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Original Article

Repellency Effect of Hydro-Alcoholic *Ricinus communis* (Castor) Leaf Extract against *Phlebotomus papatasi* Under Laboratory Conditions

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Abstract

Background: The extract of seed and leave of *Ricinus communis* (castor plant) is rich in glycerides and fatty acids, including ricin, oleic acid, palmitic acid, linoleic acid and dihydroxy-stearic. This study aimed to evaluate the repellency effect of *R. communis* leaf extract **(**castor extract, CE) on *Phlebotomus papatasi* sand flies and compare its effectiveness with a commercial insect repellent, 10% DEET spray (positive control), under laboratory conditions.

Methods: Hydro-alcoholic extract of castor leaves was prepared, and the repellency effect and mortality rates were evaluated at different doses. The study also assessed 10% DEET (positive control) and 50 µl of 70% ethanol (negative control). The modified Wirtz method was applied using the K and D apparatus.

Results: The repellency effect of various doses of hydro-alcoholic castor extract (CE) on *Ph. papatasi* sand flies were evaluated. The ED_{50} (95% CL) was calculated as 4.17 mg/cm^2, and ED_{90} (95% CL) as 7.9 mg/cm^2 after 24 hours of exposure. At 1.6 mg/cm^2, the repellency effect of hydro-alcoholic CE was greater than that of 10% DEET. However, DEET exhibited higher repellency than CE at concentrations below than 1.6 mg/cm^2 (i.e. 0.1, 0.2, 0.4 and 0.8 mg/cm^2). Mortality among sand flies was observed only at high doses (1.6mg/cm^2) of hydro-alcoholic CE, with the highest mortality rate recorded at 17.7%.

Conclusion: This study demonstrated that 10% DEET and hydro-alcoholic castor extract exhibit strong repellency effects against *Ph. papatasi* sand flies, the primary vector of zoonotic cutaneous leishmaniasis. The findings highlight castor extract's potential as an effective sand fly repellent.

Keywords: *Phlebotomus papatasi*; *Ricinus communis*; Insect repellent; DEET; Vector control

Introduction

Leishmaniasis is one of the world's most common and complex arthropod-borne diseases. The ecology and epidemiology of leishmaniasis are complex due to the involvement of sand fly vectors and other overlapping species in the disease transmission cycle. Ectoparasites such as ticks, fleas, and mites infected with *Leishmania* have been detected in their

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reservoirs (1). The causative agent, *Leishmania* spp, is also diverse and complex. About 21 *Leishmania* spp. are infectious to humans and cause different forms of leishmaniasis in humans (2). The Old World sand fly species life cycle takes place in the desert or semi-arid ecosystems. Some species of the old world sand flies, such as species under the subgenera *Larroussius* and *Adlerius*, live in high altitudes and cold areas (3). While the New World species prefer conditions in forest dwellings (2).

Some Old World sand fly species find their reservoirs in both residential and roofed places and non-residential areas, and bloodshed (4). The disease transmission by the New World species may occur among humans living in or working near the forest areas (2). The vector of leishmaniasis, the phlebotomine sand fly, is found throughout the tropical and subtropical regions of the world (2). The main vector of *Leishmania major* is *Phlebotomus papatasi*, the primary causative agent of cutaneous leishmaniasis in Iran, Uzbekistan, Turkmenistan, Azerbaijan, eastern Saudi Arabia, Jordan, Tunisia, and southern Morocco (2). *Phlebotomus* sand flies are tiny (approximately 2–3mm in length) with many body hairs. Their flight is often soundless. The female sand fly requires a blood meal to obtain the necessary protein to develop her eggs. In her search for blood, female sand flies cover a radius of a few to several hundred meters around their habitats (2). Coexistence of plants and animals dates back to over 400 million years ago, during which time plants had developed several defense mechanisms like repellency and insecticidal effects against animals (2). Natural plant extracts used as repellents degrade quickly, and so, they are considered safer for the environment than the common synthetic chemicals **(**5).

The castor bean plant (*Ricinus communis*), also known as the strange tree, belongs to the family Euphorbiaceae. It is cultivated annually in many countries, including Belgium, as an ornamental plant in gardens. The plant is native to tropical Africa and parts of southern Asia. However, it has spread in many tropical and temperate regions of both the western and eastern hemispheres (6). The seeds and pods of the castor plant have high concentrations of ricin, a toxin from lectin (7). Ricin is a glycoprotein that consists of two polypeptide chains: the A -A-chain (30 kDa) and B -B-chain (32 kDa), which are linked by a disulfide bond (8). Ricin has a molecular weight of about 63 kDa, and it strongly inhibits protein synthesis through the inactivation of ribosomes. It is estimated that the ricin content in *R. communis* changes between 1% to 5% (8, 9). Castor oil, produced industrially from castor seeds, is used in lubricating oils, paints, and varnishes, and is often prescribed orally as a medicinal cleanser (8, 9). After extracting the oil from the beans, ricin remains in the bean pomace and is inactivated if the oil separation is performed under heated conditions. It has been established that the castor oil itself does not contain ricin (8, 9). Castor oil monographs in the European Pharmacopoeia do not contain tests for the detection of plant toxins (9). Because ricin is a by-product of castor oil production, it exists in large quantities in nature and is cheap and easy to extract, making it a potential biological warfare agent (10).

Exposure to ricin can be through ingestion, injection, or inhalation (10). It has been indicated that a plant like *R. communis* (Euphorbiaceae) can also be used as a candidate for sand fly control (5).

Control of Zoonotic Cutaneous Leishmaniasis vectors is considered important due to the increasing rate at which the disease spreads to non-endemic areas of Iran in recent years (11). The use of repellents to prevent blood sucking by insects could be a valuable public health intervention. Most commercial repellents contain the chemical diethyl-3-methylbenzamide (DEET), formerly called diethyltoluamide (12). Today, the use of repellents to reduce the spread of arthropod-borne diseases provides at least 96% protection against various types of mosquitoes in the tropics (13).

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The use of plant extracts as suitable candidates for repellants in the control of insect vectors, compared to synthetic chemicals, creates risks for human health and the environment (5).

In a research by Zadeh Abbasi et al. (14), the effect of hydro-alcoholic extracts of castor, *Capparis spinosa*, and *Solanum nigrum* on promastigotes of *L. major* parasite was investigated in vitro. Results related to their study indicate that the anti-parasitic effect of the hydro-alcoholic extract of *C. spinosa* was much less than that of *S. nigrum* and *R. communis*. Extracts of *S. nigrum* and *R. communis* at doses of 500 mg/ml and 100 mg/ml killed most of the parasitic promastigotes in vitro. In the present research, the repellency effect of castor plant extract on sand flies was investigated under laboratory conditions (14, 15).

All researchers have proved the repellency effect of DEET against insects. However, before starting the study, we tested the repellency effect of three plant extracts, *C. spinosa*, *S. nigrum*, and *R. communis*, on sand flies in a pilot study, and the repellent effect of castor was more than the other two extracts.

In Iran, the castor plant is mostly distributed in Tehran, Khorasan, East Azerbijan, Khuzestan, Markazi, Sistan and Baluchistan, and Hormozgan provinces (16). In addition to ricin, a toxic alkaloid called ricinine is also found in castor seeds and leaves. The castor oil is full of glycerides and fatty acids such as ricinoleic acid, oleic acid, palmitic acid, linoleic acid, and dihydroxy stearic acid.

The main purpose of this research was to evaluate its repellency influence of castor extract (CE) on *Ph. papatasi* and compare it with that of a commercial insect repellent stick, DEET (10%), under laboratory conditions.

Materials and Methods

Castor plant

Castor plant, *Ricinus communis*, a perennial plant from the Euphorbiaceae family, was used in this study. This plant grows to an average height of 1.5 meters. Castor seeds contain a highly toxic white substance called ricin, which is a type of toxalbumin. Additionally, castor seeds and some of the leaves contain an alkaloid poison called ricinine (16).

Sampling of sand flies

Sand flies were caught from Takht-e-Khajeh in Orzuieh District (Baft County of Kerman Province), which is an endemic focus of cutaneous leishmaniasis (14, 15) situated at an altitude of 978 m (56° 59′ E, 28° 45′ N). The insects were collected using a hand-held aspirator and flashlights at sunset and at different time intervals. The adults were caught from outdoor habitats near their breeding sites at dawn and dusk. The collection of adult sand flies from walls and wooden electricity poles was done using the aspirator. After specimen collection, all adults were transferred to the cage with a piece of damp cloth hanging to provide adequate humidity. Adults were fed with a 20% sucrose solution on cotton pads. Then, the cages were covered with wet cloth to maintain humidity and were transferred to the Insectarium in the Faculty of Medicine, Kerman University of Medical Sciences.

Sand fly breeding procedure

The sand fly colonies used in this study were established based on the methods of Killick-Kendrick and Killick-Kendrick (17). The *Ph. papatasi* specimens were maintained in the insectarium by following the methods described by Shirani-Bidabadi et al. and Yaghoobi-Ershadi et al. (18, 19). The sand flies were reared using the methods of Modi and Tesh (20) and Volf and Volfova (21), at 26±2 °C temperature and 60±10% relative humidity (RH), under a photoperiod (L: D) of 14:10 hours. The female sand flies were separated using an aspirator and placed into individual pots, according to the method described by Volf and Volfa (21). The insects were then fed with a honey solution (50%) and saturated sucrose.

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Engorged females were fed on Balb/C anesthetized mice, using 0.2 cc of Ketamine and Xylazine for 30 minutes. After feeding, the blood-engorged sand flies were placed individually in oviposition vials, lined with Plaster of Paris (POP) at the bottom and covered with mesh. The vials containing the females were kept at a temperature range of 26±2 °C and a relative humidity of 60±10%. Once oviposition occurred, the females died and were removed from the vials and preserved individually in 70% alcohol. The male and female specimens were later mounted and identified to species level using appropriate entomological identification keys (22).

Non-target species were excluded from the tests. The females of *Ph. papatasi* were separated from other species for rearing. The pots were checked daily for hatched eggs. The larvae (L1) that were hatched from the eggs were fed on a mixture of rabbit food (pellets) and rabbit feces with liver powder (18). For mass rearing of sand flies, larger pots lined with POP were used, and 20–30 blood-feeding females and 5–10 males were transferred into them. The emerged adults were released into a new cage that contained wet cloth and a 20% sucrose solution (19). All specimens were kept in a plastic bag to maintain humidity and a stable temperature. Finally, emerging adults were used for experiments.

Commercial repellent

The commercial insect repellent spray, DEET (10%), was purchased from Rihan Naghsh-e-Jahan, a pharmaceutical company in Iran (Fig. 1).

Extraction of hydro-alcoholic castor extract

The roots, stems, leaves, flowers, and fruits of the castor plant were collected from Koohpayeh and Alghadir suburbs of Kerman in the Southeastern part of Iran (14) and were dried in the shade. The dried leaves were ground into fine particles. Thirty grams (g) of the obtained plant powder was produced in a ratio of 1:10 (100 g of Castor plant powder in 300 milliliters of solvent) with ethanol (solvent): water-to-volume ratio (50:50) for one week in an incubator shaker, at a speed of 1600 revolutions per minute (rpm). The solution was mixed until the extract was collected. After that, it was passed through Whatman filter paper and sterilized using a syringe head filter. The product was dried in an oven at 40 °C for 24 hours (23).

Modified Klun and Debboun apparatus

The modified Klun and Debboun (K and D) apparatus (feed chamber) used in this research was obtained from the Mahour Biotechnology Company in Iran (Fig. 2) and was used according to Wirtz (24). Rabbits between 6 to 9 months old were used in the tests to determine the Effective Doses (EDs): ED_{50} and ED_{90}, at a 95% confidence interval of the repellents (Fig. 3). The length and width of the abdomen of the test rabbits were first measured. Then, the K and D apparatus was carefully designed with the dimensions 18 cm (length), 4 cm (width), and 5 cm (height), to easily fit onto the surface of the belly of the rabbits. Each apparatus contained three cages, and each cage had a separate drawer that could easily be opened and closed. There were 4*3 cm holes at the bottom of the device that allowed exposure of the sand flies to the skin of the rabbits when the drawers were pulled outwards. A curvature at the bottom of the device helped the apparatus to fix firmly onto the rabbit's abdomen. The front wall of the device contained 1 cm-diameter holes through which sand flies were introduced into the cages via a hand-held aspirator. One of the advantages of this device is that the repellent effect of chemicals on several species of sand flies can be studied at the same time. This minimizes the possibility of interference from different doses and repellents. In this study, different doses of the hydro-alcoholic castor extract (CE) were tested at different times. The pores at the bottom of the K and D apparatus that were in contact

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with the rabbit's skin were 12 cm^2 each. The amount of plant extract that was applied to this part of the rabbit's skin was calculated to be 50 µl.

Test method

We used the technique illustrated by Wirtz et al. (24) for testing our white rabbits, *Oryctolagus cuniculus*. In the present study, white rabbits between 6 and to 9-months old were used in all experiments. Briefly, the rabbits were anesthetized with Ketamine hydrochloride (1 ml/kg) and Xylazine (1 ml/kg). The abdominal areas of the test rabbits were shaved. Then, using a cardboard template and marker pen, three sets of 12 cm^2 test areas were drawn on the rabbits' abdomen (Fig. 3).

The marked areas were treated with 50 µl of the repellents in absolute ethanol. To prevent any interference caused by different doses, in each test, only one dose of each repellent was used. Absolute ethanol was also used as a negative control. After the treated areas had dried for 5 min, the 12 cm^2 square holes at the bottom of the K and D apparatus were aligned with the marked areas on the abdomens of the rabbits treated with ethanol and repellents. Each cage contained 3–5 female sand flies. Thus, for each apparatus containing three cages, 9–15 sand flies were supplied. Each dose of the repellent was tested on rabbits only. Probing counts were recorded at 1-minute intervals for 5 minutes, and the results were pooled for statistical analysis. The tests were then repeated for different doses at various intervals. To obtain a reasonable estimate of the ED_{50} and ED_{90}, areas treated on the rabbit's abdomen were swabbed with isopropanol pads. These experiments were performed several times to reduce the heterogeneity of the sand fly population. Also, sand fly mortality was recorded 24 hours after the recovery period.

The shaved portions of the rabbits' bellies were treated with different doses of hydro-alcoholic castor extract, DEET (10%, positive control), and 50 µl of ethanol (70%, negative control) at the marked sites. To ensure accuracy in recording observations during the test, the cage doors were opened one after the other. The sand flies were denied a blood meal for 3 to 7 days, and the test materials were applied to the skin of the rabbits' abdomens using an insulin syringe.

It took 5 minutes for the test material to dry completely. Then, after placing the K and D device on the rabbits' abdomen (at the site impregnated with the test material), the sand flies were exposed to the skin by pulling the cage drawers out.

The number of probes was determined by carefully counting at one-minute intervals for five minutes with the help of an expert. The sand flies were then transported to other cages. For each concentration of the test chemicals, the test was repeated 5 times.

Statistical analyses

Data were analyzed by probit regression analysis using SPSS (version 22). Analysis yielded ED_{50}, ED_{90}, confidence limits, and slope values. ANOVA, Tukey, and Dunnett tests were used to compare means. The 95% confidence intervals between ED_{50} and ED_{90} were used to determine significant differences.

Results

Dose-response of hydro-alcoholic castor extract

The ED values of the two experimental repellents were determined (Table 1). The experiments were performed on 306 *Ph. papatasi* specimens reared in the insectarium for castor extract and DEET (10%), respectively. In the present research, the repellency effect of different doses of castor extract on sand flies was investigated, and the ED_{50} (95% CL)= 4.17 mg/cm^2 and ED_{90} (95% CL)= 7.9 mg/cm^2 were calculated after 24 hours of exposure to the sand flies. Chi-square test presented a significant difference in repellency effect between

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the different doses of castor extract (P-value< 0.001). Figure 4 shows the dose-response curve for castor extract after 24 hours of exposure.

Sand fly repellent activity of hydro-alcoholic extract of castor plant leaves

The repellency effect of different concentrations of castor extract on *Ph. papatasi* was investigated. The repellency means were remarkable, even at very low concentrations of the castor extract after 24 hours of exposure. We found that the repellency effect of 1.6 mg/cm^2 for the castor extract was higher than that of DEET (10%). The repellency effect of DEET was 84.2 % and in 1.6 mg/cm^2 of castor extract was 88.8% (Fig.5). The mean repellency effect of DEET was different from those of the other concentrations of castor extract (0.8, 0.4, 0.1 and 0.2 mg/cm^2) and the negative control (Ethanol) (Fig. 5). The ANOVA test showed a significant difference in the repellency effect between different concentrations of castor extract, DEET (10%) and ethanol (P< 0.001). Tukey's test showed no significant differences in the repellency effect between the different concentrations of castor extract, other than 1.6 mg/cm^2 (P> 0.05). After 24 hours of exposure, the repellent activity of castor extract differed in different concentrations. The Dunnett test showed significant differences between the mean repellency effect of 0.1 and 0.8 mg/cm^2 with 1.6 (P< 0.05). There were no significant differences between 0.2, 0.4 with 1.6 mg/cm^2 concentrations of castor extract (P> 0.05). The Dunnett test showed a significant difference in repellency effect between DEET (10%) and the different concentrations of castor extract (P< 0.001). Also, the Dunnett test did not show a significant difference in repellency effect between ethanol (negative control) and 0.01mg/cm^2 for the castor extract after 24 hours of exposure (P=0.994). However, there were significant differences in the repellency effect between ethanol and the other concentrations (i.e., 0.1, 0.2, 0.4, 0.8, 1.6 mg/cm^2) of castor extract after 24 hours of exposure (P< 0.001). After 24 hours of exposure, significant differences were observed in the repellency effect of different concentrations (i.e. 0.01, 0.1, 0.2, 0.4, 0.8, and 1.6 mg/cm^2) of castor extract, compared with that of DEET (10%, P< 0.001) and ethanol (P< 0.001) (Fig. 5). The mortality of sand flies after exposure to the castor extract (repellents) was only observed at high doses of the extract. The highest mortality rate was 17.7% at dosages of 1.6 mg/cm^2.

Fig. 1. Commercial insect repellent spray (10% DEET) used in repellency tests against laboratory strains of *Phlebotomus papatasi* sand flies in Kerman, Iran, 2020

Table 1. Effective Doses (EDs) of castor (*Ricinus communis*) extract against *Phlebotomus papatasi* using the modified Klun and Debboun method, Kerman, Iran, 2020

Time	ED_{50} % (95% CL)	ED_{90} % (95% CL)	Slope (±SE)	Chi-Square (df)	P- value
24 h	4.17 (2.66-12.87)	7.9 (4.86-25.71)	0.343 (±0.124)	17.1 (28)	< 0/001

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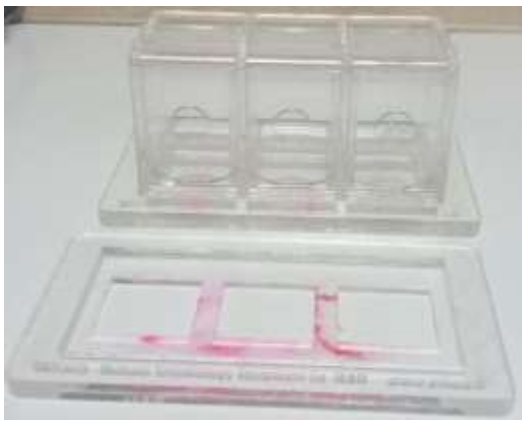

Fig. 2. Modified Klun and Debboun **(**K and D) apparatus used for the repellency effect of hydro-alcoholic extract of castor on white rabbit in Kerman, Iran, 2020

Fig. 3. A six-month-old rabbit being anesthetized for the repellency test in Kerman, Iran, 2020

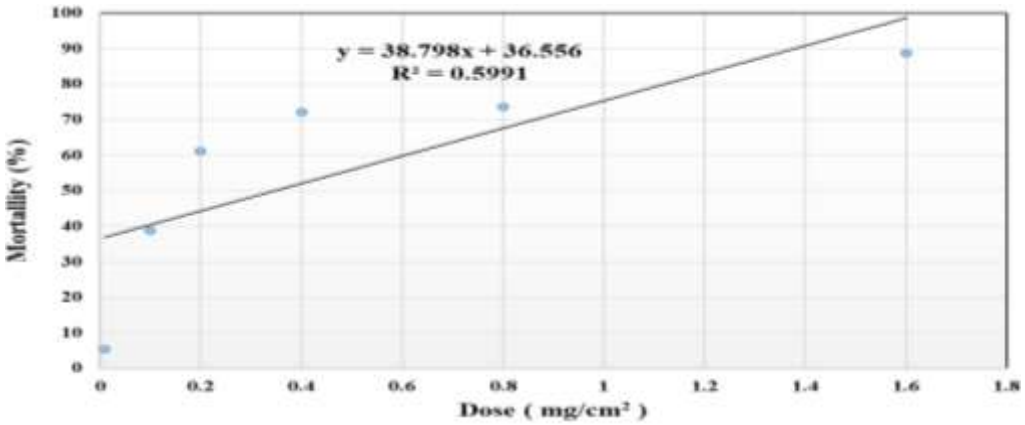

Fig. 4. Mortality dose-response curve for *Ricinus communis* (castor) leaf extract against *Phlebotomus papatasi* sand flies under laboratory conditions in Kerman, Iran, 2020

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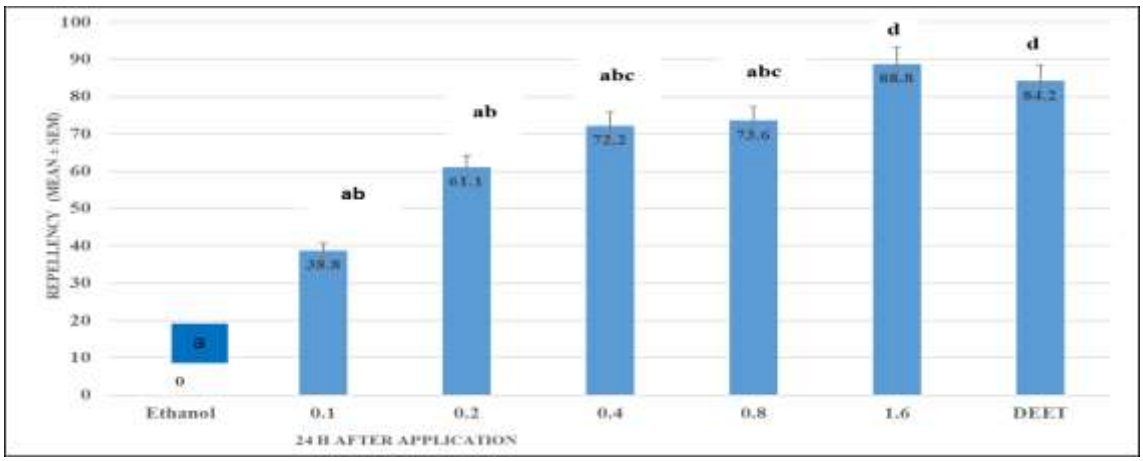

Fig. 5. Repellency effects of different concentrations of *Ricinus communis* (castor) leaf extract and 10% DEET on *Phlebotomus papatasi* sand flies. Different letters above the bars indicate significant differences Repellency at α= 0.05. (a: difference between ethanol and DEET, ab: differences between 0.1, 0.2 concentration and DEET,abc: differences between 0.4,0.8 concentration and DEET d: difference between DEET and 1.6 concentration).

Discussion

Plant-based insecticides or repellents have been found to exhibit a wide range of effectiveness against blood-sucking arthropods and are considered highly safe (5). Currently, the only known strategy for preventing leishmaniasis is the application of topical repellent compounds in various formulations for personal protection against sand fly bites (25). Traditionally, people in many parts of the world use plant-derived products such as essential oils or plant extracts to repel and kill insects (26). The repellency effect of castor oil extract was first investigated in 1917. Cross et al. (27) applied 473 ml of the oil extract on the body of camels to control stable flies, but the researchers observed no repellency effect. However, when they applied the oil extract at a rate of 1892 ml per camel for three days, they prevented persistent flies from landing on the camels and biting them (27). In 1997, Rozendaal (28) reported the repellency effect of some plant extracts and essences on mosquitoes for a period of 15 minutes to 10 hours. Plant extracts such as alkanes, terpenoids, alcohols, and aldehydes are highly volatile. Gupta and Rutledge (29) observed that the release of extracts from different repellent formulations significantly increased the protection period of the repellents.

In 1982, Buescher et al. (30) used the dose-response method to test repellents against the NW sand fly, *Lutzomyia longipalpis*, on white rabbits. Wirtz et al. (24) used the same method to test the repellency effect of eight topical repellents and one synthetic pyrethroid against *Ph. papatasi*. In addition, Mehr et al. (31) and Rutledge et al. (32) used this method to test repellents against *Aedes aegypti* and *Anopheles albimanus* (31, 32). Also in 1985, Wirtz et al. (33) and Buescher et al. (34) used this method to test repellents against *Glossina morsitans* and *Rhodnius prolixus*, respectively. Rutledge et al. (32) evaluated the laboratory rabbit model for screening topical mosquito repellents. In their study, DEET was used as a repellent against *Ae. aegypti* on humans and rabbits (32). In this study, the repellency effect of castor oil extract on *Ph. papatasi* was investigated under laboratory conditions and compared with that of DEET (10%). To the best of our knowledge, this is the first study on the repellency potential of castor oil extract on *Phlebotomus* sand flies in Iran and the rest of the world. The results of our study show that both DEET and castor oil extract had a repellent effect on *Ph. papatasi* sand flies,

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the main vector of Cutaneous Leishmaniasis in Iran. Thus, both castor oil extract and DEET could be used as potent repellents against sand flies. In the present research, we observed that the repellency potential of castor oil extract (1.6 mg/cm^2) was significantly higher than that of DEET (Fig. 5). In 2006, Yaghoobi-Ershadi et al. (11) conducted a similar study and reported that DEET was more effective as a repellent against *Ph. papatasi* sand flies than Myrtle essential oil. In our opinion, when evaluating the effectiveness of botanical insecticides and repellents, it is crucial to carefully analyze the data in comparison with control groups that include synthetic analogues. For instance, the repellency of myrtle essential oil against *Ph. papatasi* was found to be 62.2%. While this result may be considered favorable, it is important to note that the same sand fly strain demonstrated higher susceptibility to diethyl-m-toluamide (87%) (11).

The results of researchers' studies in Rajasthan and Bihar, India, showed that NEEM oil extract from *Azadirachta indica* (Meliaceae) has an 82% repellency effect against *Ph. papatasi* in India (35), 97.6% in Rajasthan (36), and 86.1% in Bihar (37). In this study, 2% Neem extract had a more repellency effect (100%) on *Ph. argentipes* (38). More specifically, neem oil repelled *Ph. argentipes* more effectively than *Ph. papatasi* (37). The ED_{50} values of their study were 0.1140 and 0.0006 mg/cm^2 for Myrtle essential oil and DEET, respectively. The laboratory tests showed that even though DEET was more effective, both Myrtle essential oil and DEET had repellency effects against *Ph. papatasi*. The authors also reported the insecticidal action of Myrtle essential oil (11). In the present study, the ED_{50} and ED_{90} values calculated for the castor oil extract were 4.17 and 7.9 mg/cm^2, respectively. Buescher et al. (30) demonstrated that the strain of sand flies used in our study (*Ph. papatasi*) was more sensitive to DEET than *Lu. longipalpis*. Kalyansundram et al. (38) also reported ED_{50} of DEET as 0.0022 mg/cm^2 against other strains of *Ph. papatasi*, while Pitasawat et al. (39) reported 0.21 mg/m^2. Castor oil extract and Myrtle essential oil are fewer effective repellents when compared with DEET. This characteristic is similar to some other botanical compounds. Previous studies have indicated that tsetse flies, mosquitoes, and Reduviid bugs seem to be less sensitive to repellents than *Ph. papatasi* (38–40).

Some of the limitations of this study included the occurrence of the coronavirus epidemic, which slowed research activities, the difficulties in anesthetizing rabbits, the challenges in rearing sand flies, and the low population of sand flies in the field. Finally, one of the strengths of the study was the investigation of the repellency effect of castor extract.

Conclusion

The use of repellents plays a crucial role in preventing sand fly bites, and consequently sand fly-borne diseases. In this study, both experimental repellents exhibited significant repellency, suggesting their potential as effective repellents. Therefore, we recommend the use of castor alcoholic extract and DEET as potent repellents against *Ph. papatasi*. Future studies should explore the durability and effectiveness of castor extract under both laboratory and field conditions.

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Ethical Considerations

This study was approved by the ethics committee of Kerman University of Medical Sciences, with the ethical code number IR.KMU.REC.1398.282.

Conflict of interest statement

The authors declare that there is no conflict of interest.

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