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Original papers

Antileishmanial and antibacterial activities of the hydroalcoholic extract of *Rhus coriaria* L.

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ABSTRACT. Leishmaniosis is one of the most serious public health concern with a worldwide distribution. Since the current treatments of leishmaniosis are toxic and expensive, frequent studies have been conducted to investigate the benefits of new resources such as medicinal plants for treatment of this infectious disease. Recent studies revealed the antiparasitic potential of *Rhus coriaria*. Here we investigated the potential antileishmanial and antibacterial activities of the hydroalcoholic extract of *R. coriaria* fruits. The fruits were extracted using 80% methanol by maceration method. The concentrations of 312, 156, 78, and 37 µg/ml of the extract were added separately to the wells containing *Leishmania major* (*L. major*) promastigotes and amastigotes. Amphotericin B was considered as positive control. Finally, the death rate was determined for the extract-treated parasites as compared to the non-treated parasite. The antibacterial activity was evaluated by measurement of the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the extract against a set of Gram-positive and Gram-negative bacteria. The extract significantly inhibited the growth of both promastigotes (60,7%) and amastigotes (59%) at the concentration of 312 µg/ml with the IC_{50} values of 147 µg/ml and 233 µg/ml, respectively. The extract showed bactericidal effects against *Staphylococcus aureus*, *Enterococcus faecalis*, *Pseudomonas aeruginosa*, and *Acinetobacter baumannii*. Totally, Gram-positive bacteria were more susceptible to the extract. Our findings show that the hydroalcoholic extract of *R. coriaria* fruits are rich in tannins and can be considered for further in vivo studies on the antileishmanial and antibacterial activities especially on dermal lesions caused by *L. major*.

Keywords: leishmaniosis, *Rhus coriaria*, antibacterial agents, *Leishmania major*, tannin

Introduction

Leishmania parasite is an obligate intracellular parasitic protozoan that is transmitted by the bites of infected female sandflies. It is distributed in North Africa, Middle East and Central Asia countries. The *Leishmania* contains more than 20 species which cause disease in both humans and animals. Leishmaniosis display several syndromes such as chronic cutaneous leishmaniosis (CL) to mucocutaneous (MCL) and visceral leishmaniosis (VL) [1]. There are two morphological forms of parasite in its life cycle; amastigote in macrophages of the mammalian host and promastigote in the gut of the sand fly vectors. Promastigote is transferred to human body by the infected fly. Then the promastigotes are phagocytosed by the host's macrophages and transformed into amastigote [2]. The major clinical manifestations include fever, jaundice, dyspnea, bacterial infections (especially in CL lesions), reduced number of neutrophils and platelets [3]. Pentavalent antimonials (meglumine

antimoniate and sodium stibogluconate), ampho - tericin B, miltefosine and paromomycin or some combination regimen have been standard treatments for leishmaniosis [4]. But the available chemo-therapeutics are highly toxic, expensive and may lead to resistance due to prolonged treatment periods. A number of well-known antiparasitic drugs such as quinine and artemisinin are plant-derived products [5].Therefore, finding effective compounds from new resources such as medicinal plants for protecting humans against leishmaniosis have been considered. *Rhus coriaria* L. *(R. coriaria*) is a shrub or small tree belonging to Anacardiaceae family. The plant is distributed in temperate and tropical regions of the world, mainly in the Mediterranean bordering countries, South Europe, North Africa, Iran and Afghanistan. It is commonly called ”sumac” and originated from ”summaq” which means ”dark red” in Arabic. The red color fruits of this plant have been used as spice and also in traditional medicines for hundreds of years [6,7]. The fruits are rich in tannins and contains phenolic acids, anthocyanins, gallic acid derivatives, flavonoid glycosides and organic acids [8]. Based on recent pharmacological studies *R. coriaria* is effective for treatment of diabetes [9], hyperlipi-demia [10] and osteoarthritis [11]. It has also showed potent antimicrobial, antioxidant [12], scolicidal [13], antiapoptotic [14] and antiangio-genic effects against cancer cell lines [15]. Based on ethnobotanical studies, some *Rhus* species are used traditionally for treatment of parasitic diseases such as malaria and schistosomosis [16, 17]. The aim of this study was to investigate in vitro antileishmanial activity of the hydroalcoholic extract of *R. coriaria* fruits against *Leishmania major* (*L. major*) and to evaluate the effect of this extract on the growth inhibition of several pathogenic bacterial strains.

Materials and Methods

Plant material. Fruits of *R. coriaria* were collected from Deh Bar village, Torqabeh and Shandiz County, Khorasan-Razavi Province, Iran. A voucher specimen was deposited at the Herbarium of the Faculty of Pharmacy, Mazandaran University of Medical Sciences, Sari, Iran**.** The dried fruits were powdered and then extracted using 80% methanol by maceration method at room temperature. The extract was concentrated by a rotary evaporator at 35°C. Finally, the remaining semisolid material was freeze-dried for 24h and stored at –19°C for further use [18]. The *R. coriaria* extract (RCE) was dissolved in dimethyl sulfoxide (DMSO) (666.6 mg/ml) up to a saturation point and kept as a stock solution at –20°C until use.

Determination of total phenolic and tannin contents. The total phenolic and tannin contents of RCE were determined using Folin-Ciocalteu method [19]. Gallic acid was used as a standard and the totals were expressed as milligram gallic acid equivalents per gram of dried extract. Total tannin content was determined in the extract after precipitation of tannins in the extract solution by polyvinylpolypyrrolidone (PVPP). The solution was then centrifuged. Finally, the phenolic content of the supernatant was measured and subtracted from total phenolic content.

Parasite. Iranian strain of *L. major* promastigotes (MRHO/IR/75/ER), were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) (Gibco, Paisley, Scotland) at 24°C with frequent passages every 3 days.

Anti-promastigote assay. The tests were carried out for 72 hours in a 96-well plate. Initially, 100 µl of RPMI-1640 medium encompassing 1×10^5 promastigotes was added to each well. The RCE, at the concentrations of 312, 156, 78, and 37 µg/ml were added separately to the wells in triplicate. Different concentrations of amphotericin B, including 0.98, 1.95, 3.9, 7.8 µg/ml were evaluated as positive control. It should be stated that the final volume of the DMSO in each well did not exceed 0.1%. The plate was then incubated at 24°C for 72 hours. Afterwards, 20 µl of each well was mixed with 20 µl of 2% formaldehyde solution in phosphate-buffered saline (PBS; pH: 7.2) and the number of promastigotes/mg/ml was calculated using a hemocytometer under light microscope [20].

Anti-amastigote assay. The tests were carried out for 72 hours using glass chamber slides. The macrophage cell line J774A.1 was cultured by seeding 2×10^5 cells to each well in a volume of 200 µl RPMI-1640 medium. The slides were incubated at 37°C, 5% CO_2, for 3 hours. Subsequently, the supernatants were discarded, and 200 µl of RPMI-1640 medium containing 10 stationary phase promastigotes was added to the wells for each cell (10:1). After 24 hours, the supernatants were discarded and the wells were washed gently with RPMI-1640 medium to remove free promastigotes. After washing, 200 µl of RPMI-1640 medium was added to each well. Then, 10 µl of RCE was added to the wells in triplicate at concentrations of 312, 156,

Table 1. Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of RCE against different organisms

Organism used	RCE (µg/ml)			MIC (µg/ml) of the isolates against ciprofloxacin
	MIC_{50}	MIC_{90}	$MBC_{99.9}$	
Staphylococcus aureus ATCC 25923	128	256	1024	0.25
Enterococcus faecalis ATCC 29212	128	256	1024	0.25
Pseudomonas aeruginosa ATCC 27853	512	1024	4096	0.5
Acinetobacter baumannii ATCC 19606	512	1024	4096	0.5
Escherichia coli ATCC 25922	512	1024	> 4096	0.5
Klebsiella pneumoniae ATCC 700603	512	1024	> 4096	0.5
Proteus mirabilis ATCC 14153	512	1024	> 4096	0.5
Serratia marcescens ATCC 14756	1024	2048	> 4096	0.5

Explanations: MIC_{50}, minimum inhibitory concentration essential to kill visually 50% of the bacteria after 24 h of incubation; MIC_{90}, minimum inhibitory concentration required to kill visually 90% of the bacteria after 24 h of incubation; $MBC_{99.9}$, minimum bactericidal concentration obligatory to kill 99.9% of the bacteria (recorded by spot inoculation on blood agar from the wells showing no growth after 24 h).

78, and 37 µg/ml. The slides were incubated at 37°C, 5% CO_2, for 72 hours. The DMSO concentration in the final volume of each well did not exceed 0.1%. For amphotericin B, different concentrations of 0.98, 1.95, 3.9, 7.8 µg/ml were used. The slides are then incubated at 37°C, 5% CO_2, for 72 hours. After incubation, the medium in each chamber was discarded and the amastigote-infected cells were stained with 20% Giemsa stain, fixed with absolute methanol, and examined under light microscope. The number of infected macrophage and also the number of amastigotes was counted for every 100 cells. Three wells were considered as negative control to which the drug was not added. To determine the percentage of amastigotes death, the number of amastigotes per 100 microphages per dilution of the sumac divided by the number of amastigotes in negative control and multiplied by 100.

Maximal LC_{50}. Inhibitory concentration of 50% (LC_{50}) for RCE was calculated for promastigote or amastigote according to the following formula: MI = Min + [˝(Max - Min)]

Where ML = maximal IC_{50}, Min = minimal death rate (%), Max = maximal death rate (%). The MI represents the death rate (%) on the Y-axis, based on which the medication value is obtained on the X-axis as IC_{50}.

Cytotoxicity concentration and selectivity index. Cytotoxicity concentration of 50% (CC_{50}) was performed similar to what was mentioned for IC_{50}, with this difference that the extract effect in different dilutions was evaluated on J774A.1 macrophages without amastigote and finally the concentration of each medication in which 50% of the macrophages were died was considered as CC_{50}. In addition, selectivity index (SI) was calculated by dividing CC_{50} by IC_{50} of amastigotes [21]. The SI > 10 represents the safety of the medication.

Data analysis. Data were analyzed by chi-square (χ^2) statistical test and two-tailed t-test using IBM SPSS v20 software.

Antibacterial assay. The double dilution technique (micro broth dilution) was performed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the RCE against some clinically important Gram-positive and Gram-negative bacteria according to the clinical and laboratory standards institute (CLSI) guidelines [22]. A final cell density of 1×10^5 CFU/ml of test strains including *Staphylococcus aureus* ATCC 25923, *Enterococcus faecalis* ATCC 29212, *Pseudomonas aeruginosa* ATCC 27853, *Acinetobacter baumannii* ATCC 19606, *Escherichia coli* ATCC 25922, *Klebsiella pneumoniae* ATCC 700603, *Proteus mirabilis* ATCC 14153 and *Serratia marcescens* ATCC 14756 was added to 96-well micro titer plates containing 100 µl Muller Hinton broth (Merck, Germany) and 100 µl RCE (in the concentration range of 0.25–4096 µg/ml) and

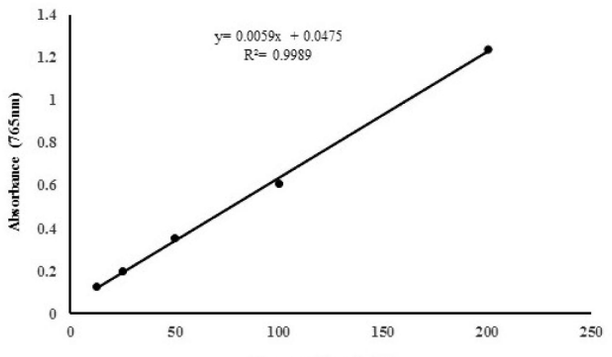

Figure 1. The calibration curve of Gallic acid

then incubated at 37°C for 24 h. The lowest concentration of RCE which has inhibited the visually growth of the bacteria was considered as MIC. Ciprofloxacin was used as a control antibiotic to compare with the RCE. Then, 20 µl of the contents of the MIC well and previous wells were cultured on blood agar and incubated at 37°C for 24 h and the MBC of RCE was documented as the lowest concentration of the extract that exhibited no growth on agar plates. Un-inoculated medium and medium without RCE were used as negative and positive controls, respectively, and all assays were performed in triplicate.

Results

Total phenolics and tannin contents

The total phenolic and tannin contents of the extract were calculated 766.45 ± 6.01 and 625.75 ± 10.82 mg gallic acid equivalents per gram of dried extract using the following calibration curve (Fig.1).

The RCE-treated promastigotes

The highest death rate was found at 312 µg/ml (60.7%) and the lowest death rate was 37 (31.82%) µg/ml. The lowest death rate for amphotericin B was found in the concentration of 0.98 µg/ml (38.4%) and the highest death rate was at 7.8 µg/ml (73.8%). There was a statistically significant difference between concentrations for sumac and amphotericin B ($P > 0.05$). IC_{50} for amphotericin B was 1.73 µg/ml and for sumac was 147 µg/ml (Fig. 2).

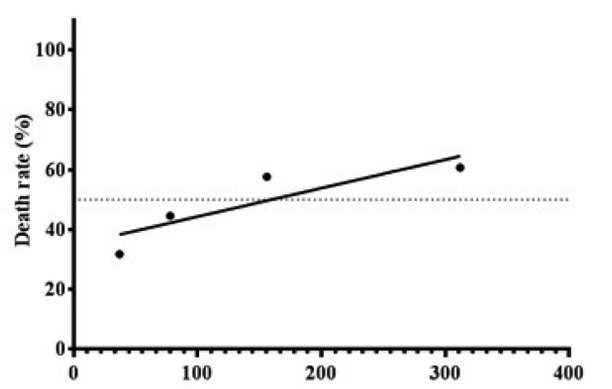

Figure 2. The RCE-treated promastigotes with different concentrations

The RCE-treated amastigotes

The highest death rate for amastigote was in 312 µg/ml (59%) and the lowest death rate was for 37 µg/ml (15%). The lowest death rate for amphotericin B was at 0.98 µg/ml (41.8%) and the highest death rate was at 7.8 µg/ml (78%). There was a statistically significant difference between different concentrations for sumac and amphotericin B ($P > 0.05$). IC_{50} was also 1.1 µg/ml for amphotericin B and 233 µg/ml for sumac (Fig. 3).

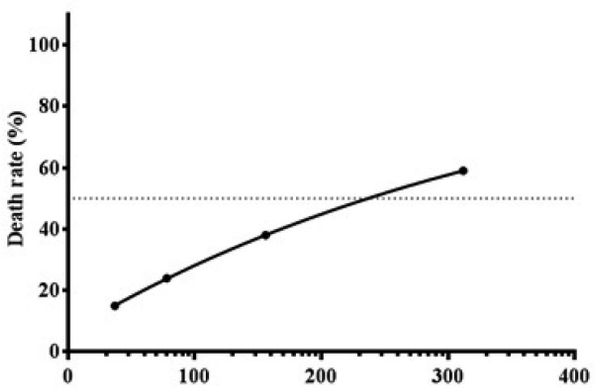

Figure 3. The RCE-treated amastigotes with different concentrations

Cytotoxicity test and selectivity index

The CC_{50} and SI values for amphotericin B were 21 µg/ml and 19.09 µg/ml, respectively. The CC_{50} of the extract was 2583 with SI of 11.08 µg/ml.

Antibacterial activity

The results of the antimicrobial activity of the extract are shown in Table 1. The extract showed better inhibitory effect against Gram-positive bacteria than Gram-negative ones. The MICs were ranged from 256–2048 µg/ml for tested bacteria, while the MBCs of the extract against them were ranged from 1024–>4096 µg/ml. Also, the extract had a bactericidal effect against *S. aureus*, *E. faecalis*, *P. aeruginosa*, and *A. baumannii*, but was found to be bacteriostatic even at the highest

concentration against other bacteria in this test. These results show that the plants extract have a potent antimicrobial activity at moderate concentrations.

Discussion

In the present study we investigated the inhibitory effects of the hydroalcoholic extract of *R. coriaria* fruits on the promastigotes and amastigotes of *L. major*. The extract significantly increased the death rate about (60%) of both promastigotes and amastigotes forms of parasite in a dose dependent manner and also showed reasonable SI (11.08) compared with the reference drug, amphotericin B (19.09). Moazeni et al. [13] reported that *R. coriaria* methanolic extract have inhibitory effect on the protoscolices of *Echinococcus granulosus*. The concentration of 50 mg/ml of the fruit extract caused 100% scolicidal effect after 10 min of exposure.

There are some reports in the literature on the antiparasitic activities of other *Rhus* species. *Rhus natalensis*, one of the medicinal plants used by the traditional healers in Kenya for treatment of malaria, showed remarkable antiplasmodial activity. The methanol extract of the leaves was moderately active against two strains of *Plasmodium falciparum* in vitro and showed the high percent parasite clearance and chemo suppression of 82% in a mouse model [17].

Naz et al. [23] evaluated the antileishmanial activity of *R. punjabensis* by MTT cytotoxic assay. The aqueous extract of the plant and hematite (Fe_2O_3) nanoparticles, synthesized using the extract, exhibited inhibitory effects against *Leishmania* amastigotes. The calculated IC_{50} value was 101µg/ml for the extract.

Based on a screening of plant extracts for antiprotozoal activity, the methanolic extract of the leaves and bark and also the methanolic and aqueous extracts of the seeds of *R. aucheri* showed the IC_{50} values of 55.85, 171, 141.87 and 323.59 µg/ml, respectively, against *Leishmania donovani* (*L. donovani*) promastigotes [24].

In our study, the spectrophotometric measurement showed the high total tannin content in the extract (625.75 ± 10.82 mg gallic acid equivalents per gram of dried extract). Recently there have been considerable attentions to the antiprotozoal properties of tannins, especially in livestock. In a research, the antileishmanial activity of several types of tannins was tested on infected cells (RAW 264.7) with *L. donovani* and *L. major*. Many of the tested polyphenols significantly reduced the survival of *Leishmania* amastigotes due to macrophage activation [25]. Phenolic compounds such as phenolic acids and tannins are one of the main constituents in the *Rhus* species [26]. Several studies have reported either direct or indirect effects of tannins from plants on intestinal parasites of some livestock such as sheep. Tannins may bind to external and internal proteins of parasites or indirectly enhance the immune responses [27,28].

Bacterial superinfection appears to be very common in cutaneous leishmaniosis. Strains of *S. aureus*, *P. aeruginosa* and *Enterobacter* sp. are usually localized in such lesions [29]. In our study, the extract exhibited considerable antibacterial activity against both Gram-positive and Gram-negative strains which the most susceptible bacteria were *S. aureus* and *E. faecalis*. Mahdavi et al. [12] investigated the antibacterial effect of *R. coriaria* fruit ethanolic extract against some gram positive and gram negative bacteria by micro dilution method. Based on the reported results, the extract showed strong antimicrobial activity. In addition, *Staphylococcus aureus* and *Salmonella enterica* were reported to be the most sensitive bacteria with a MIC of <0.78%. It was reported that the potential antibacterial effects of *R. coriaria* is due to chemical compounds such as phenolic derivatives [12,30].

In conclusion, the hydroalcoholic extract of *R. coriaria* fruits has considerable in vitro antileishmanial and antibacterial activities which may be related to phenolic compounds such as tannins. As a whole, it seems that the extract can be considered for further in vivo studies on antileishmanial activities especially on dermal lesions.

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