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Original paper

In vitro and *in vivo* effects of natural honey on *Leishmania major*

Hermineh GHOLIZADEH, Fatemeh GHAFFARIFAR, Abdolhossein DALIMI, Mohammad Saaid DAYER

Department of Parasitology, Faculty of Medical Sciences, Tarbiat Modares University, Tehran, Iran

Corresponding Author: Fatemeh Ghaffarifar; e-mail: ghafarif@modares.ac.ir

ABSTRACT. Leishmaniosis is an insect-borne disease whose clinical manifestations range from skin ulcer to visceral disease. Antimony compounds are currently known to be the main treatment for leishmaniosis, but there are limitations to their use. This study was performed to determine the *in vitro* and *in vivo* efficiency of honey on a standard strain of *Leishmania major* parasite in comparison with glucantime and amphotericin as the first line treatment. *Leishmania major* was exposed to different concentrations of honey extract at 400, 200, 100, 50, 25, 12.5, 6.25 µg/ml. The effectiveness of honey concentrations was determined by counting the parasite by Neubauer's chamber. Then, using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) colorimetric method, for promastigotes and macrophages then IC50 was calculated. A flow cytometry test was performed and necrosis and apoptosis diagrams were drawn. Next, the effect of the honey on the amastigotes inside macrophage cells was investigated. Finally, for the *in vivo* experimentation, the parasite was injected in the base of BALB/c mice tails and the resulting wounds were treated with honey. The results of all tests showed that the honey extract at 400 µg/ml concentration had the best effects on all stages. The honey has lethal effects on *Leishmania* parasite *in vitro* as well as therapeutic effects on wounds caused by the parasite. Further experiments are recommended to evaluate the performance of the extract on the parasite in volunteer human models.

Keywords: *Leishmania major*, honey, amastigotes, promastigotes, BALB/c

Introduction

Leishmaniosis is a tropical disease caused by an obligate intracellular parasite of the genus *Leishmania* [1]. This disease is distributed all over the world, including Iran [2]. The disease is transmitted by sand flies that are prevalent in many tropical and subtropical countries. It is estimated that there are about 12 million cases of cutaneous leishmaniosis in different parts of the world where 350 million people are at risk. Currently, cutaneous leishmaniosis are reported from 98 countries in the world. In Iran, however, the annually reported cases of the infection is about 20,000 cases [3]. Today, pentavalent antimony compounds are used to treat leishmaniosis, which includes two compounds, meglumine antimonite (glucantime) and sodium stibogluconate (Pentostam). However, the use of these compounds has various limitations such as lack of oral administration effect, long treatment period, toxic effects on heart and kidneys, drug resistance in 10–15% of cases and high cost [4]. This has urged the World Health Organization to encourage more research towards finding suitable alternatives with more emphasis on traditional and herbal medicines [5]. In this regard, due to its diverse climate and plant flora Iran provides a suitable ground for studying different native plants and their extracts as effective drugs [6].

Honey is a very rich and energizing food that is made of about 80% of simple sugars glucose and fructose and produces 230 calories per 100 grams. In addition, this compound has thiamine, riboflavin, nicotinic acid and ascorbic acid. It contains pyridoxine and vitamin K [7]. This compound has antibacterial properties that have long been used to treat skin disorders such as wounds and burns and known as one of the best dressings. It also has anti-

inflammatory properties. Research on honey has shown that it can fight a wide range of microbes, including *Helicobacter*, *Salmonella*, *E. coli*, and antibiotic-resistant bacteria called MRSA (methicillin-resistant *Staphylococcus aureus*). The main ingredient of honey is hydrogen peroxide, which has been used for many years to disinfect wounds. Interestingly, if honey is diluted 7 to 41 times, its effectiveness increases. This happens when honey is placed on seeping wounds and became diluted with bleeding plasma. On the other hand, hydrogen peroxide boosts the growth process of blood vessels. The presence of blood with nutrients and oxygen, cells called fibroblasts responsible of making new connective tissue, as well as monocytes which activate immune cells at the site of injury enhances the release of growth factors. The epithelial cells are also stimulated to eventually proliferate and close the wound. The monocytes then turn into other cells called macrophages, which swallow the microbes and foreign contaminants, as well as the dead cells. The shape appears faster in damaged tissue and heals faster [8].

In this study, the antiparasitic effects of honey on *Leishmania major* were investigated both *in vitro* and *in vivo* conditions.

Materials and Methods

The experimental work of this study was performed in the laboratories of the Faculty of Medical Sciences of Tarbiat Modares University under the approval ID: MODARES.REC.1397.165.

Preparation of a standard strain of Leishmania major

A standard strain was obtained from Razi Serum and Vaccine Research Center. The strain was cultured in a flask containing RPMI1640 medium (enriched with 20% of calf fetal serum) and incubated at 25°C. Under normal circumstances, the parasites were used in the stationary phase for injection into mice and macrophages [9].

Preparing a series of honey dilutions

To prepare honey extracts, first, pure and high-quality honey was obtained. The dilution of honey was prepared by distilled water at following concentrations: 400, 200, 100, 50, 25, 12.5 and 6.25 µg/ml. The solution was well shaken and kept in the freezer until use [9].

Growth inhibition of promastigotes

This test was based on inhibiting the growth of live and active promastigotes of *Leishmania major* (initial number 10^6 cell/ml) in the presence of honey at various concentrations of 12.5, 25, 50, 100, 200 and 400 µg/ml. Glucantime (100 µg/ml) and amphotericin B (1 µg/ml) were uses as positive control and RPMI media as negative control. The inhibition was observed at 24, 48 and 72 hours' time intervals. Each test was done as a triplicate in three separate 96-well plates incubated at 22–25°C. To determine the number of viable parasites, all concentrations, of the parasite were counted by Neubauer chamber and a microscope [9,10].

Cytotoxicity assay by MTT method

This test aimed to determine the *in vitro* maximum cell proliferation of promastigotes or macrophages (RAW 264.7 macrophage cell line) under different concentrations of honey. MTT is able to cross cell membranes. To prepare the MTT solution, 5 mg of yellow MTT powder is first dissolved in 1 ml of PBS buffer (5 mg/ml). To perform the MTT test, 100 µl of RPMI 1640 solution and 20% of FBS containing 2×10^6/ml promastigotes or 5×10^5 /ml of macrophages were cultured in 96-well plates. The different concentrations of honey with final concentration of 400, 200, 100, 50, 25, 12.5, and 6.25 µg/ml, glucantime 100 µg/ml and amphotericin B 1 µg/ml as positive control and RPMI media as negative control were added to the wells containing promastigotes. The final volume of each well was adjusted to 200 µl. After 72-hour incubation period, 20 µg/ml MTT solution was added to each well. The plates were re-incubated for 4 hours at 18°C for promastigotes and at 37°C for macrophages prior to centrifugation. The supernatant was then discarded,. DMSO 100 µl added to each well and OD read at 570 nm by ELISA reader [11].

Growth inhibition of amastigotes

Macrophages were cultured in 12-well plates before adding promastigotes of stationary phase at rates equal to 7 to 10 times the macrophage to each well making the final volume at 200 microliters. The plates were then incubated at 37°C under 5% CO_2 for 5 hours to allow the parasite entering the macrophage. The supernatant was finally removed to discard those which not entered the macrophage,. Honey concentrations (400, 200 and 100 µg/ml) were prepared by serial dilution and added to each

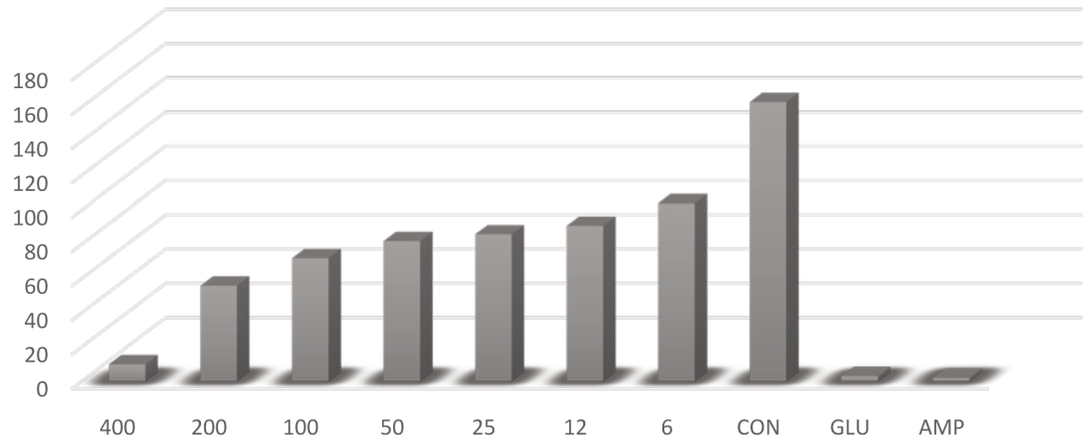

Figure 1. Average number of parasites exposed to different concentrations of honey after 72 hours in comparison with negative control (CON) and glucantime (GLU) and amphotericin (AMP) groups

well. Only media were added to control wells. This test was performed in triplicate. After 72 h, the cells on the surface of cover slips were fixed with methanol and stained with Giemsa. In the end, the percentage of infected macrophages and the number of amastigotes in each macrophage were determined [9.10].

Investigation of cell death and apoptosis by flow cytometry

Flow cytometry is based on the principle that laser light passing through a population of cells scatters in different directions and provides information about the size, shape, and structure of the cells. In this study, staining methods using annexin-V or propidium iodide (PI) were used to differentiate necrotic and apoptotic cells of promastigotes exposed to different doses of honey extract. The cells were was centrifuged and washed for 1 minute before 5 µl of annexing-V solution or otherwise PI solution was added to precipitated cells according to the protocol. The suspension was incubated for 15 minutes at room temperature and in darkness.

The dye intensity of annexing-V and propidium adsorbed on the cells was checked by the BDFACSCantoII flow cytometer and the results were analyzed by FlowJo software and displayed as a growth chart [9,10] .

In vivo assay using BALB/c mice

Fifteen BALB/c mice were infected with *Leishmania major* promastigotes. To this end, a volume of 0.1 ml solution containing 2×10^6 promastigotes in stationary phase were injected subcutaneously at the base of the mice tails. Upon lesions development (about 1 month post injection), the treatment was started, continued for 30 days and followed up for 30 days. We used three groups, each included 5 mice. One group was treated with honey (400 µg/ml), and another group was treated with glucantime (20 mg/kg/day) while the third control group was kept without treatment [9,10].

Parasitic load determination: T

The parasitic load was determined in the spleen using the serial dilution method. Four weeks after treatment, the surviving mice were killed and the spleen was removed. The spleen was then weighed and homogenized with the end of a sterile syringe in 2 ml of RPMI 1640 medium enriched with 20% of inactivated bovine fetal serum. Serial dilutions of spleen cell soup were prepared in 96-well sterile culture plates (100 microliters per well) in 20 dilutions. The following formula was used to determine the parasitic load:
(tissue weight/last dilution of the parasite with live parasite) – log10 = parasitic load

Statistical analysis

SPSS 16 software was used to analyze the data. The data were statistically judged by T-test and ANOVA (significance level was considered at 0.05). Also, using the light absorption and the following formula, the IC50 value was calculated using Excel software.
Log (IC50) = Log (x1) + ((y1-y0)/2) (y1-y2)) (log (x2) – log (x1))
Y1 = amount of parasite in concentration X1, Y2 = amount of parasite in concentration x2, Y0 = light absorption of control group.

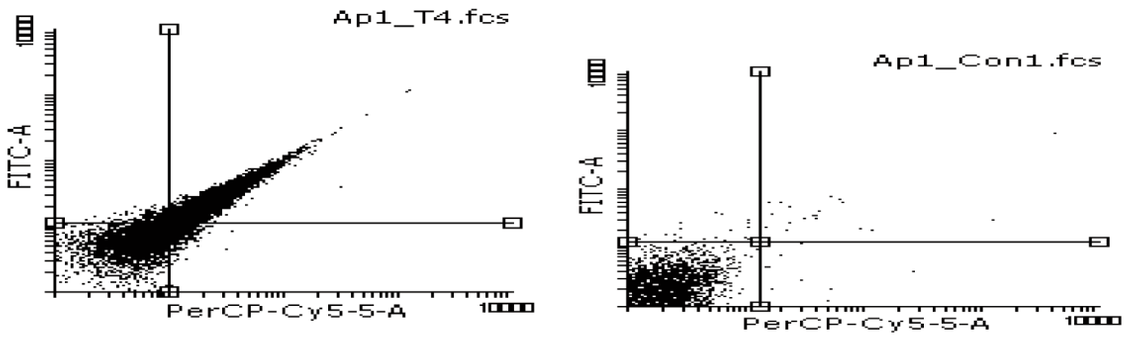

Figure 2. Comparison of flow cytometry diagram of parasite growth exposed to 50 µg/ml honey extract (left diagram) and the control (right diagram) without extract after 72 hours

Table 1. Percentage of promastigotes and macrophages inhibition in presence of various honey concentrations

Honey concentration µg/ml	Percentage of promastogotes inhibition	Percentage of macrophages inhibition
400	95	36
200	93	32
100	81	30
50	48	29
25	40	28
12.5	35	26
6.25	30	23
Control	0	0
Glucantime 100 µg/ml	70	50
Amphotericin 1 µg/ml	76	55

Results

Promastigote assay

In the first step of this study, the effects of 7 dilutions of honey extract on promastigote of *Leishmania major* parasite were compared with the first-line drugs namely glucantime and amphotericin. The result showed that after 72 hours the number of parasites in all concentrations significantly reduced compared to the negative control (P<0.05) (Fig. 1). In other words, there was a significant difference between the effect of 400 g/ml of honey extract and other concentrations.

Flow cytometry

According to calculations, the IC50 of the honey extract was considered to be about 50 µg/ml, and flow cytometry studies of the extract were evaluated at this concentration. In flow cytometry diagram, after 72 hours of exposure of *Leishmania* parasitic promastigotes to honey extract, the number of surviving cells was 38% and the number of killed cells (necrotic and apoptotic) was 60% (Fig. 2, left diagram) while in the control sample, living cells were 99% and killed ones constituted only 1% of cells (Fig. 2, right diagram). Comparison of these numbers shows a significant difference (P<0.05).

MTT

The effect of honey extracts on *Leishmania* parasitic promastigotes and macrophage cells was investigated by the MTT method. The amount of light absorption read by ELISA reader showed that the concentration of 400 µg/ml exerted the highest lethality on the promastigote cell and differed significantly with the control in this respect. Although, the same honey concentration had lower toxicity to macrophage than the positive control, the toxicities of all experimental extracts to infected macrophage were significantly higher than the negative control (P<0.05) (Tab. 1).

Amastigote assay

Also, the number of amastigotes in infected macrophages was significantly different between the test and control groups (P<0.05) (Tab. 2).

In vivo assay

The results of *in vivo* experiments indicated that 5 mice which were treated with honey showed improvement. In three mice which received complete treatment no wound recurrence was seen even after cessation of treatment. In 2 other treated mice, we recorded reduction in wound diameters,

Table 2. Mean and SD of amastigotes

	Concentration (µg/ml)	Mean±SD of amastigotes	Percentage of infected macrophages
Honey	400	6.21±0.82	4.8
	200	1±11	6.2
	100	15.21±1.09	9
Control	57.66±6.8	28.8	

while in untreated control group, wounds were purulent and fetal to 4 out of 5 mice. There was a significant difference between wound diameter in the control group (20±2 mm) and honey group (2.5±0.8) (P<0.05) in comparison to group treated with glucantime. Surviving mice were killed at the end of this experiment to determine the spleen parasitic load. The parasitic load was calculated according to the stated formula (Figs 3–5).

Figure 3. Untreated mice after 60 days

Figure 4. Mice treated with glucantime (20 mg/kg/day) after 60 days

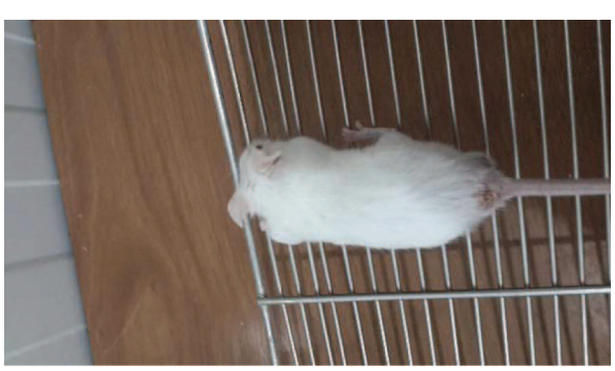

Figure 5. Mice treated with honey (400 µg/ml) after 60 days

Discussion

The results of this study showed that honey has antiparasitic compounds which are effective both under *in vitro* and *in vivo* conditions. In line with other studies on other components of honey such as propolis, we have obtained similar results. In Savoia's study, the therapeutic effects of propolis on *Leishmania major* have been observed *in vitro* conditions at different concentrations [12]. Sforcin [13] studied the effect of hydro alcoholic extract of bromine on the activity of natural killer cells. He stated that the compound increases the cytotoxic activity of killer cells and the antibacterial and anti-protozoal activity of the immune system. Hunter [14] showed increased cytotoxic cell activity against *Toxoplasma gondii*. Mustonen [15] however, attributed the role of propolis in killing amastigotes of *Leishmania donovani* to *in vitro* releasing of nitric acid and tumor necrosis factor from macrophages. Starzyk [16] described the lethal effect of alcoholic propolis extract (EEP) on *Trichomonas vaginalis* and stated that the extract could kill the parasite after 24 hours. The results of this study show the lethal effect of honey on *Leishmania major* parasite both under *in vitro* and *in vivo* conditions as well as healing property to wounds resulted in infected BALB/c mice. Our result has shown promising therapeutic effects of honey on *Leishmania major* comparable with first-line drugs.

Given the results obtained in this study, we suggest that further research should be carried out on a larger scale to test honey formulation and administration techniques such as ointment compositions or injection methods to treat leishmanial wounds. On the other hand, it may worth testing higher concentrations of honey extracts. This may increase the effectiveness and reduce the duration of treatment. It is also necessary to remember that for a better result, the honey treatment should be started immediately at the beginning of the appearance of wounds.

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