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Ficus carica hairy roots: *In vitro* anti-leishmanial activity against *Leishmania major* promastigotes and amastigotes

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ABSTRACT

Objective: To investigate the biochemical capacity, and *in vitro* inhibitory effects of hairy roots from two cultivars of *Ficus carica* L. (Sabz and Siah) on *Leishmania major* promastigotes and amastigotes.

Methods: In the hairy roots, the activity of antioxidant enzymes compared to normal leaves and roots, and the presence of some phenolic compounds in comparison with fruits were investigated. The IC_{50} values of hairy roots in promastigotes was determined by tetrazolium-dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and trypan blue assays. By calculating the infectivity index of peripheral blood mononuclear cells (PBMCs), the leishmanicidal activity (IC_{50} values) of hairy roots for amastigotes was estimated. The effects of hairy roots (IC_{50} values) treatment on the levels of *IFN-γ* and *iNOS* expression, intracellular reactive oxygen species, and iNOS protein expression in infected-PBMCs were determined.

Results: Based on antioxidant enzyme assays and high performance liquid chromatography analysis, hairy roots exhibited high antioxidant capacity and contained high levels of phenolic compounds. According to the results of tetrazolium-dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide and trypan blue assays, the hairy root extracts of both cultivars showed considerable dose-dependent inhibitory effects against *Leishmania major* promastigotes. Depending on the concentration and exposure time, treatment of infected-PBMCs with hairy root extracts caused the generation of a significant reactive oxygen species, up-regulation of *IFN-γ* and *iNOS* genes expression, and high value of iNOS protein compared to controls.

Conclusions: The findings of this study suggest that the hairy roots of *Ficus carica* can be considered as a promising natural source of antileishmanial agents.

KEYWORDS: Antileishmanial agents; Hairy roots, IFN-γ; Inducible nitric oxide synthase; Parasite; Reactive oxygen species

1. Introduction

Leishmaniasis is one of the life-threatening neglected tropical diseases reported from over 98 countries[1]. This infectious disease is predominantly induced by various species of the genus *Leishmania* and manifested in forms of cutaneous (CL), mucocutaneuous (MCL), and visceral (VL) leishmaniasis[2]. CL is the most rampant form of leishmaniasis caused by different species of *Leishmania* (*L.*) including *L. major*, *L. tropica*, *L. donovani*, *L. aethiopica*, *L. infantum*, with an approximate prevalence rate of 0.7-1.2 million new cases per

Significance

Antioxidant enzymes activity and antileishmanial ability of hairy roots, induced from two cultivars of *Ficus carica* L. (Sabz and Siah) by *Agrobacterium rhizogenes*, are presented in this paper. The findings of this study suggest that the hairy roots of *Ficus carica* can be considered as a promising natural source of antileishmanial agents.

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year and the incidence rate of 70%-75% in 10 countries, including Afghanistan, Algeria, Colombia, Brazil, Iran, Syria, Ethiopia, South Sudan, Costa Rica, and Peru[1,3]. Zoonotic CL is a serious health problem in Iran[4]. *Leishmania* parasites are transmitted to the host skin in forms of promastigote (flagellated) through the bite of female phlebotomine sand flies (vectors), and following the phagocytosis by phagocytic cells (neutrophils, monocytes, and macrophages), these parasites transform into amastigote forms (aflagellated) and infect other monocytes and macrophages to survive and replicate themselves[5,6]. The infection activates defense mechanism of the host, thereby enhancing the production of different cytokines, especially interleukin 12 (IL-12), interferon-γ (IFN-γ), and tumor necrosis factor-α (TNF-α), and also free radical molecules, including reactive oxygen species (ROS) and nitric oxide (NO)[6,7]. NO is the main effector molecule and its quantification can be done through the analysis of NO production in the supernatant of macrophages (directly) or by the expression analysis of inducible NO synthase (*iNOS*) gene in macrophages (indirectly) [8]. Establishment and proliferation of parasite within host cells depend highly on its ability to overcome host immune response. To achieve this purpose, parasite uses different mechanisms, including escape from apoptotic neutrophils before infecting the macrophages, shift in the signaling network, interference of phagosome maturation in macrophages and molecules, and change in the gene expression and metabolic processes[9,10]. Due to the lack of a leishmaniasis vaccine for this disease, chemotherapeutic drugs, such as sodium stibogluconate (Pentostam®), antimoniate *N*-methyl-glucamine (Glucantime®), meglumine antimoniate, amphotericin B or pentamidines, paromomycin, and oral miltefosine, are still applied for the treatment[11]. These chemical drugs cause many problems, such as toxicity, severe side effects, and drug resistance[11,12]. Therefore, the preference of patients to use herbal drug compounds has motivated researches to develop the herbal antileishmanial agents[12].

Ficus (*F.*) *carica*, is the most important and the only fruit species of *Ficus* genus among 800 species and belongs to the Moraceae family. This plant is one of the oldest cultivated species in the world and native to the Eastern Mediterranean and Southwest Asia[13]. Because of the high pharmaceutical values and nutritional quality, *F. carica* has tremendous value in traditional medicine, pharmaceuticals, and food industries. Numerous biological activities, including antioxidative, anti-inflammatory, antimicrobial, antifungal, antitumor, anticancer, immunomodulatory, antipyretic, antibacterial, anthelmintic and cytotoxic activities[13–16], have been reported from *F. carica* and attributed to the presence of monoterpenoids, sesquiterpenes, coumarins, sterols, flavonoids, and other polyphenolic compounds[13,17–19]. The secondary metabolites are found in all parts of this plant, but the richest source of these compounds is *Agrobacterium (A.) rhizogenes*-mediated hairy roots cultures[20]. The main purpose of the present study was to investigate the antileishmanial activity of HRs extracts from two cultivars of *F. carica*, Sabz and Siah, for the first time. Initially, we have successfully cultured the *A. rhizogenes*-induced hairy roots of *F. carica* based on our efficient protocol in previous study[20]. We compared antioxidant enzymes activity and some phenolic compounds content of HRs with other parts of *F. carica*. Next, we investigated the anti-promastigote activity of methanol extract of induced HRs compared with leaf, fruit and root extracts. Finally, after determination of IC_{50} values of HRs, their effects on the infectivity index, expression level of *IFN–γ* and *iNOS* genes, intracellular ROS level, and iNOS protein level in peripheral blood mononuclear cells (PBMCs) were evaluated.

2. Materials and methods

2.1. Plant material and extraction

The mature seeds and fruits of Sabz and Siah Estahban Smyrna-type cultivars of *F. carica* L. (Flora Iran-identified)[21] were provided by Zarringiah Co., West Azerbaijan Province, Iran. Voucher specimens (voucher numbers: FC1/15 11753, and FC2/15 11735) were deposited in the Urmia University herbarium (Urmia, Iran). After seed washing and sterilization with ethanol (70%) and sodium hypochlorite (2.5%), the *in vitro* culture of seeds was cultured under aseptic conditions. To induce HR formation, a few axenic seedlings (five weeks old) were inoculated with *A. rhizogenes* strains, ATCC 15834 for Sabz and A7 for Siah cultivars[20]. Samples, including leaves, roots, induced HRs (derived from *in vitro* plants), and fruits, were dried at (25±2) ℃ for 7-10 days. Next, the dried powder samples were extracted by methanol maceration method[22]. The methanol solvent was rotary evaporated under vacuum at 30 ℃, and obtained extracts were stored at 4 ℃ until assays.

2.2. Antioxidant enzymes

Ascorbate peroxidase (APX), catalase (CAT), and guaiacol peroxidase (GPx) activities of leaf, root, and HRs of *F. carica* (Sabz and Siah cultivars) were estimated using the following procedure. In detail, fresh leaf, root, and HRs (50 mg each) were grounded to powder form in a cold mortar using liquid nitrogen, homogenized in 2 mL of 50 mM Tris-HCl (pH 8) containing 0.4% polyvinylpyrrolidone, and centrifuged (13 400×*g*, 4 ℃, 15 min). Subsequently, the supernatant was used for the enzymes activity assays. The freshly prepared extracts were used for enzymatic assays and ice-cold conditions were maintained throughout the

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procedure. CAT enzyme activity was assessed by measuring the reduction of H_2O_2 in the reaction mixture containing the enzyme extract (20 μL), 50 mM potassium phosphate buffer (2.5 mL, pH 7), and 3% H_2O_2 (20 μL). The absorbance was measured at every 10 s for 1 min at 240 nm using a HALO DB-20 UV-VIS Double Beam Spectrophotometer (Dynamic Co., UK). APX enzyme activity was quantified by recording the absorbance of reaction mixture composed of enzyme extract (20 μL), extraction buffer (50 μL), 50 mM potassium phosphate buffer (2 mL, pH 7), 50 mM ascorbate (10 μL), and 5 mM H_2O_2 (20 μL) at 290 nm for 1 min. The reaction mixture for GPx enzyme activity contained enzyme extract (150 μL), 10 mM potassium phosphate buffer (3 mL, pH 7), pure guiacol (50 μL), and 3% H_2O_2 (50 μL). The absorbance (436 nm) was read at every 15 s for 1 min. The specific enzymes activity was determined by the following formula using extinction coefficients value of 2.6, 43.6 and 25.5 for APX, CAT, and GPx, respectively:

Specific enzyme activity for 1 min, U/mg protein=Δabsorbance value×total volume of reaction mixture/volume of enzyme extract ×ε (specific extinction coefficient for enzyme)×min×g (weight of sample for extraction).

2.3. High performance liquid chromatography (HPLC) analysis

Identification, and quantification of some phenolic compounds (gallic acid, caffeic acid, chlorogenic acid, rutin, ϱ-coumaric acid, rosmarinic acid, quercetin, cinnamic acid, and apigenin) from methanol extracts from HRs and fruits of two *F. carica* cultivars (Sabz and Siah) was performed using a HPLC machine (Agilent 1100 Series HPLC Value System of Agilent Technologies Co., USA) as described in previous study[20]. The HPLC system was equipped with a 20 μL injection loop, four solvent delivery systems, a quaternary pump, a gas stripping system, a column oven (set at 25 ℃), and a diode array detector, set at 250, 272, and 310 nm. The separation was performed by a gradient elution of acetonitrile to acetic acid onto an octadecylsilane analytical column (25 cm long, 4.6 mm diameter, and 5 μm particle size; ZORBAX Eclipse XDB). ChemStation software was employed to process the data.

2.4. Parasite cultivation

Promastigotes of *L. major* (MRHO/IR/75/ER, Iranian strain) was supplied from Department of Medical Parasitology and Mycology, Urmia University of Medical Sciences, West Azerbaijan Province, Iran. Promastigotes were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum, 100 U/mL of penicillin, 100 μg/mL of streptomycin, and 2 mM of *L*-glutamine and incubated on a shaker incubator (40×*g*) at (23±1) ℃. To maintain in logarithmic-phase growth, the cells were subcultured every week. The parasite counting was performed using a haemocytometer under a light microscope.

2.5. Tetrazolium-dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay

The toxicity of various *F. carica* extracts against *L. major* promastigotes was measured by MTT assay according to a little modified version of the method described by Shokri *et al*[23]. The *L. major* promastigotes at exponential growth phase (1×10^6 parasites/mL) were seeded in 1.5 mL tubes and incubated at 25 ℃ for 24 and 72 h after treatment with Glucantime (10 μg/mL, positive control) and different concentrations [5 000, 2 500, 1 750, 500, 250, 50, 25, 12.5, 2.5 and 0 μg/mL phosphate-buffered saline (PBS) 1×] of leaf, root, HR and fruit extracts obtained from Sabz and Siah cultivars of *F. carica*. After incubation, 100 μL of each treatment was transferred to the 96-well plates and 10 μL (10% of final volume) MTT solution (5 mg/mL) was added to each well. The plates were incubated at the growth condition in darkness for 4 h. Next, 100 μL DMSO was added to dissolve the formazan crystals (resulted from MTT reduction), and the absorbance at 545 nm was read by the enzyme-linked immunosorbent assay (ELISA) plate reader (Stat Fax 2100 ELISA Plate Reader, Awareness Technology, USA). The promastigote-free medium was read as a blank sample. The results of MTT assay were expressed as the percentage of inhibitory effect, calculated using the following formula:

$$\%\ \text{Inhibition} = [(OD_{control}-OD_{blank})-(OD_{sample}-OD_{blank})]/(OD_{control}-OD_{blank})\times100.$$

2.6. Trypan blue viability assay

Promastigote viability index was determined using trypan blue staining assay. Promastigotes (1×10^6 parasites/mL) were seeded in 96 well plates. After treatments with Sabz and Siah HR extracts, the promastigotes were exposed to trypan blue solution (1:1, trypan blue/sample volume) and the percentage of viable promastigotes (unstained) was measured by Neubauer hemocytometer.

2.7. Calculation of 50% inhibitory concentration (IC_{50})

The calculation of IC_{50} extracts was carried out based on the logarithm regression analysis of dose-response data in MTT assay. The IC_{50} values of HRs were verified using logarithm regression analysis of dose-response data in trypan blue assay.

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2.8. PBMCs isolation

From healthy donors, peripheral blood (50 mL) was collected in heparin tubes and diluted (1:1) using PBS, pH 7.2. The obtained mixture was added gently to ficol in a portion of 3:1 (v/v) and centrifuged (400×*g*) at 25 ℃ for 30 min. After the formation of the three phases above blood, the milky phase containing PBMCs between the plasma and Ficol phases was carefully transferred to another falcon tube, washed twice with cold PBS (centrifugation at 400×*g*, 4 ℃, 5 min), and re-suspended in 3 mL of RPMI 1640 medium (10% fetal bovine serum, 100 U/mL of penicillin, and 100 μg/mL of streptomycin). Viability of PBMCs was investigated, and their number was counted by a homocytometer.

2.9. Parasite infection of PBMCs

Adherent PBMCs were cultured in six-well plates (1×10^6 cells/well). To infect the cells, promastigotes in the stationary growth phase were added to each well (10:1 parasite:cell). After incubating in 5% CO_2 at 37 ℃ for 24 h, free parasites were removed by washing cells three times with PBS.

2.10. Infectivity index of infected–PBMCs

The infected cells were treated with the HRs extracts (25 and 50 μg/mL, 72 h-IC_{50} values) for 6, 12, and 24 h. Untreated infected cells were used as the positive controls. Infectivity index of PBMCs (percentage of infected PBMCs×average number of amastigotes per infected PBMC) was calculated at treatment time points of 6 h and 24 h by randomly counting 200 PBMCs in Giemsa-stained smears by an optical microscope (Nikon Eclipse 80i, Japan).

2.11. Total RNA extraction and reverse transcription

Total RNA from the non-infected-PBMCs and infected-PBMCs (untreated and treated with 25 and 50 μg/L HRs (IC_{50} values) extracts for 6, 12, and 24 h) was isolated using Trizol solution (Invitrogen, Germany) following the manufacturer's recommendations. Purity and quality of RNA were checked by agarose gel electrophoresis ethidium bromide staining. First strand cDNA was synthesized with 1 μg of total RNA using cDNA Synthesis Kit (Thermo Fisher Scientific RevertAid First Strand cDNA Synthesis Kit, USA) according to the protocol provided by manufacturer.

2.12. Quantitative real–time PCR

The quantitative real-time PCR assays were performed to analyze the expressions of *IFN*–γ and *iNOS* genes, in a real-time Mic PCR biomolecular system using gene-specific primers. Homo sapiens β-actin gene was used as the housekeeping reference gene for the normalization of mRNA levels. The specific primers of target genes are listed in Supplementary Table 1. Reactions were conducted using SYBR Green qPCR Master Mix (Thermo Scientific, USA) *via* a three-step real-time PCR program as follows: hold at 95 ℃ for 10-15 min, 40 cycles of (a) 95 ℃ for 20 s, (b) 69 ℃ (β-actin and IFN-γ)/75 ℃ (iNOS) for 10 s [Touch down: -1 ℃ per 1 cycle (s), 10 times], and (c) 72 ℃ for 10 s. The real time-PCR products were confirmed by the electrophoresis of ethidium bromid-stained agarose gels. The level of gene expression was calculated by the relative quantification ($2^{-\Delta\Delta CT}$) method.

2.13. Intracellular ROS level of PBMCs

The intracellular ROS levels of PBMCs at four conditions (1: absence of HRs treatment and absence of infection, 2: absence of HRs treatment and presence of infection, 3: presence of HRs treatment and absence of infection, and 4: presence of treatment HRs and presence of infection) were determined by using the fluorescent dye 2'7'-dichlorodihydrofluorescein diacetate (DCFDA) in accordance with the kit instructions (ROS assay kit, Kiazist Life Sciences, Iran). The non-infected and *L. major* infected-PBMCs (5×10^4 cells/well) were seeded in 24-well plates, treated with *F. carica* HRs extract (0, 25 and 50 μg/mL), and incubated at 37 ℃ for 6, 12 and 24 h. After incubation, the cells were harvested, washed with cold ROS buffer, and incubated with H2DCFDA dye for 45 min. After washing, the green dichlorofluorescein fluorescence intensity was recorded at 485 nm excitation/535 emission nm in a flow cytometer (PAS Particle Analysing System, Partec, Germany). Data were analyzed using FlowJO software, and ROS levels were expressed as geometric means and percentage of controls.

2.14. Protein expression validation of iNOS in PBMCs

The protein levels measuring of iNOS in non-infected and infected PBMCs (untreated and treated with *F. carica* HRs extracts) were carried out using ELISA kit (ZellBio GmbH assay kit, Germany) according to the manufacturer's recommendation. The OD value was read in an ELISA reader (Awareness Technology, USA) at 450 nm. Data were calculated using standard curve. The sensitivity for iNOS measurements was 0.2 ng/mL.

2.15. Statistical analysis

The values were represented as the mean (triplicate sample)±SD. The statistical analyses of the obtained data were performed using

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IBM SPSS Statistics version 22 software. Significance differences between treatments were determined by *t*-test and one-way ANOVA test followed by Duncan's multiple range tests at levels $P<0.05$ and $P<0.01$.

2.16. Ethical approval

This study was approved by the Ethics Committee of University of Mohaghegh Ardabili, Ardabil, Iran (No. 07/2020).

3. Results

3.1. Antioxidant enzyme activity

Antioxidant enzyme activity assays were performed in leaves, normal roots and hairy roots from two cultivars (Siah and Sabz) of *F. carica*. According to the results shown in Table 1, CAT, APX, and GPx antioxidant enzymes, can be significantly affected by the type of plant material. HRs derived from Siah cultivar, considerably exhibited the highest antioxidant activity of CAT (0.780±0.042) U/mg proteins, APX (20.461±8.865) U/mg proteins, and GPx (0.317±0.011) U/mg proteins in comparison to others.

Table 1. Enzyme antioxidant activity of normal parts (leaf and root) of *Ficus carica* (Sabz and Siah cultivars) compared to hairy roots (U/mg).

Samples	CAT	APX	GPx
$Sabz_{leaf}$	0.219±0.006^{c}	0.398±0.006^{b}	0.059±0.003^{e}
$Sabz_{root}$	0.093±0.004^{d}	0.371±0.022^{b}	0.119±0.004^{c}
$Sabz_{HR}$	0.158±0.003^{cd}	0.335±0.084^{b}	0.078±0.002^{d}
$Siah_{leaf}$	0.115±0.021^{d}	0.355±0.058^{b}	0.251±0.001^{b}
$Siah_{root}$	0.665±0.078^{b}	0.386±0.045^{b}	0.084±0.003^{d}
$Siah_{HR}$	0.780±0.042^{a}	20.461±8.865^{a}	0.317±0.011^{a}

The activity of antioxidant enzymes (catalase, ascorbate peroxidase, guaiacol peroxidase) was measured in leaf, root, and HRs of *Ficus carica* (Sabz and Siah cultivars). The highest antioxidant activity of enzymes was detected in Siah HR extract. CAT: Catalase, APX: Ascorbate peroxidase, GPx: Guaiacol peroxidase. Similar letters in the same column indicate no significant difference at the $P<0.05$ level according to Duncan's multiple range test.

3.2. HPLC analysis of extracts

The presence of phenolic compounds (gallic acid, caffeic acid, chlorogenic acid, rutin, ϱ-coumaric acid, rosmarinic acid, quercetin, cinnamic acid, and apigenin) in fruit and HRs from Sabz and Siah *F. carica* was determined by HPLC analysis at 272, 310, and 250 nm (Supplementary Figure 1). As represented in Table 2 (Supplementary Figure 2), Siah HR extract contained the highest amount of caffeic acid (56.2 mg/kg), chlorogenic acid (620.2 mg/kg), rutin (13.0 mg/kg), rosmarinic acid (150.2 mg/kg), and apigenin (22.5 mg/kg). Sabz HR extract showed the highest recorded content of ϱ-coumaric acid (209.2 mg/kg), quercetin (55.4 mg/kg), and cinnamic acid (3.8 mg/kg) (Table 2 and Supplementary Figure 2). Siah fruit extract contained the highest amount of gallic acid (249.2 mg/kg). The quercetin and rosmarinic acid (Table 2) were not found in fruit extracts (maybe present in form of its derivatives).

3.3. Anti–promastigote and cytotoxicity activity of F. carica

The antileishmanial activity of different parts of *F. carica* (leaf, root, fruit and HRs extracts) against promastigotes was monitored by MTT assay after 24 h and 72 h exposure to extracts. All the extracts resulted in a significant reduction of MTT, depending on the type and concentration of the extract used. Based on MTT assay results (Table 3), the highest inhibitory effects were obtained from Siah HRs [IC_{50}-24 h: (5.8±0.1) μg/mL, IC_{50}-72 h: (3.9±0.2) μg/mL], followed by Sabz HRs [IC_{50}-72 h: (12.5±0.1) μg/mL]. These results were remarkably valuable in comparison with glucantime as positive control [IC_{50}-24 h: (14.0±0.1) μg/mL, IC_{50}-72 h: (5.5±0.1) μg/mL], so the HRs extracts were selected as the best extracts for subsequent experiments.

The inhibitory effects of HR extracts were also evaluated by counting the parasites in trypan blue assay (Supplementary Figure 3). Based on trypan blue, the IC_{50} values against promastigotes were calculated as (1 750.0±2.8) μg/mL (24 h), and (50.0±1.4) μg/mL (72 h) for Sabz HRs, and (500.0±1.4) μg/mL (24 h), (25.0±2.8) μg/mL (72 h) for Siah HRs (Table 3).

In some studies, the MTT assay has been introduced as a method to quantify antioxidant activity of plant extracts[24,25], so in this study, the concentrations of 25 and 50 μg/mL, based on trypan blue assay (72 h), were elected as appropriate concentrations for anti-amastigote analyses. In order to determine the cytotoxicity activity of *F. carica*, the effect of HRs extract (25 and 50 μg/mL) against non-infected PBMCs was investigated. Fortunately, no cytotoxic effect was observed at the used concentrations on PBMCs (data not shown).

Table 2. High-performance liquid chromatography analysis of phenolic compounds from hairy roots and fruits of *Ficus carica* (sabz and siah cultivar, mg/kg).

Cultivar	Sample	Gallic	Caffeic	Chlorogenic	Rutin	ϱ-coumaric	Rosmarinic	Quercetin	Cinnamic	Apigenin
Sabz	HRs	93.0	31.7	378.8	8.0	209.2	23.6	55.4	3.8	15.2
	Fruit	100.2	3.4	4.0	2.1	7.5	-	-	0.3	1.7
Siah	HRs	120.5	56.2	620.2	13.0	55.2	150.2	49.3	3.5	22.5
	Fruit	249.2	4.2	5.2	1.9	3.7	-	-	0.3	1.9

The nine phenolic compounds including gallic acid, caffeic acid, chlorogenic acid, rutin, ϱ-coumaric acid, rosmarinic acid, quercetin, cinnamic acid, and apigenin were detected at the wavelength of 272, 250, and 310 nm. - : not detected (maybe present in form of its derivatives).

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Table 3. Inhibitory effects of different extracts from *Ficus carica* against *Leishmania major* promastigotes.

Assay	Extract	IC_{50} (μg/mL)	
		24 h	72 h
MTT	Glucantime	14.0±0.1	5.5±0.1
	$Sabz_{leaf}$	1740.0±17.0	1450.0±7.7
	$Siah_{leaf}$	1740.0±28.3	1612.5±15.9
	$Sabz_{root}$	1475.0±12.0	1262.0±9.3
	$Siah_{root}$	1785.0±21.9	532.5±11.9
	$Sabz_{fruit}$	160.0±15.5	346.0±7.9
	$Siah_{fruit}$	17.4±0.5	15.5±3.5
	$Sabz_{HRs}$	49.5±0.7	12.5±0.1
	$Siah_{HRs}$	5.8±0.1	3.9±0.2
Trypan blue	$Sabz_{HRs}$	1750.0±2.8	50.0±1.4
	$Siah_{HRs}$	500.0±1.4	25.0±2.8

IC_{50}: inhibitory concentration of 50% promastigotes based on MTT (tetrazolium-dye 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide) and trypan blue assays. In MTT assay, the highest inhibitory effects were obtained from Siah HRs followed by Sabz HRs, so the HRs extracts were selected as the best extracts for trypan blue assay, and subsequent experiments. Data are presented as the mean±SD of three experiments.

3.4. Effect of HRs on the infectivity index of infected–PBMCs

Infectivity index of infected-PBMCs was evaluated after 6 and 24 h of HR treatments. The results showed that the infectivity index of infected-PBMCs treated with HRs extracts was reduced, depending on the concentration and exposure time treatment (Table 4). The 24 h treatments resulted in the most reduction effect on infectivity index of infected-PBMCs treated with HRs. The infectivity index of infected-PBMCs decreased by 77%-82% compared to the control after 24 h treatment with Siah HRs (Table 4).

Table 4. Infectivity index of infected peripheral blood mononuclear cells treated with different dosages of the hairy root extracts at different time points.

Treatments (μg/mL)	Infectivity index (% control)	
	6 h	24 h
0 (Control)	100.0±8.3^{a}	100.0±7.9^{a}
25 (Sabz)	94.0±0.1^{ab}	41.0±8.0^{b}
50 (Sabz)	88.0±9.0^{ab}	35.0±8.4^{bc}
25 (Siah)	76.0±8.0^{b}	18.0±0.1^{c}
50 (Siah)	65.0±9.0^{c}	23.0±0.1^{c}

Infectivity index of *Leishmania major*-infected PBMCs cultures treated for 6-24 h with PBS (control), 25 μg/mL Sabz-HR, 50 μg/mL Sabz-HR, 25 μg/mL Siah-HR and 50 μg/mL Siah-HR. Data are presented as the mean±SD, and similar letters in the same column indicate no significant difference at the P<0.01 level.

3.5. Effect of HRs on the gene expression level of PBMCs

The effects of *F. carica* HRs extracts (25 and 50 μg/mL concentrations) on the expression of *IFN–γ* and *iNOS* genes (encoding enzyme inducible nitric oxide synthase and gamma

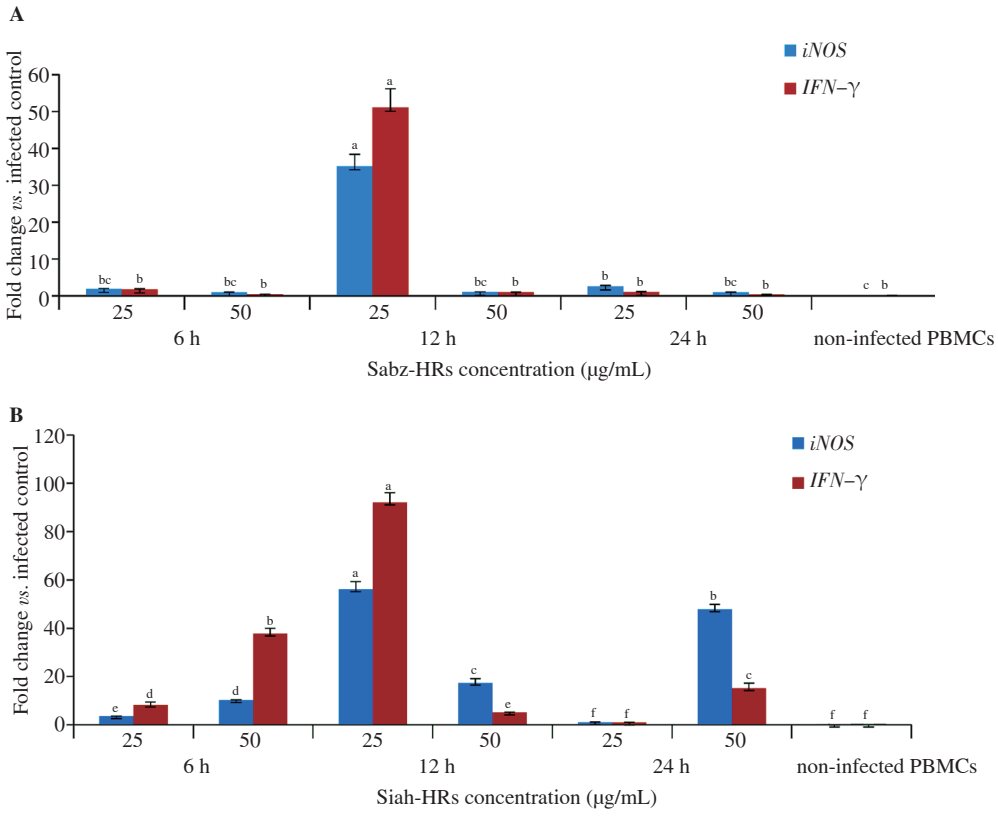


Figure 1. Expression of *iNOS* and *IFN–γ* genes in *Leishmania major* infected peripheral blood mononuclear cells affected by Sabz hairy roots (HRs) (A) and Siah (HRs) (B) extracts, and in non-infected peripheral blood mononuclear cells. The infected-peripheral blood mononuclear cells exposed to only PBS (1% in RPMI 1640) were used as control. The β*–actin* gene was used as housekeeping gene. Data was expressed as fold change of genes expression calculated by $2^{-\Delta\Delta Ct}$ method. Similar letters above the histograms indicate no statistical significant difference at the 5% level according to Duncan's multiple range test.

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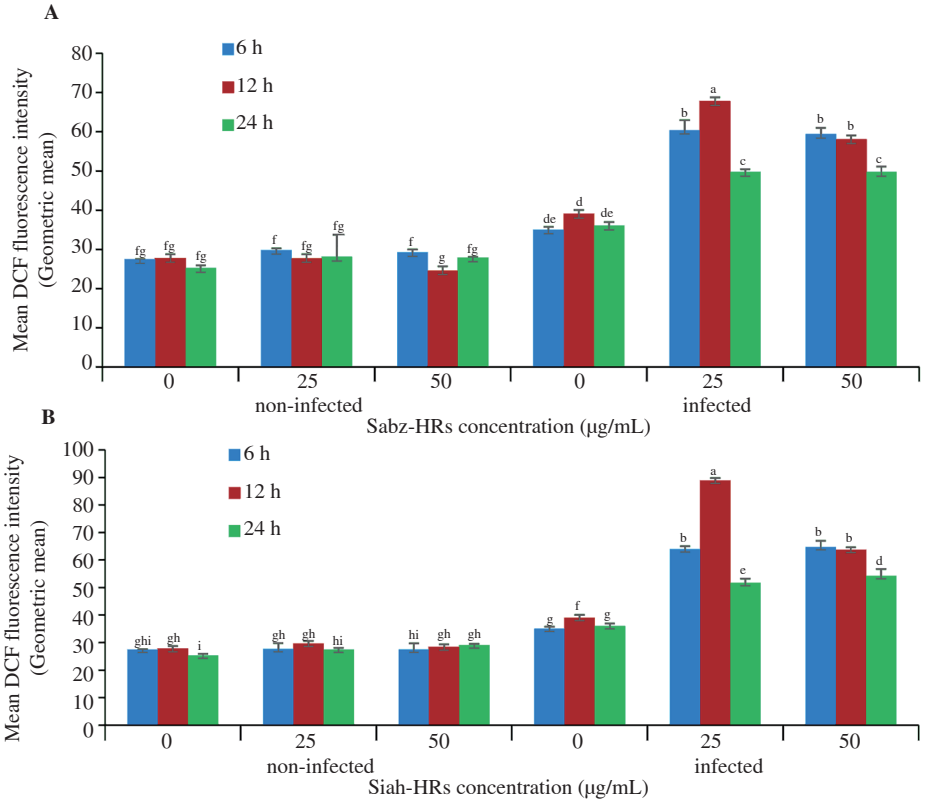


Figure 2. Geometric mean fluorescence intensity of dichlorofluorescein in non-infected and *Leishmania major* infected peripheral blood mononuclear cells at absence or presence of Sabz hairy roots (HRs) (A) and Siah HRs (B) treatment; Data were exhibited as mean±SD. Similar letters above the histograms indicate no statistical significant difference at the 5% level according to Duncan's multiple range test.

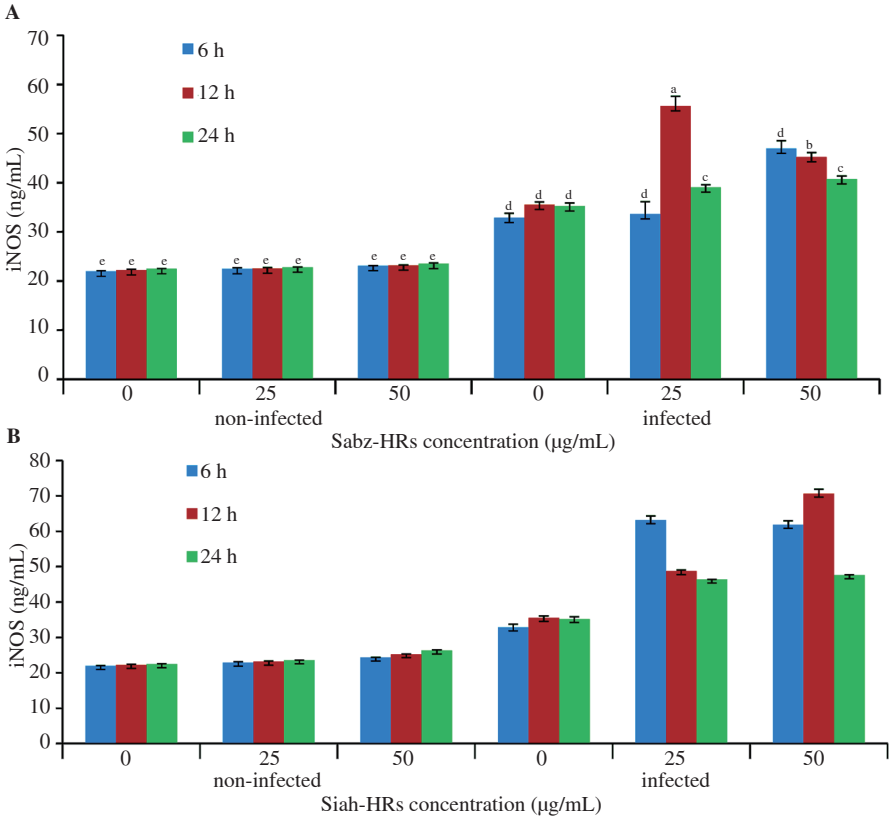


Figure 3. The levels of iNOS protein in non-infected and *Leishmania major* infected peripheral blood mononuclear cells treated with 25-50 µg/mL of Sabz hairy roots (HRs) (A) and Siah HRs (B) extracts at 24, 48, and 72-h exposure times; Similar letters above the histograms indicate no statistical significant difference at the 5% level according to Duncan's multiple range test.

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interferon, respectively) in *L. major*-infected PBMCs was estimated after 6, 12, and 24 h (Figure 1). The size of quantitative real-time PCR products was evaluated and confirmed by electrophoresis in 1.5% agarose gel stained with ethidium bromide (Supplementary Figure 4). Depending on type and exposure times, the HR extracts showed significant effects on the regulation of PBMCs genes. Our results showed that in response to the Sabz HRs, 12-h treatment with concentration of 25 μg/mL, resulted in a maximum increase in the expression of *IFN*–γ (51.1-fold) and *iNOS* (35.2-fold) genes compared to control. Similarly, the 12 h treatment with 25 μg/mL Siah HRs caused the highest expression level of *IFN*–γ (92.1-fold) and *iNOS* (56.1-fold) genes compared to control.

3.6. Effect of HRs on ROS generation in PBMCs

To measure the role of *F. carica* HRs for the induction of intracellular oxidation, the levels of ROS were analyzed in *L. major* infected PBMCs treated by HRs extracts. In the presence of HRs extracts, the maximum fluorescence value or ROS generation in infected PBMCs was observed in response to 25 μg/mL concentration with an exposure time of 12 h (Figure 2 and Supplementary Figure 5). The lowest level of ROS generation was observed in 24 h treatments.

3.7. Effect of HRs on the production of iNOS protein in PBMCs

The level of iNOS in PBMCs was significantly affected by HR extracts depending on the concentration and exposure time. In the presence of Sabz HRs extract, the production of iNOS in infected PBMCs increased by 1.5 to 2.47 times compared to non-infected PBMCs, and its maximum level (55.6 ng/mL) was related to 48-hour treatment with 25 μg/mL concentration (Figure 3). In treatments with Siah HRs extract, the iNOS level in infected PBMCs increased by 2 to 2.8 compared to non-infected PBMCs, and its maximum level (70.7 ng/mL) was related to the 48-hour treatment with concentration of 50 μg/mL (Figure 3).

4. Discussion

Leishmaniasis remains one of the main concerns of the World Health Organization[6,26]. Recently, researches are underway to find the potent plant compounds with antileishmanial activity and non-toxic properties for host cells[26,27]. Based on biochemical studies, the biological activities of various plant species have been proven effective for the treatment of leishmaniasis[28]. *F. carica*, a high-value plant species with folkloric applications, has shown varied biological properties (antibacterial, antiviral and antioxidative)[16,17,19]. Up to date, rare studies have ever been conducted on the antileishmanial properties of *F. carica*. The current study, for the first time to our knowledge, evaluated the antileishmanial activity of extracts from different parts of *F. carica* (Sabz and Siah cultivars). Since the distribution of biochemical compounds in different parts of plants is unequal, the inhibitory power of Sabz and Siah *F. carica* extracts on parasite growth was not the same due to their different metabolic profiles.

In accordance to our antioxidant enzymes assay, in both cultivars of *F. carica*, the activity of antioxidant enzymes CAT, APX and GPx was differed in leaves, roots and HRs. The activity of all three antioxidant enzymes was the highest in Siah HRs and moderate in Sabz HRs. Based on our HPLC analysis of some phenolic compounds, HRs possessed considerably a higher amount of phenolic compounds in comparison with fruits. Accordingly, *F. carica* transformation using *A. rhizogenes* and HRs induction can alter the plant's antioxidant capacity and can also turn HRs into a powerful plant tissue for the production of secondary metabolites, as reported in other plants[29,30].

According to our results of MTT assay, the inhibitory effect of the *F. carica* extracts on *L. major* promastigote growth was observed in order of HRs>fruits>roots>leaves. In some studies, the antiparasitic and antioxidant effect of plant phenolic compounds have been reported[31,32]; therefore, the antileishmanial activity of HRs may be correlated with their phenolic compounds as a powerful antioxidant. It is clear that the anti-amastigote activity of the agents depends on their role in suppressing the invasive ability of parasite through activating the macrophages, and stimulating the generation of ROS and NO[33]. ROS can be produced in response to some drugs or as a means of counteracting pathogenic infections in cells[34]. Therefore, ROS generation ability is a main basis of antiparasitic compounds to fight parasites in infected cells[34,35]. Detection of ROS can be carried out from 1 to 2 h after induction, but detecting cytokine proteins is possible after the activation of protein synthesis machinery that takes longer[36]. NO, as a short-lived major armament in macrophages against *Leishmania* parasite is produced by *iNOS* gene[35,37]. The expression of iNOS can be induced by Th1 cytokines, such as TNF-α, IL-12, and IFN-γ[5,6]. Up-regulation of iNOS leads to the release of large amounts of NO and eventually parasite killing *via* NO[5,35]. What is worthy to note is that NO, as a signal molecule, has a variety of physiological functions in cellular biology. For instance, at high concentrations, NO has a cytotoxic effect giving rise to the death of tumor cells, while at low concentrations, its inhibitory effect on the tumor cells disappears, and supports the growth of tumor cells, whose mechanism is still unknown[38]. In this study, the ROS level was 1.5-fold higher in *L. major*-infected PBMCs treated with 25 μg/mL of Siah HR than in non-treated *L. major*-infected PBMCs. ROS generation and expression level of *IFN*–γ and *iNOS* genes were positively correlated. The maximum significant positive correlation was obtained in 12-h treated infected PBMCs concomitant with the increase in ROS, mRNA expression of *IFN*–γ and *iNOS* genes

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enhanced markedly in HRs-treated infected PBMCs. The maximum value of iNOS protein in the study was achieved after 24 to 48 h treatment by Siah HRs.

It is likely from the results that HRs treatments by inducing ROS generation within treated cells through an oxidative stress, stimulating immune-stimulating cytokines production, and activating iNOS induce apoptotic cell death and, thus, could inhibit the parasite growth. In the same regard, Trun *et al.*[37] found that the application of *Pelargonium sidoides* extract could significantly increase the gene expressions of *iNOS* and some of the cytokine mRNAs in *Leishmania*-infected RAW 264.7 cells. Saha *et al.*[39] found that berberine chloride, a natural plant compound, had the ability to stimulate the production of NO and IL-12 in *Leishmania*-infected macrophages. Meanwhile, the plant hairy root culture system can also be shown antiparasitic activity[40]. The chemical constituents of hairy root culture of *Bixa orellana* L. showed antimalarial activity[40]. The diterpenes extracted from *Salvia austriaca* hairy roots had antiprotozoal activity against *Trypanosoma brucei* rhodesiense, *Trypanosoma cruzi* and *Plasmodium falciparum*[41]. The antileishmanial activity of *F. carica* leaves on *L. major* has been recently reported[42]. In this regard, the current study revealed that *F. carica* HRs extracts in optimum conditions (type, concentration and time exposure) exhibit the powerful *in vitro* antileishmanial activity against *L. major*. HRs treatment can enhance the ROS generation and induce remarkably the expression of IFN-γ and iNOS in *L. major*-infected PBMCs. Hence, *F. carica* could become a promising source of natural antileishmanial agent.

Conflict of interest statement

The authors declare no competing interests.

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Authors' contributions

SA: conceptualization, methodology, software, investigation, data curation, writing original draft, resources, validation, writing, review & editing. SK: conceptualization, methodology, software, supervision, data curation, resources, validation. MM: conceptualization, methodology, software, supervision. MJ: supervision, data curation, resources, software, validation. VK: investigation, data curation, writing - original draft, writing, review & editing.

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Ficus carica hairy roots: In vitro anti-leishmanial activity against Leishmania major promastigotes and amastigotes

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