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Plant ethanolic extracts and entomopathogenic fungi for controlling *Tetranychus urticae* Koch

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ABSTRACT

The two-spotted spider mite, *Tetranychus urticae*, is a major pest of crops worldwide. In this study the effectiveness of ethanolic extracts from wild oregano (*Lippia origanoides*) and bushy lippia (*Lippia alba*) and that of entomopathogenic fungi (*Paecilomyces fumosoroseus*, *Beauveria bassiana* and *Cladosporium* sp.) to control *Tetranychus urticae* was evaluated. Ethanolic extracts were evaluated at different concentrations (2.5, 5.0, 7.5 and 10%) using the leaf disk immersion technique. Also, the effect of conidial suspensions of the fungi on mortality and oviposition of *T. urticae* was evaluated. The presence of alkaloids, flavonoids, phenols and tannins, essential oils and saponins was verified in the plant material used in our study. Ethanolic extracts when used at a concentration of 10% showed acaricidal effects on *T. urticae*, as evidenced by the mortality rates (83.3 and 78.3% caused by oregano and bushy lippia, respectively). Similarly, a higher mortality rate was observed when the mites were treated with *Cladosporium* sp., which is 72% higher than the control, while *P. fumosoroseus* and *B. bassiana* showed similar mortality rates at around 45.5 and 54.5% higher than the control. Our results showed that ethanolic extracts and acaropathogen fungi are promising agents for the management of *T. urticae*, although their field efficacy remains to be evaluated.

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KEYWORDS: tetranychidae, sustainable strategies, pest management, two-spotted spider mite

INTRODUCTION

The two-spotted spider mite, *Tetranychus urticae* Koch, is a major pest of crops worldwide [1]. At present, control of *T. urticae* relies heavily on the use of acaricide sprays. However, resistance problems and public concerns about chemical treatments have led growers to increase their use of bio-control agents [2]. New insect control strategies have been and are being developed to fit a variety of pest management needs [3]. Alternatives include the search for new types of pesticides. These new pesticides should be effective against a limited number of specific target species, biodegradable into non-toxic products, and suitable for use in integrated pest management programs [4]. Natural plant products effectively meet these criteria and have enormous potential to influence modern agrochemical research [5]. Chemicals that are extracted from plants and denominated as botanical pesticides are now emerging as one of the prime means to protect crops and their products, and also the environment from pesticide pollution [6].

Previous studies have demonstrated the efficacy of using sustainable strategies in pest control, including plant extracts and entomopathogenic fungi, in substitution for chemical controls [7, 8, 9]. Botanical pesticides constitute a sustainable alternative for pest management since they exert

lower environmental impact and minimize the likelihood of resistances that evolve in pests [3, 10]. Various botanical families are being tested as bioinsecticides based on their effectiveness as insecticides or antifeedants [7, 11].

Agricultural pest control currently tends to minimize agrochemical use, and integrated pest management is increasing. This includes uses of natural enemies such as parasitoids, predators and entomopathogens [12]. Concerning entomopathogenic fungi, several studies have shown natural occurrences of entomopathogenic fungi species such as *Cladosporium cladosporioides* and *Beauveria bassiana* on *T. urticae* [13], and *Hirsutella* sp. and *Cladosporium* sp. [8] (van der Geest *et al*. 2002) on Eryophyoid mite species. Also, the efficacy of isolates on *T. urticae* has been determined in laboratory assays. This mite species was shown to be susceptible to the examined isolates of the entomopathogenic fungus *B. bassiana* [14]. On the other hand, mortality rates were found to increase with time and were dependent on conidial concentrations, with values ranging from 51 to 81.6% and 71.4 to 100% for *C. cladosporiodes* and *M. anisopliae*, respectively [15].

Based on previous results, our study evaluated *in vitro* effectiveness of ethanolic extracts from wild oregano (*Lippia origanoides* Kunt) and bushy lippia (*Lippia alba* Mill. N. E. Brow) and that of entomopathogenic fungi (*Paecilomyces fumosoroseus*, *Beauveria bassiana* and *Cladosporium* sp.) in controlling *T. urticae* in the state of Lara, Venezuela.

MATERIALS AND METHODS

Plant extracts

Extracts of *L. origanoides* and *L. alba* were obtained from natural populations in the Terepaima National Park or in the garden at Universidad Centroccidental Lisandro Alvarado (UCLA), in Lara, Venezuela. Mature leaves were dried and macerated and soaked in 96% ethanol. The liquid was filtered using a four-layered gauze and then concentrated in a rotary vacuum drier system (rotary-evaporator Brinkmann™, Mod. RE111) at 100 ºC. The concentrated crude extract, obtained after water and ethanol evaporation, was recovered from the rotary drier system and stored at 8 ºC until qualitative and quantitative determinations of secondary metabolites and evaluation of acaricidal properties [16].

Secondary metabolites

Qualitative determination of secondary metabolites (alkaloids, essential oils, polyphenol and tannin, antraquinones and flavonoids) in ethanolic extracts followed the methods of Marcano & Hasegawa [16], while saponins followed that of Cuéllar [17].

After dehydration, the ethanolic extract was mixed with 2.5 ml 10% hydrochloric acid and 5 ml chloroform in a separatory funnel to determine alkaloids. This procedure yielded three phases with the weak-basic alkaloids being collected in the first phase. The intermediate phase was alkalinized with ammonium hydroxide (NH_4OH) and re-extracted with chloroform in order to obtain the basic alkaloids. The third phase aimed to detect the quaternary ammonium salts. One-microliter aliquot from each of the phases was placed on a silica-gel cromoplate treated with Meyer's reagent. Alkaloid presence was evidenced by the formation of a brown-reddish coloration. Results were confirmed by adding Dragendorff's reagent.

Essential oils were determined by organoleptic procedures using characteristic odor as an indicative [16]. Total polyphenols and tannins were evidenced by characteristic brown coloration in ethanolic extract when 1% FeCl was added. The extract was evaporated and 1% agar in 1% NaCl solution was added. The presence of polyphenols and tannins was indicated by the formation of a precipitate [16].

For the determination of the presence of antraquinones, the ethanolic extract was neutralized with 5 ml 0.5% KOH, acidified with 0.5 ml acetic acid (pH 4.8) and then agitated with 5 ml benzene. Finally, NH_4OH was added and the antraquinone presence was indicated by the development of a reddish coloration.

Flavonoids were detected by a reddish coloration 20 min after the addition of 0.5 ml 10% HCl and magnesium (0.35 g) to the ethanolic extract. Flavonoids were confirmed by the development of yellow color sample spots in the 1% $AlCl_3$-treated filter papers, when observed under 450 nm ultra violet light (UVL). Finally, to detect saponins,

Table 1. Content of saponins based on foam height.

Foam height (mm)	Content
0.0	Negative
0.1-5.0	Very low
5.1-9.0	Low
9.1-14	Moderate
Greater than 14	High

From Cuéllar (1999).

1 mL of ethanolic extract was mixed with 9 mL of distilled water, filtered and then 1 mL aliquot was added in a probe tube and vigorously agitated until foam appeared. Presence of saponins was detected by the height of the foam measured with a digital Vernier after 15 min as shown in table 1.

Bands containing isolated secondary compounds were cut and weighed (silica/gel + secondary metabolite). A similar band of isolated secondary compounds was obtained from a clean silica/gel cromatoplate (silica/gel) and weighed for comparison purposes. The difference in weights indicates the amount of a particular secondary plant compound (in 7 µL extracts). Based on these weights, the contents (g per leaf material) could be calculated in a straightforward way [18].

Mite collection and rearing

Mites were collected from 40-day old black bean plants, *Phaseolus vulgaris* L., growing in the field during the dry season in Cabudare, Lara state, Venezuela. In the laboratory, the female and male mites were mounted on slides for microscope observations using the Hoyer's medium. Taxonomic identification was made by using the taxonomical key provided by Gutierrez [19], and species identification was confirmed by comparing with voucher specimens from the Acarological Collection at Universidad Central de Venezuela, especially by comparing the morphology of the aedeagus [20].

A laboratory mite culture [maintained at 27 ± 2 ºC, 70 ± 10% RH and 12:12 photoperiod] was initiated with 100 females and males from a field population, using rearing units or arenas following [21]. Males and females were transferred to *P. vulgaris* leaf disks (6 cm diameter) on each rearing unit (9 cm diameter and 1.5 cm high). They were maintained for a 24-h period to lay eggs. After this period, males and females were discarded and the eggs were reared until adult emergence.

Effect of ethanolic extracts on mortality and oviposition of *T. urticae*

Toxicity of ethanolic extracts to *T. urticae* was evaluated as per Cowles *et al*. [22]. *Phaseolus vulgaris* leaf disks (6 cm diameter) were soaked for five seconds in the ethanolic extract from both plant species evaluated (2.5, 5, 7.5 and 10%) or in distilled water as a control treatment (0%). Afterward, the excess of liquid was eliminated and leaves were left to dry at room temperature (27 ± 2 ºC, 70 ± 10% RH) for one hour, before being offered as a substratum for the rearing units. Then, 10 one-day old females were randomly selected from the laboratory culture and placed on each of the rearing units containing leaf disks from the respective plant species and extract concentration. Five replicates were run for each treatment. The evaluations were conducted at 24, 48 and 72 h after treatment to determine female mortality and oviposition. A female was considered dead when not responding to touch.

Isolation and identification of acaropathogenic fungi from *T. urticae*

Samplings on *P. vulgaris* fields were carried out in order to collect leaves showing characteristic symptoms of tetranychid feeding. Samples were taken to the laboratory and observed under magnification to confirm the presence of mites attacked by fungus. Tetranychid mites were put in a Petri dish containing potato dextrose agar (PDA) to promote fungus growth. Slides for microscope observation were prepared and the fungus was identified as *Cladosporium* sp. (Teleomorph: *Mycosphaerella*), using a taxonomical key for imperfect fungus [23, 24]. *Cladosporium* sp. is characterized by the following morphological features: conidia are long, cylindrical, septated, brown in color, with dark connections, and arranged in branched conidia chains [24]. Pure culture of *Cladosporium* sp. was obtained and applied (10^7 conidia.mL^{-1}) to evaluate its effect on mortality and oviposition of *T. urticae*. In addition,

the effect of commercial products based on *P. fumosoroseus* (PaeciloBiol) and *B. bassiana* (BeauBass) was tested at 10^7 conidia/mL^{-1} concentration.

Effect of acaropathogen fungi on mite mortality and oviposition of *T. urticae*

Effect of acaropathogen fungi on mortality and oviposition of *T. urticae* was evaluated using rearing units as described above [21]. The procedure was similar to that for ethanolic extract toxicity. Leaf disks were soaked in a 10^7 conidia.mL^{-1} suspension. Then, 10 one-day old females were randomly selected from the laboratory culture and placed on each of the rearing units containing leaf disks from the respective acaropathogen fungus species. Rearing units were observed daily under magnification to count dead females and egg number per female.

The experiments were carried out in a completely randomized design with treatment arranged in a split plot in time, with the plant extract as the main plot and concentrations as the subplots. Oviposition rate was calculated as the mean number of eggs laid per female per day. Data were corrected for mortality control [25]. Linear regression analysis was performed to describe the oviposition rate or cumulative mortality against metabolite concentration using Statistix, version 8.0.

RESULTS AND DISCUSSION

Secondary metabolites

Qualitative composition of secondary metabolites was shown to be quite similar in extracts from wild oregano (*L. origanoides*) and bushy lippia (*L. alba*), except for flavonoids being present only in wild oregano (Table 2). Although phenols and saponins were detected in both extracts, higher concentration was observed in wild oregano, while conversely alkaloids were higher in bushy lippia (Table 2). Differences in qualitative and quantitative characteristics of the essential oil of *Lippia graveolens* Kunth are affected by the prevailing edaphology [26]. Based on this, the authors evidenced two phenolic chemotypes in *L. graveolens*. The first was revealed with either carvacrol or thymol as dominant compounds, and the second was revealed with a non-phenolic chemotype dominated by oxygenated sesquiterpenes. In general, secondary metabolite production in response to stressful conditions, such as pathogen attack or herbivore feeding, is the result of ecological interactions to develop defense mechanisms [11, 27]. Finally, these variations in chemical composition could be used as a diagnostic character in chemotaxonomy [28] and they account for differences in bioactivity of ethanolic extracts [29].

Table 2. Secondary metabolites from ethanolic extracts in oregano and bushy lippia.

		Species	
Secondary metabolite		***L. origanoides***	***L. alba***
Alkaloid	Weak basic	+	+
	Basic	+	+
	Quaternary ammonia salts	+	+
	Total content	205.8	325.5
Flavonoid		+123.2	-
Phenols		+289.3	+247.9
Essential oils		+	+
Antraquinones		-	-
Saponines		+2.6	+0.4
Tanins		+	+

Units = mg/mL and saponins in mm.

Essential oils (isoprenoids) and saponins are frequently found in verbenaceous species [30], and those obtained from *Lippia* spp. have been widely used due to their potential bioactivity [31]. These two secondary metabolite groups serve as plant growth regulators and as plant defense mechanisms [32]. Flavonoids and phenols are genetically and structurally similar and confer antifeedant properties due to bitter flavor [10, 32].

Effect of ethanolic extracts on mortality and oviposition *of T. urticae*

Mortality: Cumulative mortality of mites was affected by ethanolic extracts depending on the plant species ($p < 0.001$) (Table 3). Oregano ethanolic extract provoked higher mortality at 10% concentration; however, the mortality percentage was higher than 50% even when lower concentrations (2.5%) were used. On the other hand, bushy lippia's extracts caused mortality >50% at concentrations higher than 5%.

Botanical products with activity against mites have shown promising results in the field and in laboratory conditions. High mortality levels of *Tetranychus cinnabarinus* (Boisduval) (>69.5%) were observed with extracts of *L. origanoides* [7], of *T. urticae* and *Tetranychus viennensis* Zacher (>78.8%) with extracts of *Kochia scoparia* (L.) Schrad. [33] and of *Mononychellus tanajoa* (Bondar) treated with neem extracts [34]. As in other similar studies, both wild oregano and bushy lippia yielded higher mortality levels even at lower concentrations of ethanolic extracts.

Similarly, 1% essential oils from *L. graveolens* var. *berlandieril* caused mortality (80%) in *Varroa destructor* (Anderson and Trueman) [35]. Cruz *et al.* [36] reveals that mortality, provoked in *Rhipicephalus sanguineus* Latreille by using 2 mg/mL essential oils from *Lippia gracilis* Schauer, could be related to carvacrol content. Cavalcanti *et al.* [30] did not observe significant differences in mortality caused by essential oils obtained from different applications of *Lippia sidoides Cham*. These authors suggest that all essential oil components are probably acting synergistically to achieve the acaricidal effect; hence, the action of other minor constituents cannot be fully disregarded. Similar to our findings, Yanar *et al*. [37] observed higher mortality percentage in *T. urticae* treated with *Eucaliptus camaldulensis* Dehnh. leaf extract (63.3%), *Xanthium strumarium* L. fruit or leaf extract (59.6, 57.5%, respectively), and *Solanum nigrum* L. fruit extract (51.6%). Tello *et al*. [38] observed that ethanolic extracts obtained from various plant species, including *Lampaya medicinalis* F. Phil. (Verbenaceae), provoked high mortality levels in *T. urticae* ranging from 74-88%. Sivira *et al.* [7], observed that mortality is related to the synergistic effect of alkaloids, flavonoids, phenols, tannins, essential oils and saponins content in plant ethanolic extracts. Phenolic compounds, including essential oils and alkaloids, are frequently found in ethanolic extracts from plant species and have a detrimental effect on pathogens and herbivorous animals [39], thus engendering an alternative strategy to synthetical acaricides [10, 40, 41, 42].

Table 3. Cumulative mortality (from 0 to 5 days) of *T. urticae* females caged on leaf discs and treated with different concentrations of ethanolic extracts from oregano (*L. origanoides*) and bushy lippia (*L. alba*).

Ethanolic extract concentration (%)	*L. origanoides*	%	*L. alba*	%
0	3.25 ± 1.500c	21.7	3.25 ± 1.500c	21.7
2.5	10.00 ± 1.826b	66.7	5.50 ± 1.291bc	36.7
5.0	9.00 ± 2.160b	60.0	9.75 ± 3.862ab	65.0
7.5	8.75 ± 0.957b	58.3	10.25 ± 0.957ab	68.3
10.0	12.50 ± 1.291a	83.3	11.75 ± 1.708a	78.3

Values in the columns followed by the same letter did not show significance differences according to Tukey ($p < 0.001$).

Oviposition: Significant differences were detected in *T. urticae* oviposition when this mite was exposed to the different ethanolic extract concentrations and plant species ($p < 0.01$). Lower oviposition rates were observed when rearing substrates were treated with 5, 7.5 or 10% wild oregano extracts compared to bushy lippia at the same concentrations (Table 4). Complete oviposition inhibition was observed at 10%, regardless of the plant species. The lowest concentration of wild oregano crude extract (2.5%) caused a 76.6% reduction in the oviposition of *T. urticae* at day 1, and 83.1% reduction at day 3. Oviposition reduction varied from 88.1 to 95.2 or 99.1 to 100% with 5 or 7.5% wild oregano extract.

On the other hand, bushy lippia ethanolic extracts provoked a reduction of 73.3% or 100% in the oviposition rate when treated with 2.5% or 10% on day 1. It is noticeable that when lower concentrations (2.5 and 5.0%) of bushy lippia were used, the effect on oviposition tended to diminish. At 2.5%, reduction varied from 73.3% at day 1 to 49.2% on day 3 while at 5.0% a slight increase in oviposition was observed at day 3. In general, species in Verbenaceae have demonstrated a negative effect on oviposition in tetranychid mites. Roy *et al*. [43] found that the total number of eggs laid by *Oligonychus coffeae* Nietner was significantly lower on the side of the leaf surface where 4, 6, 8 and 10 g/L of the *Duranta repens* L. extract was applied. Also, the number of eggs laid by the females of *Oligonychus afrasiaticus* (McGregor) tended to decrease from 2.2 to 1.65 eggs, 24 to 48 h after being treated with *Duranta plumieri* Jacq. (Verbenaceae) extracts [44]. As in our study, these authors observed that the effect of this alcoholic extract diminished after 72 h when oviposition reached 6.95 eggs.

Similarly, other plant species have been shown to affect oviposition of phytophagous mites. *T. cinnabarinus* oviposition decreased at a rate of 43.7% or 57% when 5% oregano or gliricidia extracts were used [7] or 66% of ethanoilic extracts of *Asphodelus aestivus* Brot. were applied, respectively [45].

Effect of the entomopathogenic fungi on mortality and oviposition of *T. urticae*

Significant differences in mean mortality rates of *T. urticae* females were detected (Figure 1). Higher mortality rate was observed when the mites were treated with *Cladosporium* sp., which is 72% higher than the control, while *Paecilomyces* and *Beauveria* yielded similar mortality rates at around 45.5 and 54.5% higher than the control. Similarly, *C. cladosporioides* isolates naturally occurring in *T. urticae* have proven to cause high mortality levels (75.25-96.25%) in India [13] and

Table 4. Daily oviposition rate (mean ± standard deviation) in *T. urticae* females treated with various concentrations of ethanolic extracts obtained from wild oregano (*L. origanoides*) and bushy lippia (*L. alba*).

	L. origanoides			***L. alba***		
Ethanolic extract concentration (%)	**Day 1**	**Day 2**	**Day 3**	**Day 1**	**Day 2**	**Day 3**
0	4.35 ± 0.39a	3.88 ± 0.63a	2.95 ± 0.67a	4.35 ± 0.39a	3.88 ± 0.63a	2.95 ± 0.67a
2.5	1.04 ± 0.24b	0.88 ± 0.60b	0.52 ± 0.44b	1.16 ± 0.41b	2.78 ± 0.48ab	3.07 ± 0.85a
5.0	0.21 ± 0.25c	0.17 ± 0.33c	0.35 ± 0.27b	0.20 ± 0.16c	1.28 ± 1.34b	1.50 ± 2.68ab
7.5	0.04 ± 0.07c	0.15 ± 0.19c	0.00 ± 0.00b	0.18 ± 0.14c	0.13 ± 0.25c	0.00 ± 0.00b
10.0	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00b	0.00 ± 0.00c	0.00 ± 0.00c	0.00 ± 0.00b

Mean values in a column followed by the same letter did not show significant differences according to Tukey test ($p < 0.001$).

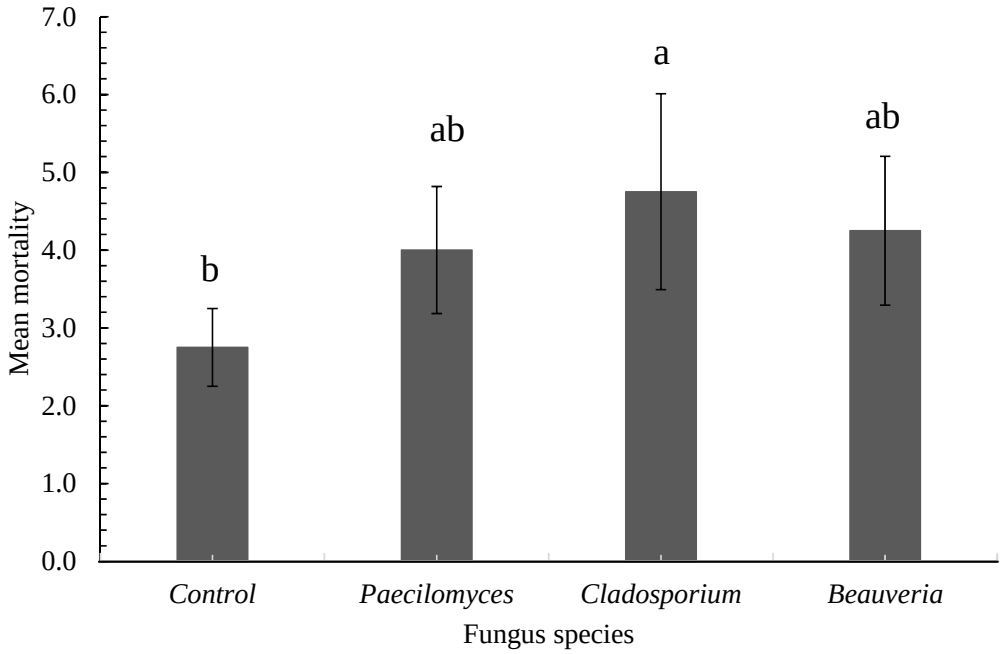

Figure 1. Lethal effect of entomopathogenic fungi on *T. urticae* after treatment with suspensions at 10^7 conidia.mL^{-1}.

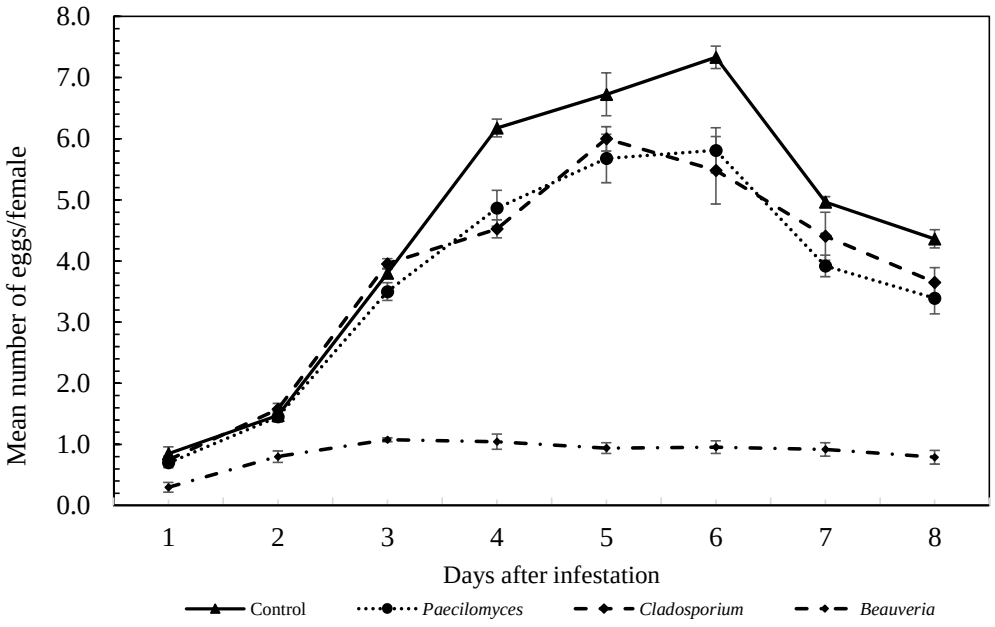

Figure 2. Effect of entomopathogenic fungi on oviposition of *T. urticae* after treatment with suspensions at 10^7 conidia.mL^{-1}.

in Turkey (50.95 to 74.76 %) [46]. On the other hand, Shi & Feng [47] observed that *B. bassiana* was more infectious to *T. cinnabarinus* eggs than *P. fumosoroseus*. Intraspecific variations in pathogenic activity of entomopathogens on arthropod pests have also been reported [48, 49]. Much of this variation is associated with the genetic diversity of the pathogen [50]. According to these

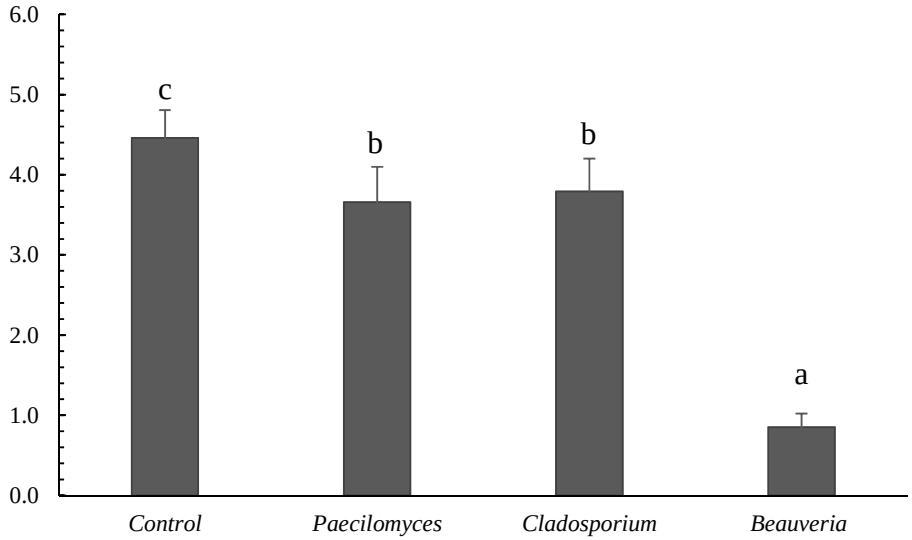

Figure 3. Mean oviposition in *T. urticae* females treated with entomopathogenic fungi at 10^7 conidia.mL^{-1}.

authors, strains of pathogens from diverse geographic origins of entomopathogenic fungi exhibit a wide range of hosts (i.e. *B. bassiana*) but, strains isolated from the same host species show a greater similarity in genetic structure than strains from the same area but isolated from different host species. This fact underlines the importance of strain selection for successful pest management when using entomopathogenic organisms as biocontrol agents [51].

In addition, significant differences were detected in *T. urticae* oviposition due to the effects of the entomopathogenic fungi (Figure 2). More severe reduction in egg numbers was observed in *T. urticae* females treated with *B. bassiana* suspensions (0.85 eggs/female/day), while no significant reduction in egg numbers was evidenced in females treated with *Cladosporium* sp. (3.79 eggs/female/day) or *P. fumosoroseus* (3.66 eggs/female/day) (Figure 3).

CONCLUSION

The impact of natural epizootics on arthropod populations caused in particular by entomopathogens suggests the potential of microbial pest control [52], constituting a sustainable alternative for minimizing the effects of conventional pesticides, and thus improving environmental quality and health [53]. Moreover, combining entomopathogenic fungi with other control methods such as bio-rational insecticides [54] and other microbial pathogens [52] or biopesticides [55] have been shown to be an effective alternative for pest control. Based on our results, it is suggested that both ethanolic extracts of wild oregano and bushy lippia and entomopathogenic fungi constitute a promising alternative as biological controls for the *Tetranychus* species. However further field studies should be run in order to evaluate compatibility of these two strategies and their relationship with environmental conditions.

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CONFLICT OF INTEREST STATEMENT

The authors declare that there is no conflict of interest regarding the publication of this paper.

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