In Vivo and in Vitro Investigations of the Effects of Iranian Honeybee (Hymenoptera: *Apis mellifera*) Venom and Propolis on *Leishmania Major*

Qasem Asgari[1], PhD; Seyedeh-Zahra Zandavi[2], MSc; Mohammad Djaefar Moemenbellah-Fard[3], PhD; Amin Hosseinpour[2], MSc; Mohsen Kalantari[3], PhD; Aboozar Soltani[3], PhD

[1]Department of Parasitology and Mycology, School of Medicine, Shiraz University of Medical Sciences, Shiraz, Iran
[2]Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran
[3]Research Center for Health Sciences, Institute of Health, Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran

Correspondence:
Aboozar Soltani, PhD;
Research Center for Health Sciences, Institute of Health, Department of Medical Entomology and Vector Control, School of Health, Shiraz University of Medical Sciences, Shiraz, Iran
Tel: +98 71 37251001-6 ext. 357
Fax: +98 71 37260225
Email: abu2sol@yahoo.com
Received: 06 October 2022
Revised: 23 November 2022
Accepted: 09 December 2022

Abstract

Background: Leishmaniasis, as a neglected health issue, is spreading in most parts of the world. It is one of the most important vector-borne diseases in Iran. Bee venom has shown a wide range of medicinal properties. The present study aimed to survey the effect of venom and propolis of *Apis mellifera* on *Leishmania major* in different environments, including in vivo and in vitro.

Methods: In this experimental study, bee venom was extracted using the modified Benton method, and propolis was prepared by the *soxhletation* method. The promastigotes of *L. major* were exposed to the different doses of the venom (0.03125-1 µg/ml) and propolis (2.5-80 µg/ml) and then evaluated by MTT assay and Flowcytometry after 24 hours. In vivo phases, 10^7 promastigotes of the *L. major* in stationary phase were intradermally inoculated into 48 mice based on the study design. After appearance of the wounds, the mice were topically treated with the lotion containing different doses (5 and 10 µg/ml) of the venom and propolis. The size of the ulcers was measured for four weeks.

Results: The results showed that propolis and BV had no significant effects on the vitality of *Leishmania* promastigotes. However, they had a high mortality effect on macrophages. The highest mortality belonged to propolis (78.39 %). *In vivo* results showed significant differences between some treated and control groups in terms of the mean ulcer size.

Conclusion: It seems that a combination of honeybee venom and propolis in a particular dosage can prevent the development of the ulcers caused by *L. major*. More studies are needed to evaluate the effects of their constituent compounds precisely.

Please cite this article as: Asgari Q, Zandavi SZ, Djaefar Moemenbellah-Fard M, Hosseinpour A, Kalantari K, Soltani A. *In Vivo and in Vitro* Investigations of the Effects of Iranian Honeybee (Hymenoptera: *Apis mellifera*) Venom and Propolis on *Leishmania Major*. J Health Sci Surveillance Sys. 2023;11(1):34-41.

Keywords: Leishmaniasis, Cutaneous, Bee venoms, Propolis, Wounds and injuries, Anti-ulcer agents

Introduction

Leishmaniasis is one of the infectious diseases that has been severely neglected, despite its high prevalence in some countries.[1] Leishmaniasis, caused by different species of *Leishmania,* is the leading health problem in some tropical and sub-tropical countries.

This zoonotic disease has different clinical forms, including Cutaneous, Mucocutaneous, and Visceral.[2] Cutaneous leishmaniasis is one of the most common parasitic diseases among humans and animals. Cutaneous leishmaniasis causes skin lesions

that remain in some cases for more than a year and, despite treatment, usually causes scars that remain on the body until the end of the life.[3] Also, secondary bacterial and fungal infections, including surface and deep tissue infections, abscesses, septicemia, and even tetanus in the ulcer can lead to an inability or even patient death. Although the incidence of these complications is negligible, due to the widespread prevalence of leishmaniasis, the number of patients with these complications is significant.[2, 4, 5] Given the importance of leishmaniasis in the world, it is essential to find out different methods of prevention and treatment. Several therapeutic methods have been proposed for the treatment of cutaneous leishmaniasis. Their purposes are to prevent secondary infection of the wounds, reduce the side effects, prevent the scars, and improve the wound healing process. Today, many treatments are used in leishmaniasis, but, because of their complications and the onset of drug resistance, the attention of researchers has focused on therapeutic compounds with natural origins.[6]

Apis mellifera is one of the most beneficial insects, the products of which have been used by humans for a long time.[7] Honeybee has a critical role in plant pollination and is also the source of some valuable services by producing materials such as honey, royal jelly, propolis, poison, etc.[8] In pharmaceutical and many other industries, honeybee products are used and formulated to treat some diseases.[9]

Bee Venom (BV) is a colorless, acidic liquid with a pH of 4.5-5.5, deactivated by ethanol. Proteins (such as the phospholipase A2 enzyme) and peptides (such as melittin and apamin) constitute the main portion of the bee venom.[9] The active part of the venom is composed of several proteins that have anti-inflammatory and anticoagulant effects and have been considered in medical, pharmacological, and pharmaceutical industries.[10] Bee venom has analgesic, anti-inflammatory, and anti-cancer effects. Various studies around the world have been done on the therapeutic effects of honey bee venom for the treatment of certain diseases, including rheumatoid arthritis, Lyme disease, cancer, osteoarthritis, HIV, multiple sclerosis (MS), Parkinson's disease, blood pressure, and skin diseases such as eczema.[8, 9] Moreover, some researchers investigated the anti-leishmanial activities of propolis in different parts of the world.[6, 11-17] The present study investigated the therapeutic effects of local honey bee venom and propolis in combination form on cutaneous leishmaniasis for the first time in Iran.

Methods

Venom Preparation

The bee venom was extracted by Benton et al.'s method with a slight modification using a modified electro-shock collecting apparatus with a cooling system.[18, 19] The beehives of the suburban areas of Shiraz city (the capital of the Fars Province) were used for BV collecting. When the device was fixed on the Beehive, wires were connected to the electricity supply, and a gentle shock was given to the bees for 10 seconds; then, the flow stopped for 20 seconds. The bees were stimulated electrically, and they started to sting the glass wall. Poisons were immediately dried in a cooling system, and then extracted powders were collected. By maintaining the cold chain, the collected venoms were quickly transferred to the laboratory for further studies.

Propolis Preparation

Propolis was collected by scraping and wire meshing the beehives floor and their walls in the spring season in Shiraz city. The hydroalcoholic extract was prepared by adding 50g of the propolis into the soxhlet cartouche set containing 600ml of alcohol and distilled water (80: 20 ratios). The extract was provided using a soxhlet extractor, and each step of soxhletation was elongated for approximately 6 hours. The extraction was concentrated with a rotary evaporator apparatus and then dried by speed vacuum for 48 hours. The extract was maintained in a dark bottle at the 2-8ºC condition.

The Effects of Honey Bee Venom and Propolis on Leishmania Promastigote using MTT Assay under in-Vitro Conditions

The cytotoxicity was evaluated using MTT assay according to a procedure adapted from the ISO10993-5 standard test method.[20] The assays were performed in triplicate. 10^5 *Leishmania* promastigotes were incubated in microplate wells containing 180µl of different concentrations of honey bee venom (0.03125, 0.625. 0.125, 0.25 and 1 µg/ml) and propolis (2.5, 5. 10, 20, 40, 80 µg/ml) in the culture medium ($RPMI_{1640}$), 10% (v/v) fetal bovine serum (FBS-Serum, Germany), 100 U/ml penicillin, and 100µg/ml streptomycin for 24, 48 and 72 hours, and 25 °C. Next, 20µl of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution (0.5 mg/ml [Sigma®, USA]) was added in each well and incubated for 4 h. Finally, to solubilize the formed formazan crystals, the medium was replaced with 200µl of DMSO and agitated on a plate shaker. The optical density (OD) of each well was read at 540 and 720 nm using an ELISA reader. The viability percentage was calculated concerning the control cells incubated in the absence of the honey bee venom and propolis. Moreover, the OD of each concentration of honey bee venom and propolis in the culture media was considered blank. Then, using the following formula, the percentage of live parasites was calculated using the following formulas:

1: OD=OD_{540}-OD_{720} 2: (OD sample-OD blank)×100/(OD control–OD blank)

The Effect of Honey Bee Venom and Propolis on Leishmania by Flow Cytometry (FCM) Under in-Vitro Conditions

2×10^5 Leishmania promastigotes were cultured in the media culture (RPMI 1640) containing different concentrations of honey bee venom and propolis (1, 2, 4, 8, 16, 32, 64, 128, and 256 µg / ml) for 24, 48, and 72 hours in 25 °C. Moreover, the promastigotes were exposed to tubes which included 70% alcohol as the positive control and PBS as the negative control. Then, the specimens were exposed to Propidium Iodide (PI) stain at a concentration of 50µg/ml for 30 minutes in dark conditions. PI staining detects the cell death and pre-determined death (Apoptosis) by means of the dye entering the cells, along with changes in the target cell membrane and DNA damage. The samples were then transferred to flow cytometry tubes. The cell suspension was transferred into polystyrene flowcytometry tubes (BD Falcon Company, USA). We performed data collection and analysis with a FACS Calibur flow cytometer (Becton-Dickinson, San Jose, USA) and Cell Quest Pro software. A total of 1000 event was acquired in the region that had been previously established as corresponding to the parasites.[21]

Macrophages Culture

Mouse macrophages (J774 cell line) were provided from the Immunology Department of Shiraz University of Medical Sciences and also cultured in DMEM, as well as RPMI 1640 medium containing 10-20% of FCS, streptomycin (100 IU/ ml), and penicillin (100 IU/ ml) in the incubator 37 °C containing 5% carbon dioxide under sterilized conditions.

Cytotoxicity Effect of Honey Bee Venom and Propolis on Macrophage

5×10^3 of the macrophages were added to each 96-well plate containing $RPMI_{1640}$, FCS 10%, 10 unit penicillin, and 10 mg streptomycin /mL and incubated at 37°C and 5% CO2. The cells were exposed to several concentrations mentioned above the honey bee venom and propolis for 24, 48, and 72 hours. The percentage of the live cells was measured by flow cytometry, as described above.

Experimental Animal Model

Sixty female BALB/c mice weighing between 18 and 22 grams, aged between 4 and 5 weeks, were prepared from the Pasteur Institute of Iran. The animals were kept for a week in the animal's house to adapt to the condition and then tested.

Inoculation of the Parasite to the BALB/c Mice

After disinfection of the mouse tail base with %70 ethanol, we intradermally inoculated 10^7 stationary-phase promastigotes of *L. major* into the tail base of 48 BALB/c female mice. After two weeks, the effects of skin lesions appeared in the form of a nodule, and after the end of the fourth week, the wounds in mouse tails in different sizes were created (Figure 1).

Figure 1: *In vivo* studies on BALB/c mice; (a) Injection of the parasite into the base of the mouse tail, (b) Measuring the area of ulcers created by the *Leishmania major*, using caliper, (c) Appearance of the ulcer, (d) Treatment process on the ulcers

Forty-eight mice were divided into eight groups; the mice in Group 1 were treated with lotion including 10 µg/ml of propolis, Group 2 mice were treated with lotion including 5 µg/ml of propolis, Group 3 were treated with lotion including 10 µg/ml of propolis and venom, Group 4 were treated using lotion including 5 µg/ml of propolis and venom, Group 5 were treated with lotion including 10 µg/ml of venom, Group 6 were treated with lotion including 5 µg/ml of venom, Group 7 mice received PBS (Phosphate buffered saline) as the negative control, and Group 8 mice were treated using amphotericin B as the positive control. Also, to evaluate the side effects, we treated the two groups of uninfected mice with lotions, including 10 µg/ml of propolis and venom. The wound size was measured with a caliper every week, and the average area of the wounds (in square millimeters) was used in the analyses.

Data Analysis

The recorded data were analyzed using SPSS software version 19. One-way analysis of variance (ANOVA), post hoc tests (LSD), and one-way repeated measures ANOVA were used to analyze the results in vivo, and also in- vitro data analysis was performed using one-way ANOVA (Kruskal-Wallis test) and Duncan's multiple range test.

Results

The flow cytometry results showed that the average concentration of propolis and venom had no significant effects on the vitality of parasite promastigote (Figure 2). However, plenty of the parasites died during 72 hours. This phenomenon may be partly due to the fact that the parasites had not been fed at this time. The result of MTT showed venom and propolis had no significant effects on Leishmania promastigote. These results were similar to flow cytometry.

The propolis and honey bee venom had a high mortality effect on the macrophages (Figure 3). The highest mortality belonged to Propolis (78.39 %). Although the treatment groups showed a significant difference from the control group, no significant difference was observed between the three treated groups.

A one-way repeated measures ANOVA was run on the results of *In vivo* assays on the mice which had undertaken various treatments to determine if they differed in the wound size during four intervals. The results showed that the size of the ulcer was not significantly different at the beginning of the study $F(8,40=1.87, P>0.05)$. However, a significant difference was found in the size of the ulcer after one $F(8,40=4.44, P<0.001)$, two $F(8,40=3.23, P=0.01)$ and three $F(8,40=3.23, P=0.02)$ weeks after the study.

In vivo results showed that, during all the tested groups, the lowest average of the ulcer size belonged to Group 3 (10 µg/ml of propolis and venom) with an 11.27 mm^2 size area. The highest average of the wound size was observed in Group 7 (PBS treated group) with a 34.04 mm^2 size area (Figure 4). In addition, the mean size of the ulcer was statistically assessed in different groups of exposure. Accordingly, significant differences were observed between some treated and the control groups (Figure 4).

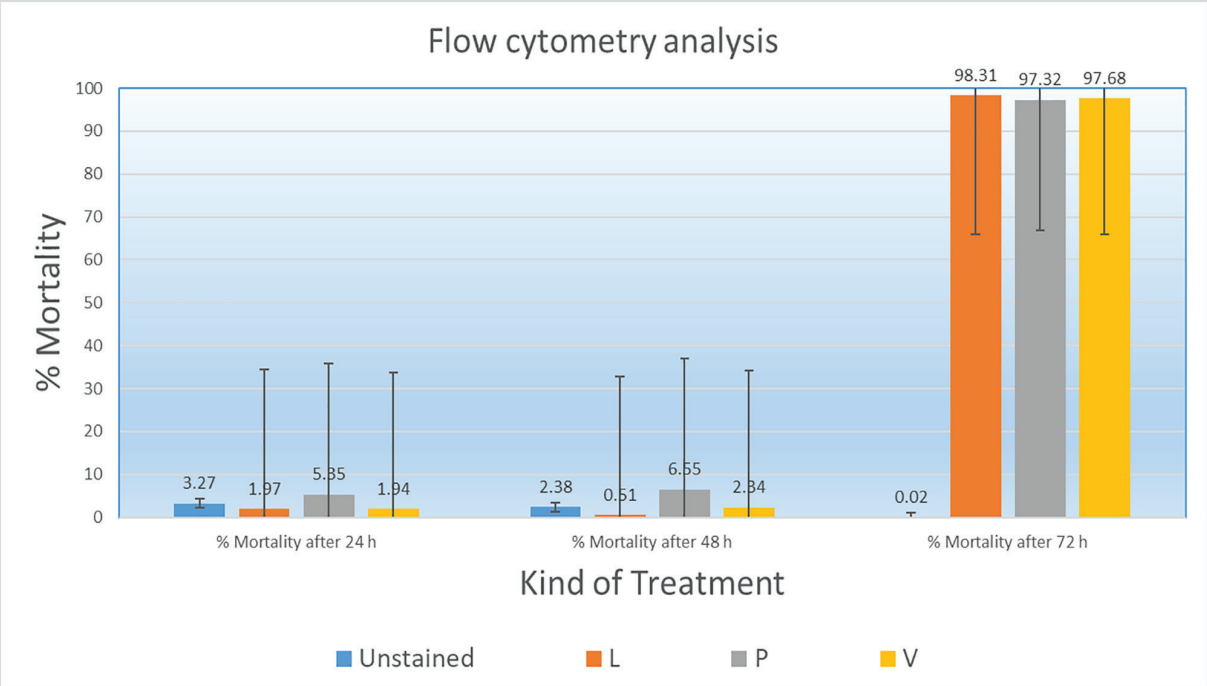

Figure 2: Flow cytometry analysis on the promastigotes of *L. major*. L: Negative control, P: Treated with propolis, and V: Treated with Bee venom.

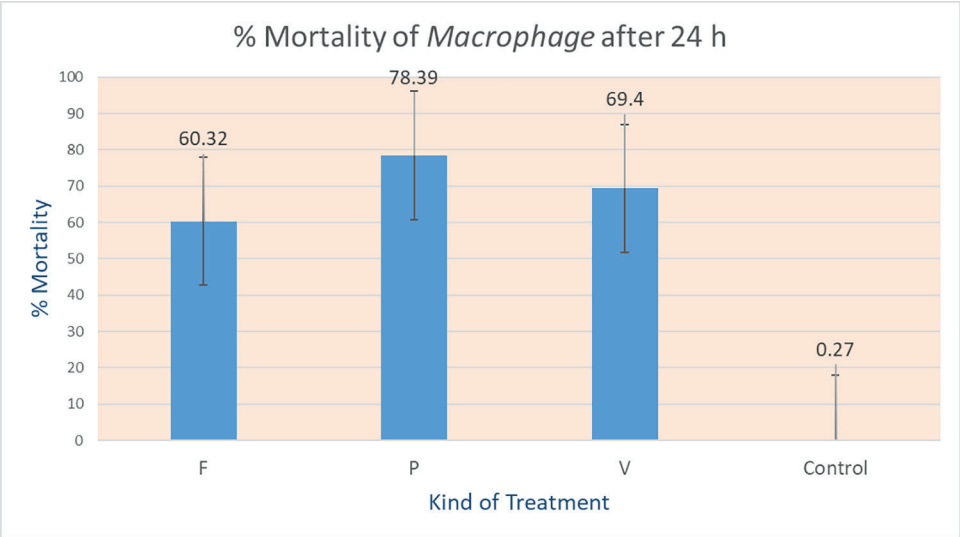

Figure 3: Mortality effects of propolis and venom on macrophages using flow cytometry**.** Negative Control, F: Exposed to Formalin as a Positive Control, P: Treated with propolis during 24 hours, and V: Treated with Bee venom during 24 hours

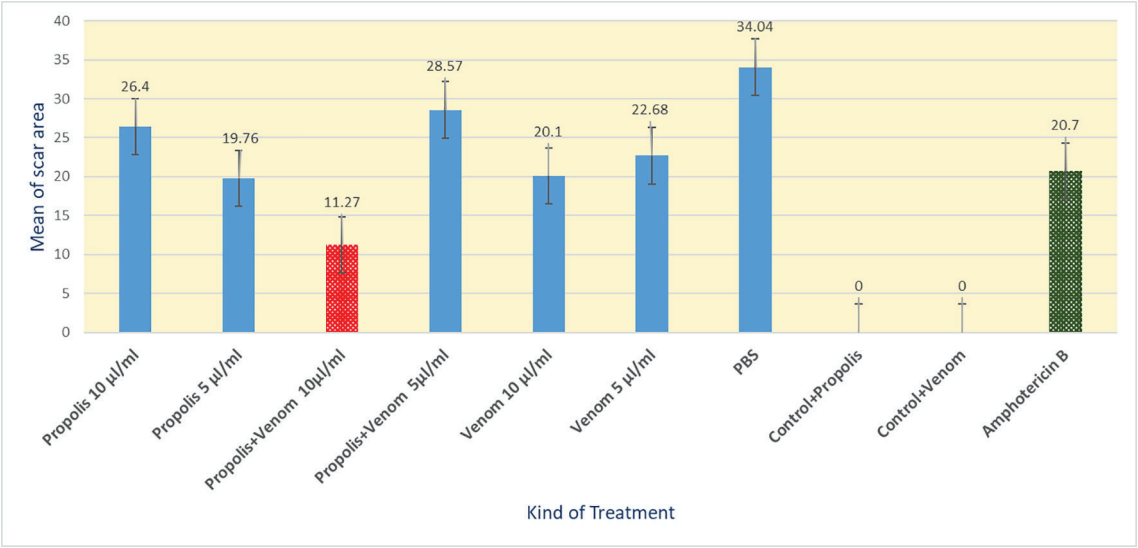

Figure 4: Relationship between the kind of treatments and mean of scar sizes (mm ^{2}) *in vivo* investigation (in control groups, all mice were without scars).

Discussion

Cutaneous leishmaniasis is one of the most common parasitic diseases among humans and animals. According to the WHO reports, leishmaniasis is one of the infectious diseases that has been severely neglected, despite its high prevalence in some countries.[1] Given the importance of leishmaniasis in Iran, it is essential to perform different methods of prevention and treatment. Several therapeutic methods have been proposed to treat cutaneous leishmaniasis to prevent wound infection, reduce the side effects, prevent scarring, and speed wound healing. Today, many drugs are used to treat leishmaniasis, but due to their complications as mentioned earlier, they have attracted the researchers' attention to natural product (NP)-derived medicines.[22] Some of these promising NP-derived medicines are those extracted from insects, such as BV, scorpion venom, liquids secreted by cantharid beetles, and other compounds found in arthropods.

The present study found that the combination of 10 µg/ml of honey bee venom (BV) and propolis could prevent the growth of the ulcers effectively in mice. This combination seems to affect the immune system of the mouse by decreasing the entry of macrophages into the position, causing less excretion of cellular fluid from the macrophages and low growth of parasites. Moreover, the *in vitro* results showed a high lethal effect of the venom on mice macrophages. It seems that the mild manifestation of the ulcer occurred due to less migration and more lethality of macrophages.

The anti-leishmanial activity of amphotericin B has been known since 1960.[23] Mice that were treated by amphotericin B, as the standard antileishmanial drug, showed significant differences in terms of the average ulcer size with PBS (P=0.005), control

+venom (P=0.01), control+propolis (P=0.014), 5 µg/ml of propolis+venom (P=0.048), and 10 µg/ml of propolis+venom (P=0.046) treated groups.

Based on the results, the therapeutic effects of bee venom and propolis when used together are pretty different from when they are used individually. It seems that the combination of BV and propolis at the dosage of 10 µg/ml is even more effective than amphotericin B in the tested mice. This phenomenon can be attributed to the possible synergistic effects of their constituent compounds. Several studies showed that BV, as an oriental medicine, could inhibit the growth of cancer cells without any side effects.[24] The considerable effect of BV has been confirmed on immune-related disorders and cancer in the lung, liver, renal, prostate, breast, and cervical cancer. Melittin and phospholipase A2, known as BV peptides, can serve as valuable, novel targets in treating some types of cancer.[25, 26]

In this study, honey bee venom did not significantly affect the parasite's vitality *in vitro*. However, Pereira et al. showed that melittin could decease promastigotes and intracellular amastigotes of *Leishmania (L.) infantum*. Furthermore, they showed that melittin could decrease the number of intracellular amastigotes due to macrophage immunomodulatory effect.[27] Also, the results of Adade et al. showed that melittin peptides killed the *Trypanosoma cruzi* parasites by inducing different cell death pathways.[28]

After drying, the BV forms a white powder and loses 70% of its weight (volatile matter and water). It is mainly prepared as a dry powder and pure dry powder or lyophilized. It can be reserved for five years or more if it is kept away from light and moisture. Bee venom color ranges from white to brownish-yellow, and if it does not retain from oxidation, some of its medical effects will be reduced.[29]

In our study, propolis alone had no significant effects on *L. major* promastigotes and prevention of ulcers growth in the tested mice. Conversely, Rebouças-Silva et al. showed that propolis extracts revealed a lethality effect on amastigotes and promastigotes of *L. (V) braziliensis*.[6]

Moreover, Brazilian propolis had a direct role in the *Leishmania* and immunomodulatory effects on murine macrophages. They claimed these effects could be associated with the presence of phenolic compounds (flavonoids, aromatic acids, and benzopyrene) and terpenes.[30] In general, propolis contains polyphenols (flavonoids, phenolic acids, and esters), phenolic aldehydes, and ketones, etc.[31]

Propolis (bee glue) produced by honeybees from substances gathered from plants are used in the building and repairing of hives for sealing cracks and openings.[32] The biological activity of the propolis sample varies due to its different geographical origins.[32] Moreover, the chemical properties of propolis vary according to plant source, season, and cultivating conditions.[33]

According to a study in the United States in 2015, bee venom and propolis accelerate the healing of wounds.[34] In 2008, the result of a study on wound healing and anti-inflammatory activities of the bee venom-chitosan blend films showed that both Chit-F and 6% BV-Chit films accelerated the wound healing compared to untreated rats.[35]

The most critical events in cutaneous leishmaniasis treatment are healing ulcers and inhibiting the secondary infection, which usually happens in this essential vector-borne disease.[36] The production of an effective drug to treat leishmaniasis has several significant limitations, including the high price of drugs and the occurrence of some health issues on patients, such as toxicity and side effects of the used treatments. Another critical issue is the drug resistance of Leishmania parasites to these drugs.

Conclusion

Bee-venom therapy has shown promising effects in the treatment of various diseases. Also, given the existence of limited therapies for treating cutaneous Leishmaniasis and the resistance of the parasite to Glucantime and other drugs, the anti-leishmania activity of BV and propolis was evaluated in this research. The results showed that a combination of honeybee venom and propolis in a particular dosage could prevent ulcer development caused by *Leishmania major*. More comprehensive studies are needed to evaluate the effects of their constituent compounds specifically.

Ethics Approval and Consent to Participate

The project was done in accordance to the ethical principles and the national norms and standards for conducting Medical Research in Iran. The study was approved by Iran national Committee for Ethics in biomedical research (Approval ID: IR.SUMS.REC. 1395. S12214). The guideline of the Institutional Animal Care and Ethics Committee of Animal Experimentation, Shiraz University of Medical Sciences was used to work on animal models in this study.

Availability of Data and Materials

The datasets used and analysed during the current study are available from the corresponding author on reasonable request.

Authors' Contribution

AS and QA designed and conceptualized the study. S-ZZ, M-DM, AH, QA and AS conducted the parasitological

and entomological surveys and analyzed data. S-ZZ, QA and AS drafted the manuscript. All the authors participated in writing the manuscript. All the authors have read and approved the final manuscript.

Acknowledgement

This research was supported by the Vice-chancellor for Research and Technology Affairs of Shiraz University of Medical Sciences, Shiraz, Iran (Grant No. 95-01-04-12214).

Conflict of Interest: None declared.

References

1 Alvar J, Cañavate C, Gutiérrez-Solar B, Jiménez M, Laguna F, López-Vélez R, et al. Leishmania and human immunodeficiency virus coinfection: the first 10 years. Clinical microbiology reviews. 1997;10(2):298-319. PMID: 9105756 PMCID: PMC172921 DOI: 10.1128/CMR.10.2.298.

2 Desjeux P. Leishmaniasis: current situation and new perspectives. Comparative Immunology, Microbiology and Infectious Diseases. 2004;27(5):305-18. PMID: 15225981 DOI: 10.1016/j.cimid.2004.03.004.

3 Bray RS. Leishmania. Annual Review of Microbiology. 1974;28(1):189-217. PMID: 4611328 DOI: 10.1146/annurev.mi.28.100174.001201.

4 Azizi K, Kalantari M, Motazedian M, Asgari Q, Mohammadpour I, Soltani A. Co-detection and isolation of Leishmania and Crithidia among naturally infected Tatera indica (Rodentia: Muridae) in Fars province, southern Iran. Asian Pacific Journal of Tropical Biomedicine. 2018;8(5):279. DOI: 10.4103/2221-1691.233010.

5 Nazari M, Taravatmanesh G, Kaveh MH, Soltani A, Ghaem H. The Effect of Educational Intervention on Preventive Behaviors towards Cutaneous Leishmaniasis at Kharameh City in 2014. Shiraz E-Medical Journal. 2016;17(10). DOI: 10.17795/semj39957.

6 Rebouças-Silva J, Celes FS, Lima JB, Barud HS, de Oliveira CI, Berretta AA, et al. Parasite Killing of Leishmania (V) braziliensis by Standardized Propolis Extracts. Evid Based Complement Alternat Med. 2017;2017:6067172. PMID: 28690662 PMCID: PMC5485350 DOI: 10.1155/2017/6067172.

7 Pucca MB, Cerni FA, Oliveira IS, Jenkins TP, Argemí L, Sørensen CV, et al. Bee Updated: Current Knowledge on Bee Venom and Bee Envenoming Therapy. Front Immunol. 2019;10:2090. PMID: 31552038 PMCID: PMC6743376 DOI: 10.3389/fimmu.2019.02090.

8 Wehbe R, Frangieh J, Rima M, El Obeid D, Sabatier J-M, Fajloun Z. Bee Venom: Overview of Main Compounds and Bioactivities for Therapeutic Interests. Molecules. 2019;24(16):2997. PMID: 31430861 PMCID: PMC6720840 DOI: 10.3390/molecules24162997.

9 Ram SKM, Jayapal N, Nanaiah P, Aswal GS, Ramnarayan B, Taher SM. The therapeutic benefits of bee venom. International Journal of Current Microbiology and Applied Sciences. 2014;3:377-81.

10 An H-J, Kim J-Y, Kim W-H, Gwon M-G, Gu HM, Jeon MJ, et al. Therapeutic effects of bee venom and its major component, melittin, on atopic dermatitis in vivo and in vitro. Br J Pharmacol. 2018;175(23):4310-24. PMID: 30187459 PMCID: PMC6240132 DOI: 10.1111/bph.14487.

11 Nina N, Lima B, Feresin GE, Giménez A, Salamanca Capusiri E, Schmeda□Hirschmann G. Antibacterial and leishmanicidal activity of Bolivian propolis. Letters in applied microbiology. 2016;62(3):290-6. PMID: 26743801 DOI: 10.1111/lam.12543.

12 Ayres DC, Marcucci MC, Giorgio S. Effects of Brazilian propolis on Leishmania amazonensis. Mem Inst Oswaldo Cruz. 2007;102(2):215-20. PMID: 17426888 DOI: 10.1590/s0074-02762007005000020.

13 Machado GMdC, Leon LL, Castro SLd. Activity of Brazilian and Bulgarian propolis against different species of Leishmania. Mem Inst Oswaldo Cruz. 2007;102:73-7. PMID: 17294003 DOI: 10.1590/s0074-02762007000100012.

14 da Silva SS, Mizokami SS, Fanti JR, Miranda MM, Kawakami NY, Teixeira FH, et al. Propolis reduces Leishmania amazonensis-induced inflammation in the liver of BALB/c mice. Parasitology research. 2016;115(4):1557-66. PMID: 26711452 DOI: 10.1007/s00436-015-4890-4.

15 Duran G, Duran N, Culha G, Ozcan B, Oztas H, Ozer B. In vitro antileishmanial activity of Adana propolis samples on Leishmania tropica: a preliminary study. Parasitology Research. 2008;102(6):1217-25. PMID: 18264819 DOI: 10.1007/s00436-008-0896-5.

16 Fidalgo LM, Ramos IS, Parra MG, Cuesta-Rubio O, Hernández IM, Fernández MC, et al. Activity of Cuban propolis extracts on Leishmania amazonensis and Trichomonas vaginalis. Natural product communications. 2011;6(7):1934578X1100600712. PMID: 21834236.

17 Ferreira FM, Castro RA, Batista MA, Rossi FM, Silveira-Lemos D, Frézard F, et al. Association of water extract of green propolis and liposomal meglumine antimoniate in the treatment of experimental visceral leishmaniasis. Parasitology research. 2014;113(2):533-43. PMID: 24292604 DOI: 10.1007/s00436-013-3685-8.

18 Światły-Błaszkiewicz A, Mrówczyńska L, Matuszewska E, Lubawy J, Urbański A, Kokot ZJ, et al. The Effect of Bee Venom Peptides Melittin, Tertiapin, and Apamin on the Human Erythrocytes Ghosts: A Preliminary Study. Metabolites. 2020;10(5):191. PMID: 32413967 PMCID: PMC7281017 DOI: 10.3390/metabo10050191.

19 Benton AW, Morse RA, Stewart JD. Venom Collection from Honey Bees. Science. 1963;142(3589):228-30. PMID: 17834840 DOI: 10.1126/science.142.3589.228.

20 ISO. 10993-5: Biological evaluation of medical devices--Part 5: Tests for in vitro cytotoxicity. 2009: International Organisation for Standardization; 2009. 34 p.

21 Asgari Q, Keshavarz H, Rezaeian M, Motazedian MH, Shojaee S, Mohebali M, et al. Direct Effect of Two Naphthalene-Sulfonyl-Indole Compounds on Toxoplasma gondii Tachyzoite. J Parasitol Res. 2013;2013:716976. PMID: 24228173 PMCID: PMC3818922 DOI: 10.1155/2013/716976.

22 Maroufi Y, Ghaffarifar F, Dalimi A-A, Sharifi Z, Hassan Z. Cantharidin-induced apoptosis in Leishmania major promastigotes and macrophages infected by Leishmania major amastigotes in vitro. Journal of Mazandaran University of Medical Sciences. 2012;22(89):32-40.

23 Escobar P, Matu S, Marques C, Croft SL. Sensitivities of Leishmania species to hexadecylphosphocholine (miltefosine), ET-18-OCH3 (edelfosine) and amphotericin B. Acta Tropica. 2002;81(2):151-7. PMID: 11801222 DOI: 10.1016/s0001-706x(01)00197-8.

24 Chaisakul J, Hodgson WC, Kuruppu S, Prasongsook N. Effects of Animal Venoms and Toxins on Hallmarks of Cancer. J Cancer. 2016;7(11):1571-8. MID: 27471574 PMCID: PMC4964142 DOI: 10.7150/jca.15309.

25 Kim Y-S, Jun H, Chae Y, Park H-J, Kim BH, Chang I-M, et al. The practice of Korean medicine: an overview of clinical trials in acupuncture. Evid Based Complement Alternat Med. 2005;2(3):325-52. PMID: 16136212 PMCID: PMC1193543 DOI: 10.1093/ecam/neh102.

26 Kim Y-W, Chaturvedi PK, Chun SN, Lee YG, Ahn WS. Honeybee venom possesses anticancer and antiviral effects by differential inhibition of HPV E6 and E7 expression on cervical cancer cell line. Oncology Reports. 2015;33(4):1675-82. PMID: 25633640 DOI: 10.3892/or.2015.3760.

27 Pereira AV, de Barros G, Pinto EG, Tempone AG, Orsi RdO, Dos Santos LD, et al. Melittin induces in vitro death of Leishmania (Leishmania) infantum by triggering the cellular innate immune response. J Venom Anim Toxins Incl Trop Dis. 2016;22:1. PMID: 26752985 PMCID: PMC4706697 DOI: 10.1186/s40409-016-0055-x.

28 Adade CM, Oliveira IRS, Pais JAR, Souto-Padrón T. Melittin peptide kills Trypanosoma cruzi parasites by inducing different cell death pathways. Toxicon. 2013;69:227-39. PMID: 23562368 DOI: 10.1016/j.toxicon.2013.03.011.

29 Ali M. Studies on bee venom and its medical uses. International journal of advanced engineering research and technology. 2012;1(2):69-83.

30 da Silva SS, Thomé GdS, Cataneo AHD, Miranda MM, Felipe I, Andrade CGTdJ, et al. Brazilian propolis antileishmanial and immunomodulatory effects. Evid Based Complement Alternat Med. 2013;2013:673058. PMID: 23762152 PMCID: PMC3670560 DOI: 10.1155/2013/673058.

31 Król W, Bankova V, Sforcin JM, Szliszka E, Czuba Z, Kuropatnicki AK. Propolis: properties, application, and its potential. Evid Based Complement Alternat Med. 2013;2013:807578. PMID: 23997799 PMCID: PMC3753737 DOI: 10.1155/2013/807578.

32 Wagh VD. Propolis: a wonder bees product and its pharmacological potentials. Adv Pharmacol Sci. 2013;2013:308249. PMID: 24382957 PMCID: PMC3872021 DOI: 10.1155/2013/308249.

33 Anjum SI, Ullah A, Khan KA, Attaullah M, Khan H, Ali H, et al. Composition and functional properties of propolis (bee glue): A review. Saudi J Biol Sci. 2019;26(7):1695-703. MID: 31762646 PMCID: PMC6864204 DOI: 10.1016/j.sjbs.2018.08.013.

34 Al-Waili N, Hozzein WN, Badr G, Al-Ghamdi A, Al-Waili H, Al-Waili T, et al. Propolis and bee venom in diabetic wounds; a potential approach that warrants clinical investigation. African Journal of Traditional, Complementary and Alternative Medicines. 2015;12(6):1. DOI: 10.4314/ajtcam.v12i6.1

35 Amin MA, Abdel-Raheem IT, Madkor HR. Wound healing and anti-inflammatory activities of bee venom-chitosan blend films. Journal of Drug Delivery Science and Technology. 2008;18(6):424-30. DOI: 10.1016/S1773-2247(08)50082-7

36 Antonio LdF, Lyra MR, Saheki MN, Schubach AdO, Miranda LdFC, Madeira MdF, et al. Effect of secondary infection on epithelialisation and total healing of cutaneous leishmaniasis lesions. Mem Inst Oswaldo Cruz. 2017;112(9):640-6. PMID: 28902290 PMCID: PMC5572450 DOI: 10.1590/0074-02760160557.