and all coats were removed and finely sliced. In the case of *T. erecta,* the flowers were processed for extraction as follows: n-hexane for *A. sativum* and acetone for *T. erecta* by a maceration process at room temperature (6, 16, 17).

2.2 *H. contortus* infective larvae (L_3)

The *H. contortus* isolate was originally obtained from a naturally infected sheep from "Las Margaritas" Experimental Sheep Farm (INIFAP) in Hueytamalco Municipality in the State of Puebla, Mexico. A *H. contortus* egg donor lamb was orally infected with an aqueous suspension containing 350 larvae/kg of body weight. Twenty days after infection, the presence of *H. contortus* eggs in fecal samples was detected with the McMaster technique (18). Infective larvae were extracted from coprocultures using the Baermann funnel technique (18). Larvae were unsheathed following the technique described by López Aroche et al. (6). The total number of L_3 in aqueous suspension was estimated by counting them in ten 10 ml aliquot drops (19).

2.3 Bioassays to determine the 50% lethal concentration (LC_{50})

Different extract concentrations of *A. sativum* n-hexane and *T. erecta* acetone extracts were evaluated to determine the LC_{50} by means of the individual and combined effects of both extracts. Initially, the solution of each extract at 40 mg/mL was prepared as a "stock solution." From this concentration, double dilutions were prepared to obtain the following concentrations: 40, 20, 10, 5, 2.5, 1.25, and 0.625 mg/mL. The assay was performed in 24-well tissue culture plates at 500 mL for three replicates. Proper controls (3% tween-20 and 3% fenbendazole and distilled water) were used, and the protocol cited by López-Aroche et al. was followed (6). Results were obtained by counting the number of live and dead L_3 in the wells and means of either live/dead larvae after 24 h and 72 h confrontation were recorded. LC_{50} was obtained using the POLO program (version 2003). The *in vitro* efficacy percentage of the extracts and LC_{50} were determined using the following formula:

$$\text{Efficacy percentage} = \frac{\text{Alive } (L_3) \text{ control} - \text{Alive } (L_3) \text{ treated}}{\text{Alive } (L_3) \text{ control}} \times 100$$

Where, Alive (L_3) Control=Mean of alive (L_3) recovered from control group

Alive (L_3) Treated = Mean of alive (L_3) recovered from treated group

2.4 Experimental animals

Forty-two male and female 3-month-old gerbils weighing an average of 40 g were obtained from a local bioterium. Animals were kept in metallic cages at 28–30°C (n=7), and a 2-week adaptation period was established to determine their health conditions. Presence of *gin* eggs in feces was diagnosed, and *gin*-positive gerbils were dewormed and other provided recommendations were followed according to de Jesús-Gabino et al. (17).

2.5 Assessing the *in vivo* oral administration of plant extracts in gerbils

The optimum dose of *H. contortus* (L_3) to achieve an infection in gerbils and the best administration route for the plant extracts were determined according to de Jesús-Gabino et al. (17). Gerbil groups were conformed as follows: I) distilled water, II) 3% tween-20 (in water), III) albendazol, IV) *A. sativum* extract, V) *T. erecta* extract, and VI) both combined extracts. The extracts were administered at 40 mg/mL contained in 100 µL volume (as a total dose). The animals were slaughtered 13 days post-infection. Necropsy was performed to obtain the stomach and to collect the nematodes presents in the gastric lumen. Then, nematodes were quantified to estimate the recovery average of parasites per group. The reduction percentage of parasitic burden attributed to the treatments was estimated using the following formula:

$$\%E = \frac{XB - XA}{XB} \times 100$$

Where, %E=Reduction percentage

XA=Mean of recovered parasites from the treated group

XB=Mean of recovered parasites from the control group

2.6 Statistical analysis

Data were analyzed using the Shapiro–Wilk statistical test ($P<0.0001$). Data were transformed to square root for data normalizing ($P<0.6035$) (20). After data normalizing, analysis of variance was used considering the average of recovered nematodes from different groups at necropsy as the dependent variable. Additionally, the minimum significance difference ($\alpha=0.05$) was used as a multiple mean comparison to determine the difference between treatments using the Statistic Analysis System (21).

RESULTS

The *in vitro* median LC_{50} after 24 h and 72 h confrontation of *A. sativum* n-hexane extract with the nematode larvae was identified as 7.9 mg/mL and 3.8 mg/mL, respectively. Meanwhile, the *T. erecta* acetone extract showed a very low *in vitro* lethal activity against the nematode from which no LC_{50} was obtained. On the other hand, the LC_{50} for the extract combinations of both plants was 3.95 mg/mL at 24 h and 1.3 mg/mL at 72 h confrontation. The results of the *in vivo* assay of extracts, either individual or combined, are shown in Table 1, which includes the total number of recovered L_4 at necropsy of gerbils as well as the average and the reduction percentage of recovered nematodes. A reduction of *H. contortus* (L_4) population of 68.7% and 53.9% were individually obtained with *A. sativum* and *T. erecta*, respectively. On the other hand, 87.5% larvae reduction was obtained with the combined extracts. Similarly, larvae reduction percentages obtained with the controls fenbendazole, 3% tween-20 (in water), and water were 97.5%, 10%, and 0%, respectively.

Table 1. Total number and average of *Haemonchus contortus* (L_4) recovered at necropsy of artificially infected gerbils after being treated with two plant extracts, either alone or combined

Treatment	Recovered *H. contortus* (L_4)	Average X (±SD)	Reduction (%)
Fenbendazole*	11	2.7 (±2.21)[a]	97.5
Allium sativum/ *Tagetes erecta*	83	11.8 (±5.9)[b]	87.5
Allium sativum	219	31.2 (±13)[c]	68.7
Tagetes erecta	308	44 (±21)[d]	53.9
Tween-20 AL 3%	572	95.3 (±42.5)[e]	10
Water	749	107 (±58)[f]	0

*=Positive control; Values followed by a different letter indicate statistical differences ($P<0.05$).
X=Average; SD=Standar Deviation

DISCUSSION

The *A. sativum in vitro* activity obtained in the present research (at 72 h) can be considered important, particularly because it is a natural extract. Similarly, the low *in vitro* lethal activity was obtained with the *T. erecta* extract after 24 h, and this percentage was increased at 72 h confrontation. The *in vitro* assays provide useful information about the lethal activity of organic plant extracts against *gin* infective larvae. However, the real effect of any plant extract as an anthelmintic compound is achieved through the *in vivo* assays (22). Both *A. sativum* and *T. erecta* individual extracts reduced the L_4 population in values higher than 50%. The *A. sativum in vivo* efficacy in the present experiment showed that it can have a very good result, and it was very similar to the one obtained at 24 h *in vitro* confrontation. This efficacy is important because such a result was obtained only by administering the plant extract as a single alternative of control. Perhaps, such efficacy could increase using other vehicles or even other doses. The *in vivo* efficacy of *T. erecta* was very similar to the one obtained in the *in vitro* assay at 72 h confrontation. Perhaps there are similar cuticle molecules between the L_4 and L_5 evaluative stages and probably they share the same cuticle receptors susceptible to act as bio-compound targets. The fact that the *T. erecta* extract removed the parasitic burden by less than 50% could be considered a low activity compared with the regular anthelmintic drugs; nevertheless, it is an acceptable reduction for a natural plant extract. The combination of both extracts achieved a higher effect in reducing the gerbil parasitic burden (87.5%). This study shows, for the first time, evidence about the anthelmintic effect of two plant extracts used, individually or combined, against *H. contortus* in artificially infected gerbils as an *in vivo* model of study. The different results obtained both in the *in vitro* assay and in the present *in vivo* assay could be due to the fact that the *in vitro* assay provides favorable conditions to allow the plant extracts to display their lethal nematocidal activity. In contrast, the *in vivo* assay has to overcome the gerbil physiological body barriers (temperature, pH, enzymes, etc.) to express their anthelmintic effect. This result motivates a continued search for increasing the efficacy of these or other bio-compounds obtained from plant extracts, either individually or combined. Similarly, these and other plants and also other organic solvents could achieve a higher anthelmintic activity in gerbils with potential use in sheep. The use of organic extracts from a number of plants, using different solvents, has been evaluated against a wide variety of parasites. The *Spigelia anthelmia*

ethanol extract showed 74% anthelmintic activity against *Nippostrongylus brasiliensis* in rats (23). In another study, the oral administration of an aqueous extract of Jazmin (*Jasminum abyssinicum*) leaves reduced *H. contortus* eggs by 69% per g of feces in artificially infected sheep (24). Similarly, 95% reduction of the gastrointestinal nematode infective larvae population in fecal cultures was obtained through the oral administration of an aqueous extract of a combination of *Mentha piperita* and *Chenopodium ambrosioides* in goats (25). The selected plant extracts assessed in the present study should be evaluated using sheep and other economically important species as possible potential natural compounds to be used in integrated control programs. The use of this technology could lead to the development of environmental friendly alternatives for the control of *gin* and can be used with other ecological measures to substantially reduce the parasitic burdens in animals and improve both animal health and production (26). The results of the present study show that the combined use of both plant extracts produces a higher anthelmintic effect than the use of the individual plant extracts. In this regard, such an increased activity could be due to either a synergic or additive effect achieved when the bio-compounds present in the two plant extracts have an interaction that leads to a higher anti-parasitic effect (27).

CONCLUSIONS

The nematocidal activity of the *A. sativum* hexane extract was demonstrated against *H. contortus* when administered in jirds (*Meriones* spp.) either alone or combined with *T. erecta* acetone extracts. The use of selected plant extracts with an anthelmintic activity offers a viable and sustainable alternative to be used as an integrated bio-control system against parasitic *gin* in economically important animal species. Obtaining plant extracts with the highest anthelmintic activity is an important alternative to obtain a natural product that replaces, at least partially, the use of chemical drugs against animal parasitic nematodes.

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