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Original Article

In Vitro Inhibitory Effect of *Berberis vulgaris* (Berberidaceae) and Its Main Component, Berberine against Different *Leishmania* Species

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Abstract

Background: Leishmaniasis has been identified as a major public health problem in tropical and sub-tropical countries. The present study was aimed to investigate antileishmanial effects of various extracts of *Berberis vulgaris* also its active compoenent, berberine against *Leishmania tropica* and *L. infantum* species on *in vitro* experiments.

Methods: In this study *in vitro* antileishmanial activity of various extracts of *B. vulgaris* also its active compoenent, berberine against promastigote and amastigote stages of *L. tropica* and *L. infantum* was evaluated, using MTT assay and in a macrophage model, respectively. Furthermore, infectivity rate and cytotoxicity effects of *B. vulgaris* and berberine in murine macrophage cells were investigated.

Results: The findings of optical density (OD) and IC_{50} indicated that *B. vulgaris* particulary berberine significantly ($P<0.05$) inhibited the growth rate of promastigote stage of *L.tropica* and *L.infantum* in comparison to meglumine antimoniate (MA). In addition, *B. vulgaris* and berberine significantly ($P<0.05$) decreased the mean number of amastigotes in each macrophage as compared with positive control. In the evaluation of cytotoxicity effects, it could be observed that berberine as compared with *B. vulgaris* exhibited more cytotoxicity against murine macrophages. Results also showed that when parasites were pre-incubated with *B. vulgaris* their ability to infect murine macrophages was significantly decreased.

Conclusion: *B.vulgaris* particularly berberine exhibited potent in vitro leishmanicidal effects against *L. tropica* and *L.infantum*. Further works are required to evaluate the antileishmanial effects of *B.vulgaris* on *Leishmania* species using clinical settings.

Introduction

Leishmaniasis is a protozoan parasitic disease found in 16 developed and 72 developing countries with 12 million cases (1). Cutaneous leishmaniasis (CL) is the most common type of leishmaniasis affecting 1.5 million people annually, worldwide. About 90% of cases are reported from countries such as Iran, Afghanistan, Pakistan, Iraq and Saudi Arabia (2). In Iran, the principal pathogenic species of CL are *Leishmania tropica and L. major* (3). Visceral leishmaniasis (VL) is the most severe form of leishmaniasis in the world, which is responsible for an estimated 500,000 cases each year, globally. VL is endemic in various parts of Iran which is caused by *L. infantum* (4,5). At present, there is no efficacious vaccine against leishmaniasis and chemotherapy remains the only choice. However, existing drugs are associated with adverse effects including toxicity, high cost, long duration of treatment and emergence of resistance (6-10). Therefore, the development of new drugs against leishmaniasis is an urgent need. Recent researches showed that plant extracts and plant-derived compounds due to having less side effects, low cost and high availability are a successful approach to treat a wide range of diseases, such as leishmaniasis (11).

European barberry, *Berberis vulgaris* L. (Berberidaceae), grows in Asia and Europe, which is well known in Iran and most countries in the world. The different parts of the plant including root, leaf, bark and fruit have been used widely as traditional medicine for the treatment and preventation of different disease conditions including cardiovascular, gastrointestinal, respiratory, skin, renal and infectious diseases (12). Previous studies have also been undertaken on chemical composition of the *B. vulgaris* which showed the main important components of this plant are isoquinoline alkaloids such as berbamine, palmatine and particularly berberine (12-14).

So far, in the various studies, antibacterial and antifungal activities of *B. vulgaris* and also its main constituent, berberine against several pathogenic strains have been proven (15-17). Moreover, the recent studies have demonstrated high antiparasitic potential of *B. vulgaris* and its main component, berberine against *Entamoeba histolytica, Giardia lamblia, Trichomonas vaginalis* and also some *Leishmania* spp. (18-19).

The present study was aimed to evaluate the *in vitro* antileishmanial activity of various extracts of *B. vulgaris* and also berberine against promastigote and amastigote stages of *L. tropica* and *L. infantum* species.

Materials and Methods

Chemicals used

MTT powder [3-(4.5-dimethylthiazol-2-yl)-2.5-diphenyl tetrazolium bromide)], fetal calf serum (FCS) and RPMI-1640 medium with L-glutamine were purchased from Sigma-Aldrich, St Louis, MO (USA). Meglumine antimoniate (MA, Glucantime) as control drug was purchased from Rhône, Poulenc, France. Penicillin and streptomycin were prepared from Alborz Pharmacy, Karaj, Iran and were stored at room temperature (25°C) until testing. All other chemicals and solvents were of analytical grade.

Leishmania strains

Leishmania tropica standard strain (MHOM/IR-/2002/Mash2) was obtained from the Center for Research and Training in Skin Diseases and Leprosy (Tehran, Iran). *Leishmania infantum* standard strain (MCAN/IR/07/Moheb-gh) was prepared from Laboratory for Leishmaniasis, Department of Medical Parasitology, Tehran University of Medical Sciences, Iran. The parasites were cultured in NNN medium, subcultured in RPMI-1640, supplemented with penicillin (200 IU/mL), streptomycin (100 µg/mL), and 15% heat-inactivated FCS.

Preparation of murine macrophages

For investigation of cytotoxicity effects of *B. vulgaris* and berberine, murine macrophages

were collected from male healthy BALB/c mice (4-8 weeks old) by injecting 2-5 mL of cold RPMI-1640 medium into mouse peritoneal cavity, then aspirated macrophages were washed twice and resuspended in RPMI-1640 medium. The experimental procedures carried out in this survey were in compliance with the standard guidelines of the Kerman University of Medical Science (Kerman, Iran) for the care and use of laboratory animals.

Collection of plant materials

The *B. vulgaris* root was collected from Baft district in September 2012, Kerman province, Iran. The identities were confirmed by the botanist at the Botany Department of Shahid Bahonar University, Kerman, Iran. Voucher specimen (KF769) of the plant materials was deposited at the Herbarium of Department of Pharmacognosy of School of Pharmacy, Kerman University of Medical Science, Kerman, Iran.

Preparation of the aqueous extract

Fifty grams of plant material was ground and boiled gently with 500 mL distilled water for approximately 1 h. The filtered aqueous extracts were concentrated in a rotary vacuum evaporator and dried by exposure to hot air to yield solid material and then were stored at -20°C, until testing.

Preparation of the methanolic extract

The dried plant materials (500 g) were ground and extracted by percolation method by methanol for 72 h. in room temperature. Solvent was removed in a rotary evaporator and extracts were concentrated to dryness and stored at -20 °C, until testing.

Preparation of the berberine

Berberine (2,3-methylenedioxy-9,10-dimethoxyprotoberberine chloride), as active principle of of *B. vulgaris* obtained from Sigma-Aldrich, (St. Louis, MO, USA), was dissolved in the dimethyl sulfoxide (DMSO). Final concentration of DMSO was never exceeded 1% either in control or treated samples

Evaluation of inhibitory effects against promastigote forms

In order to evaluate the antipromastigote activities of various extracts of *B. vulgaris* and berberine, 100 µL of the promastigotes of each species (10^6 promastigotes/mL) harvested from logarithmic growth phase were plated into a 96-well plate (Lab-Tek, Nalge Nunc International NY, USA). Then 10 µL of extracts or berberine was added to wells at a final concentration of 5-50 µg/mL and 0.5-10 µg/mL, respectively. Plates were incubated at 37 ±1°C for 72 h. After incubation, 10 µL of MTT solution (5 mg dissolved in 1 ml saline solution) was added to each well and were incubated for additional 4 h. The medium was removed and formazan crystals were dissolved by addition of 100 µL of sulphoric acid. Promastigotes were cultured in complete medium with no drug used as positive control, and complete medium with no promastigotes and drugs as blank (20). Finally, absorbance was measured by an ELISA reader (BioTek-ELX800) at 490 nm. We also calculated the 50% inhibitory concentrations (IC_{50} values) for all tested extracts by probit test in SPSS software. All tests were performed in triplicate.

Evaluation of inhibitory effects on intra-macrophages amastigote forms

At first, $1cm^2$ cover slips were placed in the wells of 6-chamber slides (Lab-Tek, Nalge Nunc International NY, USA). Then Peritoneal macrophages collected from BALB/c mice were plated at 10^6/mL in each well and incubated for 4 h at 37 °C in 5% CO2. Non adherent cells were removed, and stationary-phase promastigotes of both species were added at a 5: 1 parasite/ macrophage ratio. Cultures were added for further 4 h and free parasites were removed by washing with RPMI1640 medium. In the next step, 990 µL of the RPMI complete medium and 10 µL of the different extracts and berberine were added, following serial dilutions 1: 2, to obtain final concentrations between 5 to 100 µg/mL

and 1 to 10 µg/mL, respectively. Then, plates were incubated for 48 h at 37 ˚C (21). Cultures containing parasite without extract and cultures with no parasite and extracts were considered as positive and negative controls, respectively. At the end, dried slides were fixed with absolute methanol, stained by Giemsa and tested under a compound light microscope. Anti- intramacrophage amastigotes activity of extracts was assessed by counting the number of amastigotes in each macrophage by examining 100 macrophages (% amastigotes viability) in comparison with those obtained with positive control. Also the IC_{50} values of extracts and berberine were calculated by probit test in SPSS software. All experiments were carried out in triplicate.

Inhibition of infection in murine macrophages

To investigate the inhibitory effect of various extracts of *B. vulgaris* and also berberine against the promastigotes of *L. tropica* and *L. infantum* invasion of macrophages, promastigotes of both species were pre-incubated in aqueous and methanolic extracts (5 µg/mL) and also berberine (1 µg/mL), for 2 h at room temperature. Then promastigotes were washed with RPMI-1640 medium and incubated with murine macrophages for 4 h. After washing the cells again, the macrophages were stained by Giemsa and studied by a light microscope, to evaluate the frequency of infection by counting 100 macrophages. All tests were carried out in triplicate.

Cytotoxicity effects on murine macrophages

For assessment of cytotoxicity activities on murine macrophage cells, we determined the CC_{50} (cytotoxicity concentration for 50% of cells) of various extracts of *B. vulgaris* and also berberine on peritoneal murine macrophages. Macrophage cells were plated at 10^6 cells /mL in 96- well Lab-Tek (Nunc, USA) and left to adhere for 2 h. at 37 ◦C in 5% CO2. Non-adherent cells were removed by washing with medium after 2 h. of incubation at similar conditions. In the next stage, 190 µL of complete RPMI medium was added in each well, and later 10 µL of extracts dilutions, previously prepared in medium, was added. Macrophages were treated with the extracts from 10 to 500 µg/mL for 72 h. The cytotoxicity rate was evaluated using the colorimetric assay with MTT as previously described in the promastigote sensitivity assay. In this stage, similar to previous stages, all experiments were carried out in triplicate.

Statistical analysis

All tests were performed in triplicate, and IC_{50} and CC_{50} values were directly determined by probit test in SPSS software. In addition, the results were expressed as their average and standard deviation. The selectivity index (SI), calculated based on the equation of CC_{50} for murine macrophage cells / IC_{50} for promastigote forms of both species, was used to compare the toxicity and activity of the aqueous and methanolic extracts and berberine as described by Weninger et al. (22). Moreover, *t*-test was used to compare the IC_{50} values of extracts, berberine and control drug and $P<0.05$ was considered as significant.

Results

Inhibitory effects against promastigote forms

In the evaluation of various extracts of *B. vulgaris* and also berberine against promastigote forms of *L.tropica* and *L.infantum*, both extracts and especially berberine significantly ($P<0.05$) inhibited the growth of promastigotes in a dose-dependent manner. Whereas, berberine as active principle of *B. vulgaris* showed more potent anti-promastigote activity than methanolic or aqueous extracts. However methanolic extract in compare with aqueous extract showed better antileishmanial effects against promastigotes of both species. Moreover, the IC_{50} value of berberine , methanolic and aqueous extracts of *B. vulgaris* against promastigote forms of *L.infantum*

was 2.7 µg/mL, 13.2 µg/mL and 21.6 µg/mL, respectively, while the values for *L. tropica* were 2.9 µg/mL, 16.1 µg/mL and 26.6 µg/mL, respectively (Table 1).

Table 1: IC_{50} values of methanolic and aqueous extracts of *B. vulgaris* and berberine against the growth rate of promastigotes and intramacrophage amastigote forms of *Leishmania tropica* and *Leishmania infantum*. Data are expressed as the mean ± SD (n = 3)

Sample	IC_{50} value (µg/ml) [a]			
	L. infantum		*L. tropica*	
	Promastigotes	Amastigotes	Promastigotes	Amastigotes
Methanolic extract	13.2 ± 1.17	32.6±2.52	16.1 ±1.15	39.4±2.0
Aqueous extract	21.6 ± 2.08	52.8±3.08	26.6 ±2.51	59.2±3.08
Berberine	2.7 ±0.05	3.9±0.1	2.9±0.05	4.7±0.1
MA[b]	9.3 ±1.17	21.3 ±3.08	11.6 ±0.05	26.3 ±2.15

[a]: Concentration of drug that caused 50% of growth inhibition of promastigotes
[b]: Meglumine antimoniate (Glucantime) as control drug

Inhibitory effects against amastigote forms

Similar to promastigote stage the anti-amastigotes effects of various extracts of *B. vulgaris* and also berberine were based on a dose-dependent response. Berberine as main component of *B. vulgaris* exhibited much higher activity against intramacrophage amast-igotes of both species than crude extracts of *B. vulgaris*. In contrast, aqueous extract of *B. vulgaris* indicated the lowest antileishmanial activity against intra-macrophage amastigotes of both species of *Leishmania* tested. Furthermore, the IC_{50} values of methanolic and aqueous extracts of *B. vulgaris* and its active principle, berberine were 32.6 µg/mL, 52.8 µg/mL and 3.9 µg/mL for *L. infantum*, respectively. Whereas the values for *L. tropica* were 39.4 µg/mL, 59.2 µg/mL and 4.7 µg/mL, respectively (Table 1). Meanwhile, MA used as a positive control, indicated significant inhibition in the growth of the two *Leishmania* species examined.

Inhibition of infection

Infectivity is one of the most important pathogenic and biological criteria of *Leishmania* parasites. In this survey the effects of various extracts of *B. vulgaris* and also berberine on the infectivity of promastigotes of both species of *Leishmania* to murine macrophage cells were examined. The findings indicated that promastigotes forms of *L. infantum* and *L. tropica* with no drugs were able to infect 83.3% and 79.6% of the murine macrophages, respectively. In contrast, promastigotes forms of *L. infantum* treated with the methanolic or aqueous extracts of *B. vulgaris* and also berberine had potency to infect 39.6%, 44.3% and 17.3% of the murine macrophages, respectively (Table 2). Similarly, promastigotes forms of *L. tropica* treated with the above extracts and berberine were able to infect only 36.3%, 42.3% and 15.6% of the murine macrophages, respectively (Table 3). Our findings showed that pre-incubation of promastigote forms of *L. infantum* or *L. tropica* with crude extracts of *B. vulgaris* and especially its active component, berberine significantly ($P<0.05$) inhibited their invasion to macrophage cells.

Cytotoxicity effects

The crude extracts of *B. vulgaris* showed no significant cytotoxicity on murine macrophage cells, while the berberine as active constituent displayed a more cytotoxicity effect at high concentrations ≥25 µg/mL on murine macrophages. The CC_{50} value for different extracts of *B. vulgaris* and also berberine against murine macrophage cells and the selectivity index (SI) are shown in Table 4. The SI for berebrine, methanolic and aqueous extracts of *B. vulgaris* are also shown in Table 4.

Table 2: Inhibition of the infection in murine macrophages after treatment of *Leishmania infantum* promastigotes with the methanolic and aqueous extracts of *B. vulgaris* and berberine. Data are expressed as the mean ± SD (n = 3)

Sample	Percentage of infected macrophages by non-treated promastigotes	Percentage of infected macrophages by treated promastigotes	Percentage of Infectiveness Reduction
Methanolic extract	83.3± 3.51	39.6±2.15	52.5±2.52
Aqueous extract	83.3± 3.51	44.3±2.52	48±2.08
Berberine	83.3± 3.51	17.3±1.17	79.2± 3.08

Table 3: Inhibition of the infection in murine macrophages after treatment of *Leishmania tropica* promastigotes with the methanolic and aqueous extracts of *B. vulgaris* and berberine. Data are expressed as the mean ± SD (n = 3)

Sample	Percentage of infected macrophages by non-treated promastigotes	Percentage of infected macrophages by treated promastigotes	Percentage of infectiveness Reduction
Methanolic extract	79.6± 3.08	36.3±2.15	54.4±2.52
Aqueous extract	79.6± 3.08	42.3±2.52	46.9±2.52
Berberine	79.6± 3.08	15.6±1.17	80.4±3.08

Table 4: CC_{50} values of methanolic and aqueous extracts of *B. vulgaris* and berberine on murine macrophage cells and selectivity index (SI) against promastigote forms of *L. infantum* and *L. tropica*. Data are expressed as the mean ± SD (n = 3)

Plant extracts	CC_{50}[a] ± SD (µg/ml)	SI [b]	
		L. infantum	*L. tropica*
Methanolic extract	203.3±3.08	15.4	12.6
Aqueous extract	362.6 ±4.6	16.8	13.6
Berberine	27.3±2.08	10.1	9.4

[a]: Concentration of extracts that caused 50% mortality in BALB/c mice peritoneal macrophages.
[b]: Selectivity index (CC_{50}/IC_{50})

Discussion

The results of the present study revealed an *in vitro* inhibition of the growth of promastigote and amastigote stages of *L. tropica* and *L. infantum* by the methanolic and aqueous extracts of *B. vulgaris* and its main constituent, berberine. Natural products due to having less side effects, low cost and high availability are potential sources of new and selective agents for the treatment of a wide range of diseases, such as leishmaniasis (11). Advent of the industrial and synthetic antimicrobials agents in the last century led to lack of interest in plants as a natural and valuable source for antimicrobial drugs (23). In the recent decades, with the emergence of some limitations in the use of these drugs the situation has changed and the field of ethnobotanical research has expanded (24). At persent, the standard drugs for the treatment of leishmaniasis are pentavalent antimonials including meglumine antimoniate (3).

The use of these drugs are limited due to high cost, toxicity, long term treatment and the emergence of drug resistance (7, 25-26). These reasons indicate urgent need for the development of new effective and safe antileishmanial drugs from natural resources. In the present study, results of the optical density (OD) and consequently IC_{50} values for promastigote stage showed that various extracts of *B. vulgaris*, especially its main constituent, berberine significantly inhibited the growth of promastigote forms of both species. This activity is probably due to some morphological and biochemical changes as previously demonstrated in other pathogenic protozoa, elsewhere (19). Moreover, the aforementioned extracts and berberine were toxic to intramacrophage amastigotes and significantly reduced the mean infection rate and subsequently the viability of amastigotes in the murine macrophages. Our results demonstrated that promastigote forms were more susceptible to *B. vulgaris* and berberine than amastigote forms. This difference in susceptibility of promastigote and amastigote stages against various extracts of *B. vulgaris* and berberine might be related to structural, biochemical and morphological features as previously shown by other researchers (20).

As mentioned, in the previous studies antibacterial and antifungal activities of *B. vulgaris* and also its main constituent, berberine against several pathogenic strains have been demonstrated. However, in the other studies high antiparasitic potential of *B. vulgaris* and its main component, berberine against some pathogenic parasite strains have been shown. Fata et al. (27) has demonstrated that the ethanolic extract of *B. vulgaris* significantly decreased the ulcer size of ZCL in BALB/c mice after 2 weeks. In addition, the study was conducted by Kaneda et al. (19) showed that berberine significantly reduced the growth of *Entamoeba histolytica, Giardia lamblia* and *Trichomonas vaginalis* on *in vitro* experiments, and caused morphological changes in their structure. Besides, Sheng et al. (28) reported that in chloroquine resistant malaria, the combination of berberine and pyrimethamine indicated a synergistic effect in the elimination of parasites and it was more effective than other drugs such as tetracycline or cotrimoxazole. In the case of antileshmanial effects of *B. vulgaris* and its main constituent, berberine, Vennerstrom et al. (18) showed that berberine derivatives significantly suppressed the parasite load in liver or ulcer size in golden hamsters infected with *L. donovani* and *L. braziliensis,* when compared with meglumine antimoniate. The present findings are consistent with those reported by others. In this investigation, we exhibited that various extracts of *B.vulgaris* showed no significant cytotoxicity effect at low concentrations in the murine macrophage cells, while berberine indicated moderate cytotoxicity effects on these cells. Similar to these findings, Peychev (29) reported that the administration of *B.vulgaris* as oral is moderately toxic in mice (LD50 = 2.6 ±0.22 g/kg b.w. in mice). In contrast, Lin et al. (30) showed that berberine has a strong inhibition on the proliferation of both hepatoma and leukemia cell lines on *in vitro* experiments. However, it has been proven that berberine is not considered toxic at the doses used in clinical situations, nor has it been shown to be cytotoxic or mutagenic, whereas, its side-effects can result from high dosages (12). In addition, $SI_s \geq 10$ of extracts showed their safety to the macrophages and specificity to the parasite according to Weninger et al. (22). Thus, we offer that the *B.vulgaris* extracts are safe for mammalian cells, considering that at high concentrations showed significant cytotoxicity in the host cells.

Conclusion

B. vulgaris and particularly its main component, berberine exhibited potent antileishmanial activity against *L. tropica* and *L.infantum* species on *in vitro* model. In addition, further clinical studies are required to evaluate exact effect of *B. vulgaris* on other *Leishmania* species in animal models as well as the volunteer hu-

mans as a new therapeutic agent against leishmaniasis.

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