Original Article

Evaluation of the Entomopathogenic Fungus *Beauveria bassiana* on Different Stages of *Phlebotomus papatasi* (Diptera: Psychodidae), Vector of Zoonotic Cutaneous Leishmaniasis in Iran

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Abstract

Background: Zoonotic cutaneous leishmaniasis is a major public health problem in Iran with the main vector of *Phlebotomus papatasi*. The use of entomopathogenic fungi for biological control of the vector is a potential substitute for the current methods which are being used. The purpose of the current study was to assess the virulence of two local isolates of *Beauveria bassiana* (OZ_2 and TV) against *Ph. papatasi*.

Methods: To perform the bioassay test, fungal suspensions were applied for every stage of the sand fly life cycle. The mortality rate, longevity, and number of eggs laid were determined. Also, the probability of fungal survival on the surface of rodent's body was assessed.

Results: The longevity of infected adult sand flies with both isolates of *B. bassiana* was significantly lower ($P< 0.05$) in comparison to the negative control. The estimated Lethal concentration 50 (LC_{50}) values for adult female and male sand flies treated with OZ_2 isolate were 1.4×10^6 and 2.2×10^7 conidia/ml, respectively, while they were 6.8×10^6 and 2.3×10^8 conidia/ml for TV isolate, respectively. Both isolates of *B. bassiana* exhibited nonsignificant mortality rates in sand fly larvae and pupae and fecundity rate ($P> 0.05$). According to our findings for both isolates, the fungus continued to spread throughout the surface of the rodent's body for 144 hours after spraying.

Conclusion: The current study demonstrated that both isolates of *B. bassiana* have considerable biological control capacity against adult sand flies.

Keywords: Biological control; *Beauveria bassiana*; Sand fly; *Rhombomys opimus*; Burrow

Introduction

Leishmaniasis is a vector-borne disease, caused by protozoan parasites (genus *Leishmania*), transmitted to humans and animals through infected female sand fly bites (1). Approximately 700,000 to 1 million new cases and between 20,000 and 40,000 deaths are reported annually throughout the world from this disease (2). There are reports of two types of

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leishmaniasis in Iran, visceral and cutaneous leishmaniasis. The last one is more prevalent and has two forms of anthroponotic cutaneous leishmaniasis (ACL) and zoonotic cutaneous leishmaniasis (ZCL) which are different in their reservoirs (3–5). *Leishmania major* and *Phlebotomus papatasi* are the causative agent and primary vector of ZCL in Iran, respectively. One of the main foci of this disease is in the Esfahan area in central Iran where the main reservoir host is *Rhombomys opimus* (3–9).

Adult sand flies are recognized as small, fragile, nocturnally active insects with weak fly capability. Both sexes of sand flies require sugar for energy, primarily from the plants, and female sand flies require a blood meal to mature their eggs. During the day, adult sand flies seek refuge in dark, humid areas such as tree holes, animal burrows, and beneath rocks. The eggs are placed in a terrestrial microhabitat rich in organic matter, which feeds the larvae (10). Several methods are used to collect adult sand flies, whether foraging at night or resting during the daytime. Immature stages are hard to find and much continues to be not understood about sand fly breeding habitats, a knowledge gap that limits vector control options (11). The main method for vector control is the residual spraying by different insecticides which is recommended by WHO in regions associated with human cases; nevertheless, this method has not proven successful (12, 13). Furthermore, the usage of these chemical insecticides may cause environmental and toxicological issues as well as insecticide resistance (14). As a result, new vector control strategies must be developed to improve human life quality (15). Biological control agents (BCAs) are widely regarded as a safer alternative rather than conventional insecticides and many of BCAs are suitable for use in organic production systems (16). Sand fly control with BCAs has huge potential in terms of developing sustainable and environmentally beneficial options (17,18). Entomopathogenic fungi are emphasized as the primary agents in pest control. Currently, about 700 species and 90 genera representing nearly all the major fungal classes are thought to be insect-infecting. Hyphomycete fungi mass manufacturing is not particularly expensive. To manage a variety of harmful insects to crops, insect pathogenic fungi are being developed and mass-produced throughout the world. The specificity of entomopathogenic fungi varies greatly within, across, and among species. The most researched fungi are *Beauveria bassiana* and *Metarhizium anisopliae*, which have a broad host range that includes hundreds of insect species. Numerous other hosts, such as species of Coleoptera, Lepidoptera, Diptera, Homoptera and Hymenoptera, have been documented in recent years (19).

Beauveria bassiana has a global distribution and can be retrieved from various sources such as soil and insects, where it can live for a very long time and infect the host at any stage of development (20–22, 23). According to several studies, some leishmaniasis vectors are also susceptible to this fungus (24, 25). Although it is still in the early phases of the study, using the entomopathogenic fungus as a biological control for phlebotomine dipterans may be beneficial for the integrated management of sand flies and ultimately improving public health (23). To our knowledge, limited studies considered the fungus as a biological control of sand flies. So, the present study investigated how two isolates of the *B. bassiana* fungus affect *Ph. papatasi* at immature and mature stages, as well as how well the rodent skin could keep the fungus and probably transfer to the burrows where the sand flies breed.

Materials and Methods

Phlebotomus papatasi collection, identification, and rearing

Phlebotomus papatasi adults were captured from Matin Abad area (33°45'15.2"N 51°59'34.1"E) in Isfahan Province (Fig. 1) and transferred to the sand fly insectary, De-

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partment of Vector Biology and Control of Diseases at Tehran University of Medical Sciences. Sand flies were maintained under suitable conditions at 26±2 °C, 75% RH, and 10:14 hours L: D). After acclimatization, female sand flies were allowed to feed on anesthetized mice (ketamine 60 mg/kg and xylazine 15 mg/kg) for 1 h. Adult females and males were placed into plastic pots measuring 5 cm in diameter and 7 cm in height that were coated with sterile plaster to ensure the preservation of moisture for 24 hours following feeding to induce oviposition. After oviposition, female specimens were mounted and subsequently identified using morphological keys (26, 27). The larvae were fed with a diet, consisting of rabbit faces and rodents' pellet at regular intervals until they reached the pupal stage. When the adults emerged, they were placed in special cages measuring 25×25×25 cm. For feeding of adults, cotton soaked in saturated sucrose and 50% honey was used, and 3–5-day-old adults took blood. Following this stage, the blood-fed females were placed into pots with a diameter of 7 cm and a height of 8 cm for mass rearing (28, 29).

Culture and suspension preparation of *Beauveria bassiana*

Two isolates of *B. bassiana*, TV and OZ_2, used in this study were isolated from soil of different regions of Karaj, Alborz Province, Iran and it was generously provided by Biological Control-Insect Pathology lab, University of Tehran. *Beauveria bassiana* isolates were cultivated in standard petri-dishes (8 cm diameter) on Sabouraud dextrose agar containing 1% yeast extract (SDAY) (Quelab) at 25± 1 °C in the dark. After 14–16 days of growth, conidia were scraped and diluted with 0.05% v/v Tween80®. The suspension was vortexed for 15 minutes to ensure homogeneity before being filtered. The conidial numbers were determined using a Neubauer® hemocytometer. The concentrations 1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/ml were obtained through serial dilutions (30).

Conidial viability

Before each bioassay, the viability of conidia was assessed by spreading 1 ml of the 1 $\times10^4$ conidia/ml suspension onto SDAY plates and incubation at 25±1 °C for 24 h for germination. The viability was determined by inserting sterile microscopic coverslips on each plate and duplicating each plate four times, it was possible to calculate the percentage of germination from 100-spore counts. More than 90% of conidia germinated were considered as acceptable for doing the test (31).

Bioassay tests

To assess the virulence of these two isolates, four fungal concentrations (1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/ml) were used for the larval and pupal stages and five fungal concentrations (1×10^5, 1×10^6, 1×10^7, 1×10^8 and 1×10^9 conidia/ml) for the adult stage with two control groups: 0.05% v/v sterile Tween 80 (negative control) and 0.05% w/v Deltamethrin (BAYER AG) for adults, as well as Diazinon 60% v/v (RAJA) for larval and pupal stages (positive control). The samples for the testing were chosen randomly. Twelve female adults, 12 male adults, 12 larvae and 10 pupae were considered for every one of the 5 repeats (23).

Treatment of early 2nd instar larvae

Twelve larvae of the second instar, were placed in the bottom of each plastic pot similar to rearing pots. Using a Hand spray, each fungal suspension (3 ml), Diazinon (positive control), or Tween 80 (negative control) was applied to the inner walls and bottom of each pot. The treated pots were then housed in a dedicated chamber with a temperature of 26± 2 °C, a humidity of 70%, and a light-dark cycle of 10: 14 h. They were fed the same diet as the colony, and larval mortality numbers were counted daily until the pupal stage or the death of all larvae.

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Published Online: Sep 30, 2023

Treatment of pupae

Ten pupae were placed and subjected to fungal infection using the same procedure employed for larvae. Daily counts of pupal mortality were done until the emergence of adults or until all pupae had died.

Treatment of adults

Twelve males and 12 females, 24 h post-blood feeding were used in each replicate. The adult sand flies were put into plastic pots, chilled to -6 ºC for 2 min to immobilize them, and then were treated with 3 ml of either fungal concentration, Deltamethrin, or 0.05% Tween 80. They had access to saturated sucrose and 50% honey solution for feeding. Daily adult mortality was recorded until all of them died, and if an adult survived, the number of eggs deposited by treated females was recorded.

Beauveria bassiana* growth and microscopic features post-passage in *Phlebotomus papatasi

In order to facilitate sporulation, the larvae, pupae, and dead adults were sterilized using 70% ethanol, 3% sodium hypochlorite, and sterile distilled water. They were then put on sterile petri dishes with moist sterile filter paper (21). The fungal isolates were mounted into microscopic slides using the slide-culture method and were identified.

Isolation of fungus from the rodent body surface

Preparation of *Rhombomys opimus* colony

The animal house at the Esfahan Health Research Center was used to maintain *R. opimus* after capturing by Sherman's live trap from its natural habitat in the Matin Abad region, Esfahan Province. These rodents were housed in glass enclosures measuring 1 m^3, with the soil from their habitat. In each chamber, three *R. opimus* were released. They were fed pellets as the main diet and carrots as a supply of water.

Determining of reservoir's skin fungal contamination

This experiment was conducted in a semi-field setting. This was accomplished by using the chambers consisting of rodent colonies. For fungal isolates, separate chambers were chosen, and one was used as a control. Each chamber contained 3 rodents, which were considered as 3 replications. The concentration used for each isolate was 10 times the lethal concentration 50 (LC_{50}) obtained from the adult stage. The amount of spraying in each hole was 15 ml and the spraying was done using a hand spray. Twenty-four, 72 and 144 hours after spraying, rodents were caught and using a swab soaked with sterile distilled water, sampling was carried out from the rodent's body surface. For each rodent, the belly, back, back of the head, muzzle and ventral surface of the tail were sampled in triplicate. These samples were cultured on SDAY medium under the hood. For fungus growth, the culture plates were incubated at 25±1 °C.

Data analysis

The Kolmogorov-Smirnov test was used to determine the normality of the data. Using probit regression, the LC_{50} and lethal time (LT_{50}) values were estimated. One-way and Two-way ANOVA tests were used to analyze the effects of fungal infection on egg production, larval, pupal, and adult mortality. Means were then separated using Tukey HSD at a significant level of 5%. Parametric testing was performed by applying Z-transformation as a variable change test. SPSS 25 was utilized to carry out all the analyses.

Results

Adult bioassays

Our results indicated that both fungal isolates are capable of causing lethal disease in *Ph. papatasi* adults (Fig. 2). Data analysis showed that the highest concentration (1×10^9 conidia/ml) of OZ_2 isolate resulted in a mortality rate of 91.80±0.78% in female adults

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during 4 days and 73.40±0.86% in male adults during 5 days. Both groups had significant differences with the negative control (P< 0.001). Lethal concentration 50 values equal to 1.43×10^6 and 2.16×10^7 (conidia/ml) for females and males respectively.

For the *B. bassiana* TV isolate, in the highest concentration (1×10^9 conidia/ml) the mortality rates were estimated to be 80.33±1.19% for females with LT_{50} of 5 days and 75.00± 1.03% for males with LT_{50} of 6 days. Both groups demonstrated a significant difference from the negative control group (P< 0.001). The LC_{50} for this isolate was calculated to be 6.81×10^6 conidia/ml for female and 2.29×10^8 conidia/ml for male. According to the study's findings, the OZ_2 isolate caused higher mortality and a lower LC_{50} for both males and females than the TV isolate; it also showed stronger virulence (Tables 1 and 2). The mean percent mortality of adult sand flies exposed to the two *B. bassiana* isolates (OZ_2, TV) was dose-dependent and increased with conidial concentration. Additionally, the findings demonstrated that the longevity in male and female adults treated with the OZ_2 isolate, following exposure to the fungi in all concentrations, was substantially different from the negative control group, and that the longevity declined with increasing concentration (P≤ 0.001). The results from the adults treated with the TV isolate also demonstrate that the longevity of female adults following exposure to the fungi was significantly different in all concentrations (P< 0.001), but that of male adults was only significantly different from the negative control group (P< 0.05) in the three highest concentrations; and in the lowest concentration, due to the absence of fungal death, the longevity was similar to the negative control. While death occurred in all adults exposed to 0.05% deltamethrin in the first hours after exposure (Table 3).

Egg laying

The results of this study showed that the total mean number of laid eggs per female in the groups treated with OZ_2 isolate was 24.22 ±0.89 (F= 6.144, df= 5, P> 0.05) and at the highest concentration due to the 100% mortality rate in adults, it was not possible to compare the number of eggs laid per female with the negative control group and other concentrations did not have significant differences with the negative control group. In adults treated with TV isolate, the total mean of laid eggs per female was 26.59±0.71 (F= 0.565, df= 5, P= 0.725) and at the highest concentration, the mean laid eggs per female was 24.00±0.20 which did not show any significant difference with the negative control group (P= 0.964) (Table 3).

Larval bioassays

The results of the present study showed that larval mortality caused by two isolates (OZ_2 and TV) of *B. bassiana* was dose-response dependent, but that none of the isolates was capable of causing a high level of mortality in sand fly larvae. The maximum percentage of larval mortality at the highest concentration for OZ_2 isolate was observed to be 21.80±0.56, which was significantly different from the negative control group (P= 0.001) and for the TV isolate it was reported to be 15.20±0.52%, which was significantly different from the negative control group (P= 0.001). The LC_{50} and LT_{50} obtained for larvae treated with two fungal isolates, OZ_2 and TV, were 3.8×10^{10} conidia/ml in 144 days and 3.9×10^{11} conidia/ml in 363 days, respectively (LC_{50} and LT_{50} values for these isolates are software generated and have only theoretical importance).

Pupal bioassays

The outcomes from this stage were almost similar to those from the larval stage. The highest mortality rate in *Ph. papatasi* pupae treated with *B. bassiana* OZ_2 isolate were equal to 22.00±3.74% and 22.00±4.91% in concentrations of 1×10^8 and 1×10^9 conidia/ml, respectively, and both were significantly different from the negative control group (P= 0.004). The OZ_2 isolate's LC_{50} and LT_{50} were determined to be 8.7×10^{11} conidia/ml and 15 days, respectively. The maximum pupae mortality

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rate for the TV isolate was found to be 30.00± 4.48% at the highest concentration (1×10^9 conidia/ml) and was significantly different from the negative control group ($P< 0.001$). This isolate acquired LC_{50} and LT_{50} values were 7.6×10^{11} conidia/ml and 24 days, respectively. (LC_{50} and LT_{50} values for these isolates are software generated and have only theoretical importance).

Isolation of fungi from the rodent body surface

Based on the results obtained in this part of the study, the rodent can move the OZ_2 and TV isolates of the *B. bassiana* fungi sprayed in the burrow opening with its body surface and may even take it deep inside the burrow. According to the findings for both isolates, the transmission of the fungi with the surface of the rodent's body continued until 144 hours after spraying. The OZ_2 isolate's highest growth percentages of the fungus on culture media were back of the head and back (27.3%), back of the head (33.3%), and back (35.7%) at intervals of 24, 72, and 144 hours after spraying, respectively. These percentages for the TV fungal isolate were 33.3% in the belly, 23.5% in the back of the head, the belly, the back, and the muzzle, and 40.0% in the back at intervals 24, 72, and 144 hours after spraying, respectively. The samples taken from the ventral surface of the tail showed the least amount of fungal growth in the culture media for both isolates (OZ_2, TV) (Fig. 3).

Table 1. Lethal concentration 50 (conidia/mL) values of the entomopathogenic fungus *Beauveria bassiana* against female and male adults of *Phlebotomus papatasi*

Fungi	Isolate (s)	Female		Male	
		LC_{50}	FD[a]	LC_{50}	FD[a]
Beauveria bassiana	**OZ_2**	1.43×10^6	7.22×10^5-2.78×10^6	2.16×10^7	1.11×10^7-4.40×10^7
	TV	6.81×10^6	2.99×10^6-1.52×10^7	2.29×10^8	9.37×10^7-6.53×10^8

[a]Fiducial limit.

Table 2. Mortality percentage and lethal time 50 (days) values of the entomopathogenic fungus *Beauveria bassiana* against female and male adults of *Phlebotomus papatasi*. The data presented are means ±SE

Fungi	Isolate (s)	Female			Male		
		Concentration (conidia/ml)	%Mortality	LT_{50}	Concentration (conidia/ml)	%Mortality	LT_{50}
Beauveria bassiana	OZ_2	1×10^5	33.20±2.75a	16	1×10^5	15.20±0.52a	58
		1×10^6	50.20±2.75ac	10	1×10^6	23.60±1.4a	29
		1×10^7	68.20±1.41bc	6	1×10^7	51.60±3.36a	9
		1×10^8	81.40±0.46b	5	1×10^8	61.40±1.63b	7
		1×10^9	91.80±0.78b	4	1×10^9	73.40±0.86c	5
	TV	1×10^5	19.67±0.59a	58	1×10^5	0.00±0.00a	-
		1×10^6	36.33±2.23a	15	1×10^6	11.00±0.67a	100
		1×10^7	41.67±4.35ab	12	1×10^7	14.00±0.67ab	41
		1×10^8	80.33±1.58b	5	1×10^8	39.00±0.67b	13
		1×10^9	80.33±1.19b	5	1×10^9	75.00±1.03b	6

The means in the same column that are preceded by the different lowercase letter differ significantly (Tukey HSD test, $P< 0.05$)

http://jad.tums.ac.ir
Published Online: Sep 30, 2023

Table 3. Effects of two isolates of *Beauveria bassiana* on longevity and number of laid eggs in *Phlebotomus papatasi*. Data presented are means ± SE

Treatments (conidia/ml)	OZ_2			TV		
	Longevity days		Number of laid eggs per female	Longevity days		Number of laid eggs per female
	Female	Male		Female	Male	
1×10^5	4.00±0.09a	3.40±0.07a	29.20±0.89a	4.33±0.07a	5.00±0.12ab	28.67±0.81 a
1×10^6	3.40±0.11ab	3.40±0.07a	37.33±2.47a	4.00±0.12a	3.33±0.07ab	21.33±2.73a
1×10^7	2.80±0.06abc	3.20±0.06a	22.75±0.71a	3.33±0.07a	2.67±0.15ac	32.00±2.08 a
1×10^8	2.80±0.06abc	2.60±0.07a	18.00±1.77a	3.00±0.12a	2.67±0.07ac	18.33±0.99 a
1×10^9	1.80±0.06c	2.40±0.07a	-	2.33±0.07ac	2.67±0.07ac	24.00±0.20 a
Tween80 0.05%	6.80±0.10d	5.00±0.09b	43.00±0.89a	8.00±0.12b	5.33±0.19b	34.33±1.11a
Deltamethrin 0.05%	0.00±0.00e	0.00±0.00c	-	0.00±0.00c	0.00±00c	-

The means in the same column that are preceded by the different lowercase letter differ significantly (Tukey HSD test, P< 0.05)

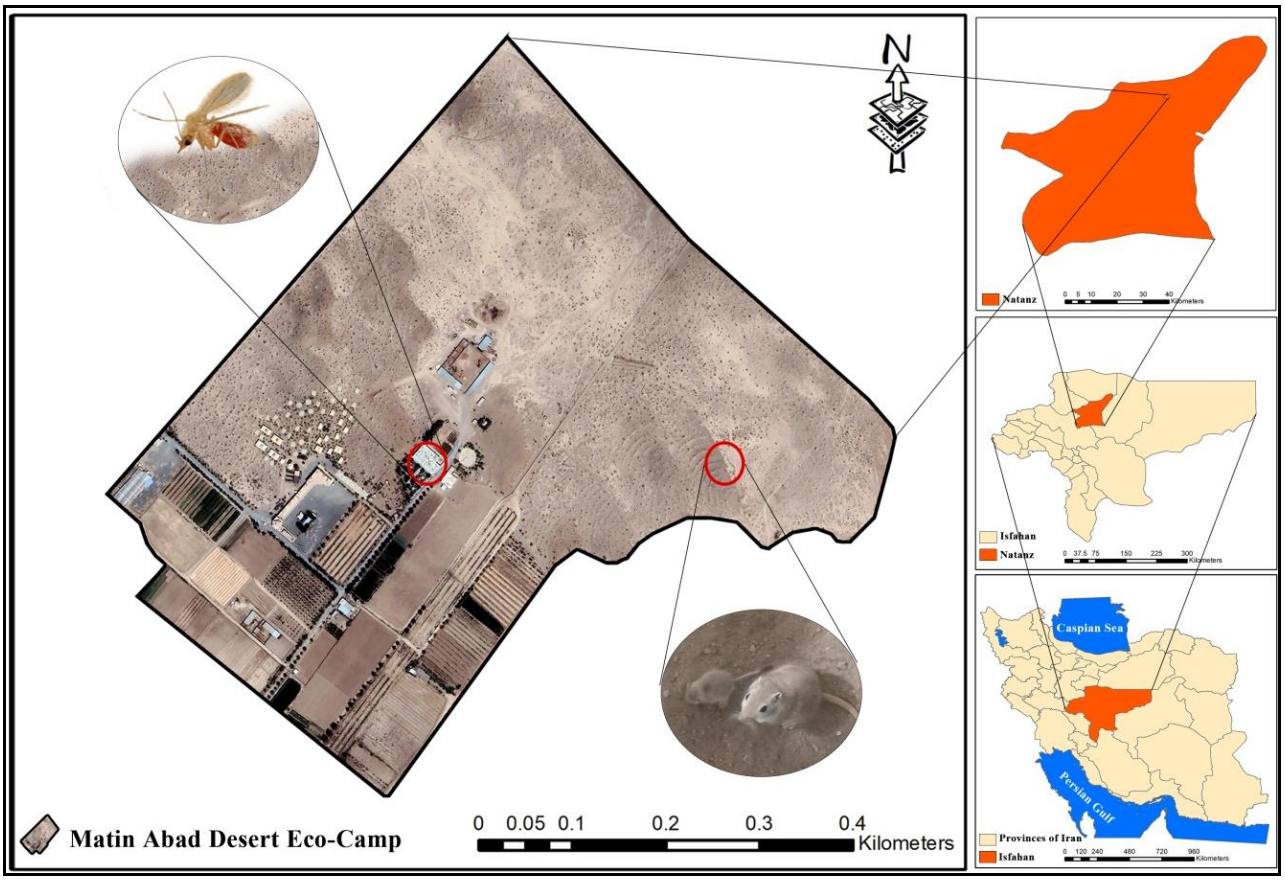

Fig. 1. Geographical map of collection site for *Phlebotomus papatasi* and *Rhombomys opimus* specimens, Matin Abad area, Isfahan Province, 2020–2021

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Published Online: Sep 30, 2023

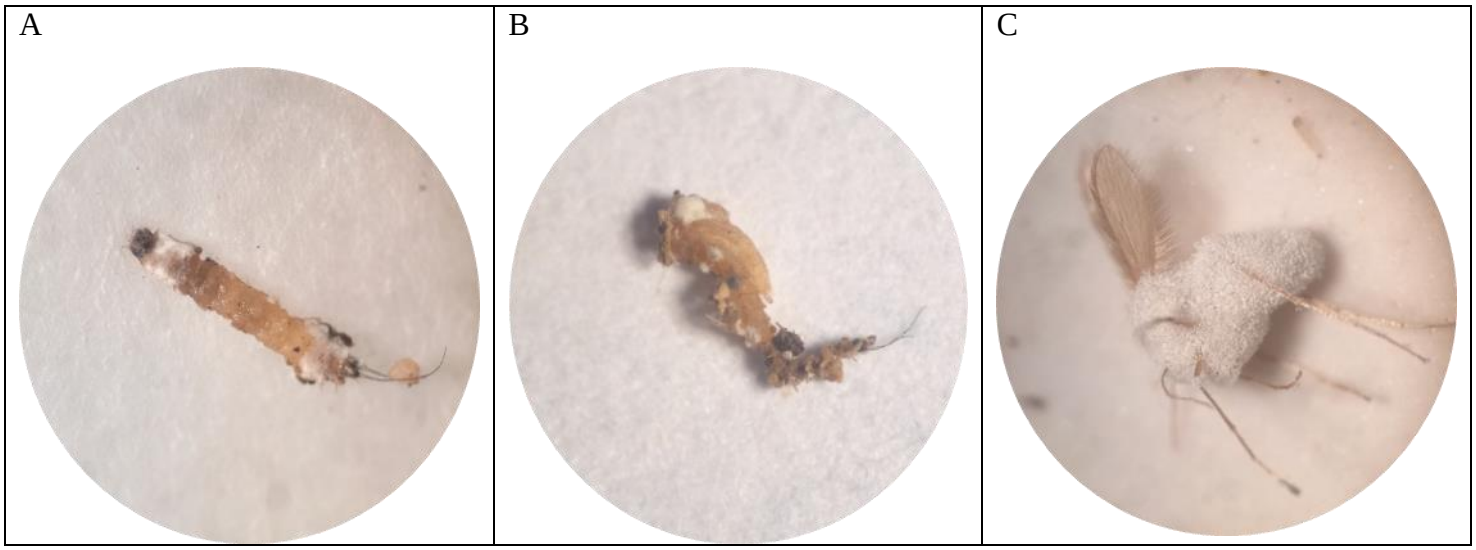

Fig. 2. The infection of sand fly *Phlebotomus papatasi* treated with *Beauveria bassiana* (OZ_2 isolate) A, larva, B, pupa and C, adult

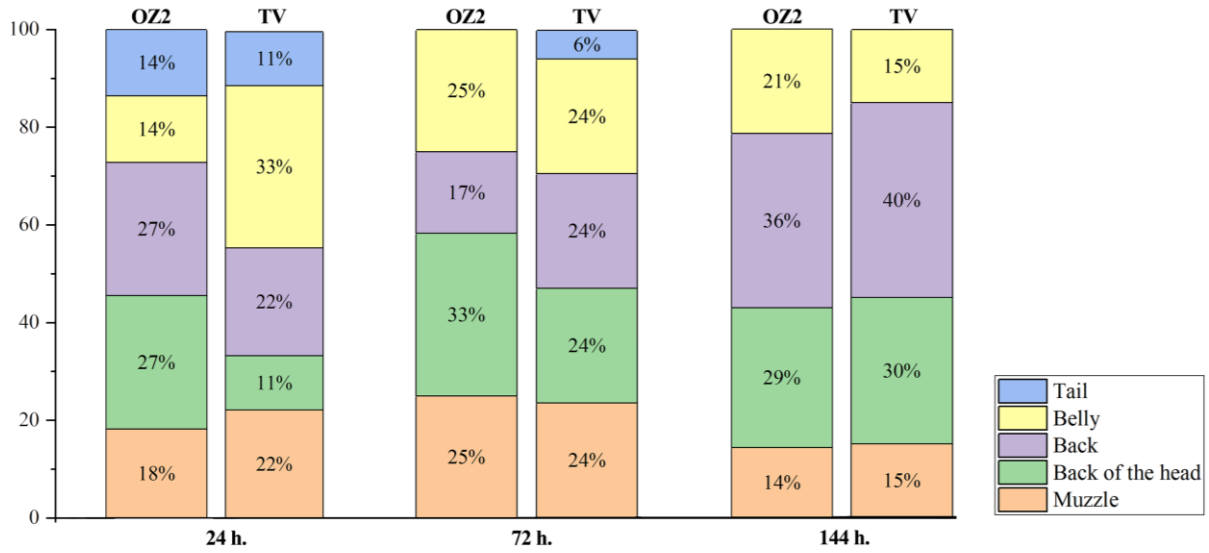

Fig. 3. The mean percentage of positive culture media for *B. bassiana* OZ_2 and TV isolates inoculated with swabs from different parts of the rodent's body where contaminated by the released fungus on the soil of open rodent burrow, in semi-field condition, Esfahan

Discussion

Although various studies on the insecticidal activities of *B. bassiana* fungus on various types of arthropods such as Dipteran, Coleopteran and Acari have been published (32–36), few investigations on the insecticidal effects of this fungus on sand flies have been Conducted. The current study sought to assess the virulence of two local isolates of *B. bassiana* against *Ph. papatsi* in mature and immature stages. The current investigation found that, although OZ_2 and TV fungal isolates did not have strong pathogenic power for larvae and pupae, they

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did have high insecticidal power in the adult stage and could considerably decrease the longevity of adults following exposure to the fungus. Overall, in terms of adult bioassays, the results show a significant difference between the two isolates of OZ_2 and TV (longevity and mortality). According to our results, the OZ_2 and TV isolate have demonstrated high virulence for adults *Ph. papatasi*.

These results are in line with earlier research conducted on the harmful effects of *M. anisopliae* and *B. bassiana* fungi on the adult stage of *Phlebotomus duboscqi* under lab circumstances (37). They demonstrated that all isolates of these two fungi for *Ph. duboscqi* adults were pathogenic and that three isolates of *B. bassiana* had mortality rates ranging from 89.4 to 96.7%. According to their investigation, the 50% and 90% lethality periods varied from 5.0 to 5.7 days and 11.6 to 13.3 days, respectively. These results are compatible with those from our own study. The way the sand flies were infected in that study differs from our investigation. In that study, the sand flies were infected through a velvet cloth that contained dried conidia (37).

Both male and female sand flies experienced a shortening life under the influence of fungal concentrations. Like the findings from other research on sand flies, such as the impact of *B. bassiana* and *M. anisopliae* on *Lutzomyia longipalpis*, and *B. bassiana* on the longevity of *Ph. papatasi* and *Lu. longipalpis* (22, 38, 23).

The longevity means of *Lu. longipalpis* sand flies, the main vector of visceral leishmaniasis in Brazil, exposed to the CL1 (URM-3447) isolate of *B. bassiana* in all concentrations was significantly different from that of the negative control group. The mean longevity at the maximum concentration (10^8 conidia/ml) was approximately 4.1 days, whereas it was 7.2 days in the negative control group, indicating that the fungus decreased adult longevity by 3.1 days. Their other study demonstrates that the *M. anisopliae* fungus could substantially shorten adult longevity, bringing the mean longevity down to 7 days. But these results could not overcome the deaths that occurred due to cypermethrin as a positive control (38, 23).

The mean of adult longevity following exposure to the fungus was determined separately for males and females in the current investigation. In comparison to the negative control group, the results showed that OZ_2 and TV isolates were able to shorten the mean of adult male longevity at the highest concentration (1×10^9 conidia/ml) by 2.6 and 2.66 days and mean of adult female longevity by 5and 5.67 days. Even in females and males exposed to the highest concentration of TV isolate, the results had no significant differences with the deltamethrin treated group as a positive control.

The fact that these findings, as noted by Amora et al. (38), differ from those of Reithinger et al.'s study (24) on the impact of *B. bassiana* fungus on the longevity of *Lutzomyia youngi* - the vector of cutaneous leishmaniasis in Colombia- is intriguing. They have demonstrated that the longevity of sand flies treated with the fungus was higher than the negative control group, except for the highest commercially tested concentration of the fungus (3.5×10^{10} conidia/ml) (24).

Regarding the number of laid eggs, the results are like Warburg and Amora et al studies and there was no significant decrease in infected female oviposition (25, 38, 23).

In this regard, if we look at other studies conducted on flies, the results of one of the studies show that the longevity of house fly adults treated with sublethal dose (1×10^6 spores/mL) of three isolates Bb-01, Bb-08 and Bb-10 of *B. bassiana* fungi has decreased by 15.43, 12.03 and 11.73 in males and by 16.49, 13.6 and 12.51 days in females respectively compared to the negative control group. The mean number of eggs/female in adults treated with sublethal doses of entomopathogenic fungi ranged from 120.45 to 212.7, while the mean number of eggs/female in the negative control group was reported to be 462.68 which was contrary to our findings (39).

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Published Online: Sep 30, 2023

In a study that assessed the fecundity and blood-feeding behavior of adult *Aedes aegypti* infected with *B. bassiana* fungus in laboratory and semi-field settings, the findings revealed that fungal infection was able to minimize mosquito-human contact by 30%. In lab settings, the fecundity of female mosquitos dropped by 29.3±8.6 eggs. Additionally, fungal infection decreases mosquito survival in semi-desert settings by 59–95% in large cages against 61–69 % in small cages (40).

Another study that attempted to control *Aedes albopictus* and *Culex pipiens* simultaneously with various isolates of *B. bassiana* revealed that 12 and 23 isolates of the fungi were pathogenic for *Ae. albopictus* and *Cx. pipiens*, respectively. The JN5R1W1 isolate was finally introduced as the most effective and efficient isolate for the simultaneous control of *Ae. albopictus* and *Cx. pipiens*. The measured LT_{50} of this isolate was 4.4 days for *Cx pipiens* and 4.5 days for *Ae. albopictus*, which is comparable to the results of our investigation. According to other studies, *M. anisopliae* caused adult *Ae. aegypti* mortality between 3.1 and 4.1 days (41, 42). Among other studies conducted regarding the effects of entomopathogenic fungi on different life stages of *Ae. aegypti* mosquitoes, we can mention the results of Rochaet al. (43), that all showed high lethality and reduced longevity in adults exposed to fungi, which was similar to our results. On the other hand, their studies have shown the lethal effects of entomopathogenic fungi on the larvae of *Ae. aegypti* mosquitoes, which is contrary to the findings of this study (43–45). In terms of the larval and pupal stages, our findings demonstrate that the fungus does not produce high virulence, which is consistent with the outcomes noticed by Warburg (25) regarding the effect of the fungus on the larval stages of *Ph. papatasi* and *Lu. longipalpis* in laboratory conditions. However, the mortality values were dose-dependent. The findings of Amora and her colleagues (38, 23) showed that the *M. anisopliae* fungus had a significant effect on the mortality of the larval stage of *Lu. longipalpis* at a concentration of 1×10^8. Moreover, the results obtained in their other study regarding the effect of *B.bassiana* on the larval stage of *Lu. longipalpis* showed that larval mortality was not only dose-dependent but also had a significant difference with the control group at the lowest concentration, but in our study none of the two isolates of *B.bassiana* could not have a significant effect on the mortality of the larvae, these differences may be related to the type of fungal isolate or the sand fly species. The studies of Lecouna and Geden (46, 47) on the house fly, which both demonstrated that none of the isolates of the fungus *B. bassiana* have been able to affect house fly larvae and pupae, are among the other studies that have been conducted on other flies and are consistent with the findings of the present study.

In line with previous research, Zayed et al. (48)'s findings revealed that pupal stage mortality was highest in the groups treated with *M. anisopliae* fungus. Also, the highest larval mortality occurred in the highest concentration. Another remarkable point is that they showed that the percentage of mortality decreases with increasing temperature from 26 to 31 °C.

Discrepancies between studies can be attributed to several variables, including methodology, host sensitivity, fungal concentration, and fungus severity. By using the immersion approach in the field, it is entirely impossible to infect insects with pathogenic fungi (39), and bringing the fungus to the depth of the reservoir's burrow is one of the challenges associated with using the entomopathogenic fungi against sand flies that transmit rural cutaneous leishmaniasis, such as *Ph. papatasi*, because all stages of the sand fly life cycle, until the emergence of adults occur in the depth of the rodent's burrow where sufficient food and moisture are available, and access to the depth of the rodent's burrow is practically impossible (49). As evidenced by the results, both fungal isolates were obtained after spraying on soil of rodent

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Published Online: Sep 30, 2023

nest opening from various locations on the rodent's body at different times. This suggests that an effective method may involve transferring the fungus with the rodent to the depth of the burrow and then introducing it to both the mature and immature stages of the sand fly. One of the noteworthy points in the results of this part of the study is that with the passage of time, the stability of the fungus in the back and back of the head increases, which can be said to be the reason for the greater movement of rodents and as a result, more body surface contact with the soil of the nest opening. On the other hand, due to the grooming behavior of rodents, the possibility of the rodent accessing the back of the head and back is far less than other parts of the body, and the probability of survival of the fungus in these parts increases.

Although *B. bassiana* fungus could not have high virulence for immature stages, but the presence of fungus in the rodent nest and surface contamination of larvae and its transfer to the next stages can increase the chance of adult infection with the fungus through the pupal shell. On the other hand, the appearance of adults in the nest of rodents happen and then they leave the nest with interrupted flights, which also helps to infect the adults with fungi. Despite the fact that the rodents are constantly cleaning their nests, we could detect the fungus from the surface of the rodents up to 6 days after spraying, which shows that even if the rodents move the fungus out of the nests, they are still able to return it with the surface of its body to the depth of the nest and even in this way help the fungus to spread. Additionally, based on the findings of our study, it was demonstrated that the rodent is capable of disposing of the fungus added to the bait through its feces, which will be a very dependable approach to introduce the fungus to the depth of the burrow (Unpublished data). The findings of this investigation demonstrated that OZ_2 and TV isolates of *B. bassiana* were not pathogens to *Ph. papatasi* larvae and pupae, suggesting that these pathogens cannot be a reliable alternative for managing the immature stages. However, positive outcomes in terms of adult longevity and mortality, demonstrated that *Ph. papatasi*, the primary vector of ZCL, is extremely susceptible to this fungus. Since the period of metacyclogenesis of the *L. major* in the digestive canal of the sand flies lasts at least 7 days (50), and taking into account that in the present study, the longevity was calculated in females that were exposed to the fungus 24 h after blood feeding, even if the interval between intervention with entomopathogenic fungus and mortality of adults is 3 to 5 days, this method can be beneficial. Because, it prevents the parasite from completing its metacyclogenesis cycle and thus is a more acceptable approach than chemical ones, which can occasionally result in resistance over time but it needs more studies in field conditions. Because in field conditions, various factors such as humidity, temperature and UV of the sun may affect the survival rate and infectivity of the fungus and it may even affect the concentration of fungi used in the field (51).

Finally, since one of the reasons for the lack of widespread use of entomopathogenic fungi is their slow pathogenicity process for the host, new studies are more towards genetic engineering and the introduction of desirable traits in order to accelerate the pathogenicity of fungi (52). Therefore, it is suggested that the next studies be based on the identification and introduction of these traits to these fungi.

Conclusion

The findings of the current study demonstrated the effectiveness of *B. bassiana* as a biological control against adult sand fly, *Ph. papatasi*, particularly when high concentrations of conidia are sprayed. Additionally, the most potent *B. bassiana* isolate (OZ_2) present a promising avenue for forthcoming mycoinsecticidal research. However, it is important to assess *B. bassiana* field efficacy, particularly in sites with rodent colonies.

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Ethical considerations

Our project was approved by the Tehran University of Medical Sciences (IR. TUMS. SPH. REC. 1400.088).

Conflict of interest statement

The authors declare that there is no conflict of interest.

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