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Original Article

Investigating in Vivo and in Vitro Effects of Ethanolic and Aqueous Extracts of Myrtle (*Myrtus communis*) on *Leishmania major*

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Abstract

Background: The extract of myrtle plant contains polyphenolic compounds that show antibacterial, antiviral, and anti-parasitic properties. We aimed to investigate the therapeutic effect of aqueous and ethanolic myrtle extract against leishmaniasis caused by L. major in vivo and in vitro conditions.

Methods: This study was carried out in Tarbiat Modares University, Tehran, Iran in 2018. Aqueous and ethanolic extract of myrtle plant at 6.25 to 400 mg/ml concentrations were tested on *Leishmania major* promastigotes, non-infected macrophages, and macrophages infected with amastigotes in vitro using counting, MTT and flow cytometry techniques. Then, BALB/c mice were treated with ethanolic, aqueous and a mixture of both extracts of myrtle plant. The treatment was carried out for four weeks. Then, the effectiveness of the herbal medicine was assessed by measuring wounds diameters, mice weights and their mortality rate on weekly basis.

Results: The IC50 values of aqueous and ethanolic extracts for promastigotes were 7.86 and 11.66 µg/mL respectively. The IC50 values of the aqueous and ethanolic extracts for amastigotes were 12.5 and 47.2 µg/mL respectively. Flow cytometry indicates 62.88% and 60.16% apoptosis induced by ethanolic and aqueous extract of myrtle plant respectively. The lowest parasitic load was seen in the group treated with ethanolic extract.

Conclusion: The lesion sizes for treated groups with extracts were similar to those treated with glucantime. Oral administration instead of injection is another advantage of myrtle plant over glucantime, which makes the herb easy and more practical.

Introduction

Leishmania major is recognized as an important parasite of humans causing cutaneous lesions and wounds. However, this disease poses a new challenge every day in terms of its treatment. The solution seems to be the production of an effective medicine with the lowest side effects and the most enhancing effects on the immune system (1-6).

Myrtle plant contains antioxidant, anti-inflammatory, anti-protozoan, anti-bacterial, anti-fungal and anti-viral chemicals. Researches have shown the existence of 32 compounds in myrtle extracts including alpha-Pinene, Limonene, 1,8-cineole, and Linalool. In addition, its antibacterial effects against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans* has been shown using disk diffusion, MIC (minimum inhibitory concentration) and MBC (Minimum bactericidal concentration) methods (7-9). The more known species of myrtle with medicinal effects is *Myrtus communis* (10). Extracts and essential oils of *M. communis* leaves have antimicrobial and antioxidative activity (11). The plant belongs to Myrtaceae family with about 145 genus and 5500 species (12-16). Myrtle extract includes polyphenolic compounds, which are anti-bacterial and astringent for the skin. (8, 17).

A comparative study to evaluate anti-protozoal effects of herbal extracts of Shirazi Thyme, Peganum harmala, and Myrtle showed advantage of the later for the treatment of *Leishmania* parasite at in vitro condition (18).

The most important compound of the myrtle plant is its essence which contains 1,8-cineole Myrtenol, Pinene, Geraniol, Linalool, and Camphene. Moreover, Acyl phloroglucinol compounds are found in myrtle, which is responsible for its anti-inflammation properties. (10). Myrtle plant has antiseptic properties used in internal and external situations (19-20). Myrtle can also be used to treat cutaneous diseases and disorders during which skin flakes are formed which are small and white. Myrtle can be used topically in the treatment of herpes as a disinfectant, as well as an anti-inflammatory agent in nasal mucosal inflammation (21). The topical application of the plant is helpful in healing wounds (19-20).

The objective of this study was to investigate the therapeutic effect of aqueous and ethanolic myrtle extract against leishmaniasis caused by *L. major* in vivo and in vitro conditions.

Materials and Methods

This study was conducted in the Department of Medical Parasitology at Tarbiat Modarres University. The survey was approved by the Ethical Committee of Tarbiat Modares University. The Code of Ethics is: IR.TMU.REC13950447.

L. major parasite and the animal of the test

The parasitic strain used for this investigation, MHRO/IR/75/ER, was obtained from the Parasitology Laboratory of Tarbiat Modarres University in culture medium and BALB/c mice. This study was carried out in Parasitology Department, Tarbiat Modarres University, Tehran, Iran in 2018.

For experimentation, female pure BALB/c mice aged 6 to 8 wk were obtained from Royan Research Institute and kept at the Laboratory Animal Care Center of Tarbiat Modares University (6).

In vitro experimentation

L. major preparation

The parasite was transferred from -70 ºC environment to 25 ºC water bath. In the next stage, the RPMI1640 medium (enriched with 20% Fetal Bovine Serum (FBS)) was added to

a plate containing parasites and incubated at 25 ºC (6).

Preparing the aqueous and ethanolic extracts of myrtle plant

We prepared the myrtle plant from Fars province in the south of Iran. We used the leaf of plant. The fresh Myrtle plant was identified by an expert from the Herbarium School of Pharmacy at Shahid Beheshti University. The Herbarium code of green myrtle used in this study was 8024. Concentration of 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml were prepared using the stock solution.

Aqueous extract

First, 50 gr of dried myrtle plant was crushed and 200 ml diluted water was added to it. It was then placed on medium heat for 30-60 minutes. After that, it was left for 5 min and filtered by We used Whatman filter paper (Grade 1: 11 µm). Then, 20-25 ml of the filtered and were stored in -70 ºC freezer, then lyophilized.

Ethanolic extract

First, 50 gr of dried myrtle plant was crushed and 500 ml of 80%-85% ethanol was added. The beaker was shaken for 4-6 h, and left at room temperature for 72 hours. The solution was centrifuged at 5000 rpm (2800 gr) for 30 min. The extracts were freeze-dried for 24 h and the obtained dried powder was stored at -20 ºC until used.

Promastigote inhibition test

The growth of promastigote (initial count 2×10^6 promastigotes) was tested in the presence of different concentrations of aqueous and ethanolic extracts of myrtle (12.5, 25, 50, 100, 200, and 400 µg/ml. We used glucantime 100 µg ml^{-1} and amphotericin B (GILEAD UK) at 1µg ml^{-1} as positive control (6).

The inhibitory effects were tested after 24, 48 and 72 h post-incubation, in addition, each plate accommodated 3 wells containing only promastigotes with no medicine added which served as control (6). We used GraphPad prism5 software to determine IC50 of Leishmania growth.

MTT Test for promastigotes

Three 96-well plates were used to culture 2×10^6 promastigotes in 100 microliters of RPMI 1640 solution fortified with 10% FBS. to prevent vaporization. The extract were added to promastigote-containing wells at 6.25, 12.5, 25, 50, 100, 200, and 400 µg/ml concentrations. The plates were then incubated at the temperature of 24-25 ºC for 72 h before 20 µl of MIT (3-(4,5-dimethyl thiazolyl-2)-2,5-diphenyl tetrazolium bromide) solution was added to each well. The plates were incubated for further 4 h at 18 ºC before being centrifuged at 1000 gr for 10 minutes. The supernatant was removed. Each well was filled with 100 µl of DMSO and absorbance was read at 540 nm using ELISA reader.

MIT test for macrophages

The macrophage cell line J774 was cultured in the way as done for promastigotes.

Amastigotes growth inhibition test

Stationary phase promastigotes at a ratio of 10 times that of macrophages were added. The plates were incubated at 37 ºC with 5% CO2 for 5 h so that the parasites invade the macrophages. In the next stage, non-penetrating parasites were removed by discarding supernatants under sterile conditions. Serial dilution of aqueous and ethanolic extracts of myrtle was prepared by adding 6.25, 12.5, 25, 50, 100, 200 and 400 µg/ml to infected macrophages (6). We used GraphPad prism5 software to determine IC50 of *Leishmania* growth.

Cell apoptosis as revealed by Flow Cytometry

Annexin-V staining method was used to differentiate necrotic and apoptotic promastigote cells exposed to different doses of aqueous and ethanolic extracts of myrtle. The kit of

Roche Company (Germany) was used to conduct the test as follows (6).

1. Cells exposed to different concentrations of aqueous and ethanolic extracts for 72 h, as well as control cells were washed using cold PBS solution and centrifuged at 1400 gr for 1 minute.
2. To ensure complete removal, the washing was performed again using RPMI 1640 medium.
3. Based on the protocol, 5 microliters of Annexin-V were added to the settled cells.
4. Again, 5 ul of the PI solution was added.
5. The suspension was incubated at room temperature and in a dark place for 15 minutes.
6. The staining intensity of Annexin-V and Propidium adsorbed by the cells were evaluated by the BDFACSCantoII Flow cytometer.
7. The results were analyzed using FlowJo software.

In vivo experiments

To infect BALB/c mice with *L. major* parasites, a volume of 0.1 ml of the solution containing 2×10^6 promastigotes in stable phase was subcutaneously injected to the base of the tale using Insulin syringes.

After 36 d post-injection, the mice developed completely visible ulcers. The mice were then treated with aqueous and ethanolic extracts of myrtle plant by oral administration for 28 d as follows.

Grouping of mice

Mice were divided into 7 groups each including 5 individuals as follows:

1. Infected control group receiving no treatment.
2. Infected group treated with 0.5ml (200 µg/ml) of ethanolic extract
3. Infected group treated with 1ml (200 µg/ml) of ethanolic extract
4. Infected group treated with 0.5ml (200 µg/ml) of aqueous extract
5. Infected group treated with 1ml (200 µg/ml) of aqueous extract
6. Infected group treated with a mixture of ethanolic and aqueous extracts of myrtle (200 µg/ml) 0.5 ml from each extract.
7. Infected group injected with Glucantime (60 mg/Kg/day) for 28 days.

Determination of parasitic load

Six weeks after treatment, 2 mice from each group were sacrificed and their spleens were removed and weighed. Serial dilutions of the spleen cell suspension were prepared in sterile 96-well culture plates (100 microliters in each well). Twenty dilutions (1/10 to $1/10^{20}$) were prepared. After incubation at 26 ºC, wells were observed under 40× magnification of invert microscope for 3 wk at 3-day intervals to investigate and the presence of viable promastigotes. The last shot was the last dilution with one living parasite. The count of parasites was calculated using the following formula:

Parasitic load= (last parasite dilution along with living parasite) ×-log10

Statistical analysis

One-way ANOVA was used to compare the means of the studied variables. In addition, Tukey post hoc test was used to determine significant differences between groups. Kolmogorov-Smirnov test was used to test the assumption of normality. Data were analyzed using SPSS software version 16 (Chicago, IL, USA). The value of $P<0.05$ was considered significant.

Results

Aqueous and ethanolic extracts of the myrtle plant caused significant reduction of mean counts of infected macrophages compared to control groups ($P<0.05$). The average counts of amastigotes in each macrophage treated with the aqueous extract of myrtle after 24 h exposure to 400 µg/ml and 6.25 µg/ml concentrations were 16 and 42 respectively compared to control which was 68 (Table 1). The average counts of amastigotes in each macrophage treated with ethanolic extract of the same concentrations were 8.66 and 45 respectively compared to control which was 67.66 (Table 1).

Table 1: Effect of different concentrations of aqueous and ethanolic extracts of myrtle on the average infection of macrophages and the average count of amastigotes per 100 macrophages, 72 h after treatment

Extract concentration µg/ml	*Aqueous extract*		*Ethanolic extract*	
	Mean ± SD of amastigotes per 100 macrophages	Average macrophages infected with amastigote per 100 macrophages	Mean ± SD of amastigotes per 100 macrophages	Average macrophages infected with amastigote per 100 macrophages
400	16 ±1*	8	8.66 ±1.52*	4.3
200	20 ±1*	10	14±1*	7
100	20.66±3.51*	10.5	20 ±2*	10
50	21.66±2.08*	10.3	27.33±2.51*	13.6
25	31.33±1.52*	15.6	32.66±1.52*	16.3
12.5	34±1*	17	37.66±2.51*	18.8
6.25	42±4.35*	21	45 ± 2*	22.5
Control	68±2.64	34	57.66± 6.80	28.8

*There was a significant difference between the average number of amastigotes in treated macrophages and the test and control groups ($P<0.05$).

Fig. 1(A) presents the results of flow cytometric analysis of experimentations using different concentrations of ethanolic extracts of myrtle plant.

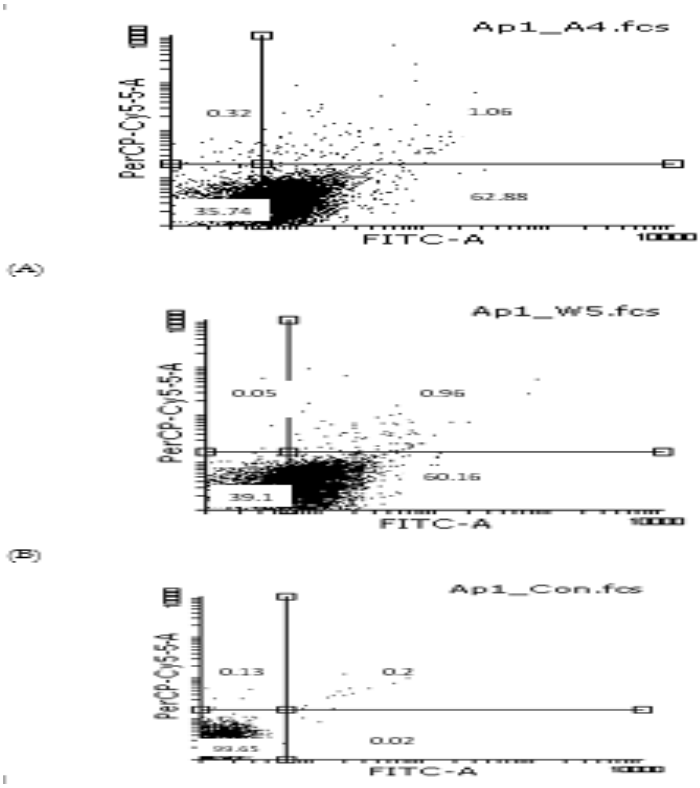

Fig. 1: Flow cytometry results of promastigotes treated with ethanolic extract of myrtle plant (A), and at aqueous extract of myrtle plant (B) at a concentration of 100 µg/ml, and control group (C), 72 h after treatment. Regions of quadrants show; left bottom: alive promastigotes, right bottom: early apoptosis, left top: necrosis, right top: late apoptosis

Ethanolic extract of myrtle plant at 100 µg/ml concentration induced 62.88% apoptosis, 35.74% live parasites, 0.32% necrosis, and 1.06% secondary apoptosis. Whereas aqueous

extract of myrtle plant at 100 µg/ml caused 60.16%, initial apoptosis, 39.1% live parasites, 0.05% necrosis, and 0.96% secondary apoptosis (Fig. 1.B). Results from flow cytometry test of the control group indicated 99.65% live parasites, 0.02%, initial apoptosis 0.13% necrosis, and 0.2% secondary apoptosis (Fig. 1.C).

Following the incubation of parasites for 24, 48, and 72 h in the presence of different concentrations of aqueous and ethanolic extracts of the myrtle plant, the count of the promastigotes decreased, so that IC50 of the aqueous extract was 182.6, 13.73, and 7.86 µg/ml, for the abovementioned incubation periods respectively (Table 2). Whereas, these values for the ethanolic extract, were was 122.4, 12.36, and 11.66 µg/ml, respectively. Application of ANOVA test showed significant differences between the effects of different concentrations of both extracts with control treatment (Table 3).

Table 2: The effect of aqueous extract of myrtle plant on mean± SD of promastigotes after 24, 48 and 72 h

Concentration of aqueous extract (µg / ml)	***Number of promastigotes ×10⁴***					
	After 24 hours		After 48 hours		After 72 hours	
	Mean ± SD	Inhibition rate (%)	Mean ± SD	Inhibition rate (%)	Mean ± SD	Inhibition rate (%)
400	31±3.60	80.98	14±2	88.94	11±5.29	82.53
200	49.33±1.15	69.79	20±2	84.20	17.66±1.52	71.96
100	64±4	60.81	37.33±2.51	70.52	21.33±3.21	66.14
50	73.66±4.04	54.90	42.66±2.51	66.31	12.33±1.52	80.42
25	89±1.00	45.50	46.66±3.21	63.16	29.66±2.51	52.99
12.5	96±5.29	41.22	47.66±2.08	62.37	40.33±2.51	35.98
6.25	110±10	32.65	59.33±4.04	53.15	47±3	30.55
Control	163±23	0	126±5.77	0	63±3	0
Glucantime 100 µg ml^{-1}	10±2	93.87	4.33±1.15	94	3.33±0.57	94.71
Amphotericin B 1 µg ml^{-1}	10.33±5.50	93.67	2±1	95.33	2±1.73	96.82

The difference between all groups in comparison with the control group was significant (P <0.05)

Table 3: The effect of ethanolic extract of myrtle plant on promastigotes after 24, 48 and 72 h

Concentration of ethanolic extract (µg / ml)	***Number of promastigotes ×10⁴***					
	After 24 hours		After 48 hours		After 72 hours	
	Mean ± SD	Inhibition rate (%)	Mean ± SD	Inhibition rate (%)	Mean ± SD	Inhibition rate (%)
400	4.33 ±3.05	97.11	1.33±1.52	98.94	0.33±0.57	99.39
200	23.33±4.16	84.24	14±1	88.88	9.66±2.51	82.22
100	42.66±2.51	71.56	21±1	83.33	23.33±4.16	57.05
50	84±4	44	25.33±1.52	70.90	33.66±3.21	39.62
25	95±5	36.66	36.33±1.52	65.07	45.33±2.51	17.06
12.5	113±4.35	24.66	47.66±2.51	62.69	43.66±8.14	20.12
6.25	120±2.08	20	49.33±1.15	60.84	52.66±6.80	3.65
Control	150±10	0	126.66±5.77	0	54.33±4.04	0
Glucantime 100 µg ml^{-1}	10±2	93.33	9±2	93.83	3±2	94.51
Amphotericin B 1 µg ml^{-1}	10±5	93.33	7±5	95.33	2±1.73	96.82

The difference between all groups in comparison with the control group was significant (P<0.05)

The results of MTT tests for various concentrations of aqueous and ethanolic extracts of myrtle on promastigotes are presented in Table 4, whereas those for the concentrations of the extract on macrophages are shown in Table 5. The results showed lower toxicity of both extracts to macrophages than to promastigotes.

Table 4: The effect of aqueous and ethanolic extracts of myrtle on promastigotes in the MTT test

Concentration of aqueous extract (µg / ml)	***aqueous extract***		***ethanolic extract***	
	Mean ± SD absorption rate	Percentage of inhibition	Mean ± SD absorption rate	Percentage of inhibition
400	0.23 ±0.01	69	0.24±0	67
200	0.25 ±0	66	0.27±0.02	63
100	0.28 ±0.01	62	0.31±0	56
50	0.33±0.03	54	0.33±0	54
25	0.41±0.01	43	0.34±0	52
12.5	0.44±0	38	0.39±0.01	45
6.25	0.51±0.02	29	0.43±0	40
Control	0.72±0.01	0	0.72±0.01	0
Glucantime 100 µg ml^{-1}	0.21±0.01	70	0.21 ±0.01	70
AmphotericinB 1 µg ml^{-1}	0.17±0	76	0.17 ±0	76

The difference between all groups in comparison with the control group was significant (P <0.05).

Table 5: The effect of aqueous and ethanolic extracts of myrtle on macrophages in the MTT test

Concentration of aqueous extract (µg / ml)	***Aqueous extract***		***Ethanolic extract***	
	Mean ± SD absorption rate	Percentage of inhibition on macrophage	Mean ± SD absorption rate	Percentage of inhibition on macrophage
400	0.44 ± 0	41	0.62 ± 0	20
200	0.48 ± 0	36	0.65 ± 0	16
100	0.51 ± 0	32	0.70 ± 0.01	10
50	0.63 ± 0.02	16	0.71 ± 0	8.9
25	0.68 ± 0	9.3	0.72±0.01	7.6
12.5	0.69 ± 0	9.2	0.74±0	5.1
6.25	0.70±0	6.6	0.76±0	2.5
Control	0.75 ± 0.15	0	0.75±0.15	0
Glucantime 100 µg ml^{-1}	0.39 ± 0.03	48	0.39±.0.03	48
Amphotericin B 1 µg ml^{-1}	0.53±0	55	0.53 ± 0	55

The difference between all groups in comparison with the control group was significant. (P<0.05)

The untreated control group underwent continuous mortality from the first week until day 60 when most died. The survival rate of mice under treatment with myrtle ethanolic extract was better than other groups. The untreated control group showed significant dif-

ferences from the treated groups in terms of average wound diameter and average weight (P<0.05) (Table 6). The control group had higher value for average wound diameter and lighter weight during 60-day experimentation.

Table 6: Mean and standard deviation of alternations in wound diameter (millimeter) due to *Leishmania major* in untreated control mice, and mice treated with extracts of myrtle

Group / ***Week***	***Mean ± SD of wound diameter (mm) in treated and control groups***					
	Ethanolic extract of myrtle 200 (µg)	ethanolic extract of myrtle 100 (µg)	Aqueous extract of myrtle 100 (µg)	ethanolic and aqueous extracts of myr-tle100+100 (µg)	The control group (with-out treat-ment)	Glucan time injected group
First *	3.81±0.41	3.85±0.68	3.97±0.33	4.08±0.08	3.67±0.88	4.13±0.25
Second *	3.81±0.41	3.85±0.68	3.98±0.33	4.08±0.08	5.49±0.91	4.13±0.25
Third *	3.82±0.41	3.85±0.68	3.98±0.33	4.09±0.08	7.51±0.67	4.14±0.25
Fourth *	3.82±0.41	3.85±0.68	3.98±0.33	4.09±0.08	8.71±0.41	4.14±0.25
Fifth *	3.82±0.41	3.86±0.68	3.98±0.33	4.09±0.08	9.93±0.83	4.14±0.25
Sixth *	3.82±0.41	3.86±0.68	3.98±0.33	4.09±0.08	12.22±0.56	4.14±0.25

* Significant difference compared with the control group (P <0.05)

The results of parasitic load tests are presented in Fig. 2. The lowest parasitic load was observed in the group treated with the ethanolic extract (200 µg). The difference between the treated groups and the control group was statically significant (P<0.05).

The difference between the treated and untreated groups was significant (P<0.05) as tabulated in Table 1 and Fig. 3.

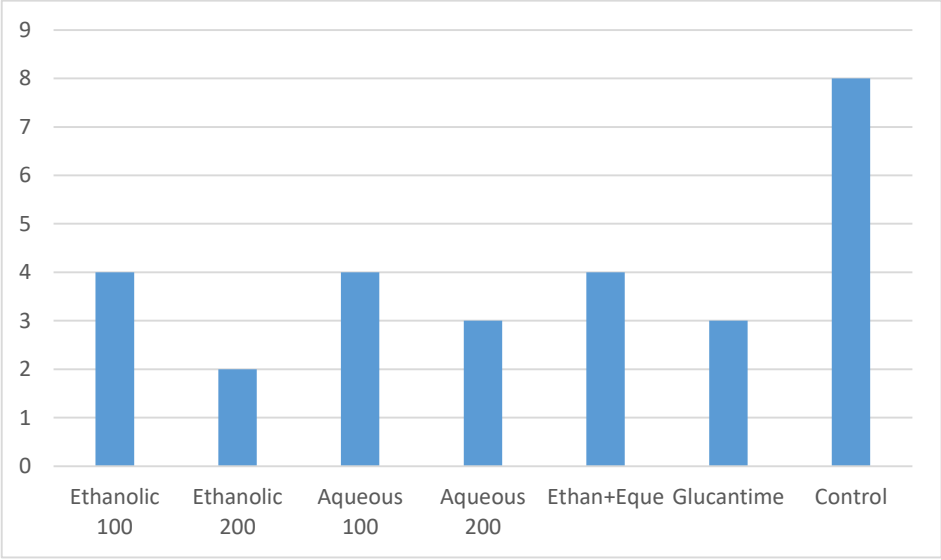

Fig. 2: -log10 the dilution of parasitic load evaluated from using the spleen culture by serial dilution (1/10 to $1/10^{20}$) method six weeks after treatment

A B

Fig. 3: **A**) The lesion of treated mice with aqueous extract of myrtle. **B)** The lesion of treated mice with ethanolic extract of myrtle, 4 wk after treatment

Discussion

Considering the side effects of the medicine, efforts are being made to provide a new medicine that can treat the lesions of the disease faster with minimum side effects but leave no scar after healing. Given these facts, we focused to investigate the effectiveness of the extract of the myrtle plant both in vivo and in vitro conditions. The aqueous and ethanolic extracts of myrtle include polyphenolic compounds, which are mostly anti-bacterial, antiviral and skin astringent.

A study revealed the inhibitory effect of myrtle extract on the proliferation of herpes simplex type I virus in neonatal hamster kidney cell culture. There was a significant relationship between the concentration of myrtle extract and cell fatality so that increased concentrations lead to higher cell death (21). In Iran, anti-malaria, anti-*Leishmania*, and anti-*Trichomonas* effects of the myrtle plant have been studied. To the contrary of our finding, Babakhoo et al. reported no decrease in the diameter of the wound in infected mice when they used 10% and 20% extracts of myrtle plant. Instead, they observed a significant increase in the diameter of the wound even after continuing the treatment for 14 d using extracts of 20% concentration. Their parasitology tests also showed that the count of parasites was not decreased (22). However, our results showed that treatment using aqueous and ethanolic extracts of myrtle plant leads to significant decrease in wound diameter compared with the control group. The differences were significant after the second week. A study conducted on the anti-parasitic effects of extracts of thyme, *Peganum harmala*, and myrtle, including green myrtle in treating *Leishmania* parasite in laboratory indicated better effects compared with the control medicine (18). The study conducted on the same extract showed good results against *Toxoplasma gondii* parasite (23). These results encouraged us to investigate the anti-*Leishmania* effects of this extract.

The research with essential oil and methanolic extract of *M. communis* L. on promastigotes of *L. major* showed that the IC50 values for essential oil and methanolic extract was 8.4 and 28.9 µg/ml against promastigotes, respectively and against the amastigotes the IC50 were 11.6 and 40.8 respectively (24). In our study, the IC50 values of aqueous and ethanolic extracts for promastigotes were 7.86 and 11.66 µg/mL respectively, and against the amastigotes the IC50 were 12.5 and 47.2 µg/mL respectively. The results of two research seem very close.

Conclusion

In both groups treated with aqueous and ethanolic extracts, the lesion sizes were similar to those treated with glucantime. Both aqueous and ethanolic extracts were less toxic to normal macrophages than glucantime and oral

administration instead of injection is another advantage of myrtle plant over glucantime, so this method of treatment can be used as an alternative method for treatment of cutaneous leishmaniasis.

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Conflict of interest

The authors have no conflicts of interest regarding this paper.

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