



Antileishmanial Effect of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* against *Leishmania major* and *Leishmania tropica* Promastigotes and Their Cytotoxicity and Antioxidant Activities

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Authors' contributions

This work was carried out in collaboration between all authors. Authors F. Sharififar and IS designed the study. Author MP performed the statistical analysis. Author F. Sharifi wrote the protocol and wrote the first draft of the manuscript. Author MP managed the analyses of the study. Author IS managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Aims: This study was aimed to use *Coffea arabica*, *Salvia rhytidea* and *Bonium persicum* extracts against *Leishmania major* and *L. tropica* promastigotes to compare various concentrations of these plant extracts. In addition, their cytotoxicity and antioxidant activities were also evaluated.

Study Design: This study was performed experimentally using various concentrations of three medicinal plant extracts compared to control groups.

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Methodology: Fresh seeds of *C. arabica* and *B. persicum* and leaves of *S. rhytidea* were powdered and each plant material was extracted by ethyl alcohol via warm maceration method. Complete medium was used to prepare nine final concentrations (1-1000 µg/ml) for experiments. The cytotoxicity and antioxidant activities of the ethanolic extracts were evaluated using colorimetric cell viability WST1 and DPPH assays. All experiments were performed in triplicate and analyzed by t-test. The optical density (OD) values as measured by enzyme-linked immunosorbent assay (ELISA) were used to calculate the IC₅₀ values. Selectivity index (SI) of the plant extracts was not attributed to cytotoxicity when it was ≥ 10.

Results: The results indicated that *B. persicum* extract had potent antileishmanial activity against the promastigotes of both *Leishmania* species based on a dose-dependent response ($P= 0.001$) followed by *C. arabica* and *S. rhytidea* extracts. The IC₅₀ values for *C. arabica*, *S. officialis* and *B. persicum* against *L. major* and *L. tropica* were 79.2µg/mL and 40.8µg/mL; 98.7µg/mL and 45.5µg/mL and 49.4 and 23.9µg/mL, respectively. These extracts were safe for macrophages as the SI values of *C. arabica*, *S. officialis* and *B. persicum* for *L. major* and *L. tropica* were 12.1 and 23.5; 9.04 and 19.6 and 14.0 and 28.9, respectively. Results also confirmed the great antioxidant activities for these plant extracts.

Conclusion: The present findings indicated that the extracts possess potential antileishmanial activities and remarkable antioxidant levels with an excellent safety index.

Keywords: *Coffea arabica*; *Salvia rhytidea*; *Bunium persicum*; antileishmanial; cytotoxicity, antioxidant.

1. INTRODUCTION

Leishmaniasis is an important global vector-borne and complex disease which is caused by more than 22 *Leishmania* species. Over 1 billion people living in endemic areas of 101 countries and territories are currently at risk of infection [1, 2]. This disease comprises a spectrum of three distinct clinical forms including cutaneous (CL), mucocutaneous (MCL) and visceral (VL). CL constitutes approximately 70-75% of the total cases [3,4]. Current statistics indicated that the burden of CL has drastically increased particularly in the conflict zone of the Middle East countries notably in Iraq, Syria, and Afghanistan and has reached hyperendemic levels [1]. Hence, the CL burden of disease in terms of active and inactive cases has increased by a factor of 6 to 10 [5].

Over the past decades, despite extensive efforts, chemotherapy by antimonial agents (meglumine antimoniate, Glucantime and sodium stibogluconate, Pentostam) has been the first-choice for treatment of all types of leishmaniasis. Unfortunately, at present, these drugs alone have sufficiently lost their activities to some extent due to high toxicity index, long duration of use and development of resistance [4,6,7]. Presently, there is no efficacious vaccine against all forms of leishmaniasis.

Medicinal plants and herbal-derived materials are the rich source of bioactive products and lead molecules against various infectious agents

including microbial diseases [8]. Medicinal products have been traditionally used throughout histories worldwide. These natural products [9-11] due to high safety index, availability in leishmaniasis-endemic countries and low price are valuable sources of active compounds which are traditionally employed to treat a wide spectrum of disease conditions [12].

Salvia rhytidea Benth known as maryam goli lalezari belongs to Lamiaceae family and unlike other *Salvia* species, has been studied less. This plant has been widely distributed through Kerman province, in southeastern of Iran. Aerial parts have antioxidant, anticholinesterase, antidiabetic and alpha-glucosidase inhibitory effects [13-15]. Recently we have reported antifungal effects of a standardized extract of the plant [16].

Zire Kermani, *Bunium persicum* (Boiss.) Fedtch. is an odorous plant which is endemic to different regions of Iran, especially in the highlands of Kerman province. This plant has been used traditionally as carminative and antispasmodic. This biennial plant contains a considerable content of flavonoids and has antioxidant and anticonvulsant effects [17,18]. The effect of the plant has been reported against toxoplasmosis and protoscolecetes too [19,20].

In fact, such natural sources because of having a variety of bioactive complexes and lead molecules including alkaloids, terpenoids, antioxidants, and flavonoids could make

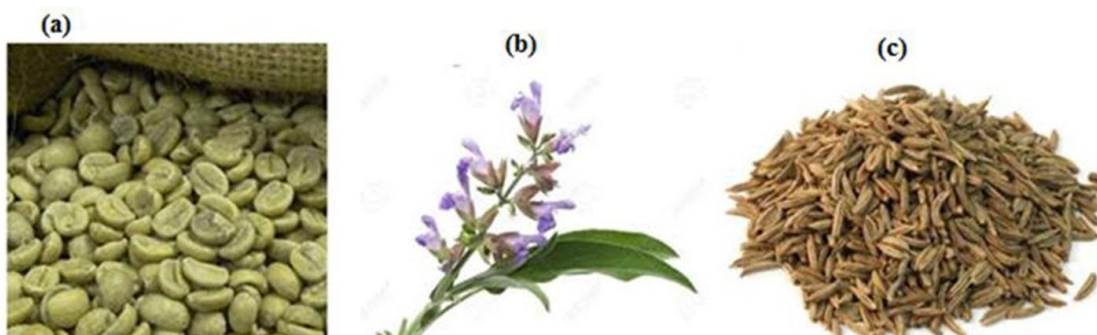


Fig. 1. a: *Coffea arabica*, b: *Salvia rhytidea* and c: *Bunium persicum*

available important preliminary screening to help select plant extracts with potential antileishmanial properties for planning future investigations [12, 19-28]. Therefore, this study aims to use three plant extracts against *L. major* and *L. tropica* promastigotes, the two primary causative agents of CL in the Old World, zoonotic and anthroponotic types (ZCL and ACL, respectively), as a model to compare various concentrations of these plant extracts with the first-line drugs, Glucantime as comparative positive control. Fig. 1 (above) shows the seeds of *Coffea arabica* (a) *Bunium persicum* (c) and the leaves of *S. rhytidea*.

2. MATERIALS AND METHODS

2.1 Chemicals and Plant Materials

2.1.1 Chemicals

Glucantime (meglumine antimoniate) was used as the positive control, purchased from the Provincial Health System (Sanofi-Aventis, Paris, France). WST1, Fetal Calf Serum (FCS), Penicillin/Streptomycin, DMEM and RPMI1640 media were purchased from Sigma-Aldrich.

2.1.2 Preparation of the crude extracts

Fresh seeds of *C. arabica* and *B. persicum* and leaves of *S. rhytidea* were gathered from Kerman province, southeast of Iran and kindly identified by Professor Fariba Sharififar, Department of Pharmacognosy, Kerman University of Medical Sciences, Kerman, Iran. A voucher specimen of each collected plant as reference was provided in the Herbarium of the Faculty of Pharmacy, Kerman University of Medical Sciences. Ethanolic extractions were performed for each plant; each powdered plant material was

extracted by warm maceration of plant powder in 70% ethyl alcohol for 72 h at room temperature with gentle shaking. The medicinal parts of each plant were milled and passed through a Whatman filter paper (No. 3, Sigma, Germany) to remove excess materials and concentrated under reduced pressure in a rotary evaporator at 30°C. The extracts were then concentrated in a vacuum chamber at 50°C in a rotary evaporator (Heidolph, Germany) and stored at -20°C to be used as the source of materials for experimentations [29].

2.2 Parasite Culture

Standard strains of *L. tropica* (MHOM/IR/2002/Mash2) and *L. major* strain (MRHO/IR/75/ER) were provided by Leishmaniasis Research Center, Kerman University of Medical Sciences. Promastigotes were inoculated into Novy-MacNeal-Nicolle (NNN) medium and sub-cultured in RPMI1640 (Sigma-Aldrich, Germany) supplemented with 100 IU/ml of penicillin and 100 µg/ml of streptomycin (Sigma Germany) at 24±1°C and 10% v/v heat inactivated (56°C) fetal calf serum (FCS) for 30 min. It was kept at -20°C until further use. The parasites were incubated at a temperature of 24 ±1°C. The multiplication rate of *Leishmania* promastigotes was observed every 24 h, and the number of parasites was calculated by counting the parasites in a drop of medium (10 µl) by using a Neubauer slide.

2.3 Macrophage Culture

The murine macrophage cell line (J774-A1) was obtained from the Pasteur Institute of Iran (Tehran, Iran). The cells were cultured and maintained in RPMI1640 medium supplemented with Pen/Str, 10% FBS at 37°C and 5% CO₂.

2.4 Anti-promastigotes Assay

The effect of *C. arabica*, *S. rhytidea* and *B. persicum* on promastigotes was evaluated by colorimetric cell viability WST1 assay using the method described elsewhere [26]. Briefly, 100 μ l of the promastigotes (10^6 cells/ml) harvested from logarithmic growth phase were added into a 96-well microtiter plate. Then 100 μ l of various concentrations (1, 5, 10, 25, 50, 100, 250, 500 and 1000 μ g/ml) of each extract was added to each well and incubated at $24^\circ\text{C} \pm 1^\circ\text{C}$ for 72 h. After incubation, 10 μ l of WST1 solution (10 μ g/ml) was added into each well and they were incubated at 25°C for 4 h. Promastigotes were cultured in complete medium with no drug used as untreated control, and complete medium with no promastigotes and drugs as blank. All the experiments were performed in triplicate. Finally, absorbance was measured by an enzyme-linked immunosorbent assay (ELISA) reader (BioTekELX800) at 490 nm. 50% inhibitory concentrations (IC_{50} values) were also calculated by Probit test in SPSS software.

2.5 Cytotoxicity Effects on Macrophages J774 Cell Line

For evaluation of cytotoxicity activities on macrophages J774 cell line, we determined the CC_{50} (cytotoxicity concentration for 50% of cells) for various concentrations of extracts of *C. arabica*, *S. rhytidea* and *B. persicum* on macrophages. Macrophage cells were plated at 10^6 cells /mL in 96- well Lab-Tek (Nunc, USA) and left to adhere for 24 h at 37°C and 5% CO_2 . After removing the non-adherent cells by washing with DMEM medium, the cells were incubated at similar conditions as mentioned. Thereafter, 190 μ l of complete DMEM medium was added in each well, and then 10 μ l of each extract dilution was added (as previously prepared in medium). Macrophages were preserved with the extracts from 1 to 1000 μ g/ml for 72 h. The cytotoxicity rate was evaluated using the WST1 colorimetric cell viability assay as previously defined in the promastigote sensitivity assay [30,31]. All experiments were carried out in triplicate like in previous stages.

2.6 Antioxidant Activity

2.6.1 DPPH assay

The antioxidant assay was performed by DPPH scavenging method. This method measured the

ability of the plant extract to scavenge DPPH free radicals [32,33]. Briefly, at first, a 0.1 mM of DPPH radical solution in methanol/water (8:2, v/v) was prepared. Then 1 ml of this solution at concentrations of 0-400 μ g/ml was mixed with sample solution (3 ml) in methanol/water (8:2, v/v). After 30 min, the absorbance (OD) was measured at 517 nm. The decrease in the DPPH solution absorbance shows an increase of the DPPH radical scavenging activity [34]. This activity is given as % DPPH radical scavenging which is calculated by the following equation:

$$\% \text{DPPH radical scavenging} = (\text{control absorbance} - \text{sample absorbance}) / \text{control absorbance} \times 100.$$

The DPPH solution without sample solution was used as a control and butylated hydroxyanisole (BHA) was used as a standard.

2.7 Statistical Analysis

Student's t-test was used to analyze intergroup differences. Experiments were performed in triplicate and data were represented as the mean \pm SD. A *P* value of less than 0.05 was considered to be statistically significant. The IC_{50} value (50% inhibitory concentrations) was calculated by Probit test in SPSS.

3. RESULTS

3.1 Anti-promastigotes Assay

The results indicated that the *B. persicum* extract had significant antileishmanial activity against the promastigotes of both *Leishmania* species based on a dose-dependent response ($P= 0.001$) followed by *C. arabica* and *S. rhytidea* extracts which showed antileishmanial effects against promastigotes of *L. tropica* and *L. major*, respectively. Optical density and inhibitory effect of three extracts and untreated control compared to Glucantime are shown in Figs. 2 and 3. The IC_{50} values for the extracts and Glucantime against promastigotes of *L. major* were 49.4 μ g/ml and 79.2 μ g/ml, 98.7 μ g/ml and 28.07 μ g/ml, respectively and against promastigotes of *L. tropica* were 23.9 μ g/ml and 40.8 μ g/ml, 45.5 μ g/ml and 14.5 μ g/ml as shown in Table 1.

3.2 Cytotoxicity Effects on Macrophages J774 Cell Line

In the evaluation of *in vitro* cytotoxic activity of three extracts, we observed that these extracts

had no significant cytotoxicity effects against macrophages (J774 cells). CC_{50} values for *C. arabica*, *S. rhytidea*, *B. persicum* and Glucantime were 957.5, 892.6, 691.9 and 287.1 $\mu\text{g/ml}$, respectively (Table 2) which showed that these extracts were safe for mammalian cells as

determined by safety index (selectivity index; SI) and displays the cytotoxicity effects among extracts, Glutamine and untreated control. As indicated, the extracts were all safe with no cytotoxicity effects, as far as $SI \geq 10$ was concerned [35,36].

Table 1. IC_{50} values of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* and Glucantime against the growth rate of promastigotes of *Leishmania major* and *L. tropica*. Data are expressed as the mean \pm SD

	IC_{50}^a ($\mu\text{g/ml}$)			
	<i>L. major</i>	<i>P value</i>	<i>L. tropica</i>	<i>P value</i>
Glucantime ^b	28.07	NA	14.5	NA
<i>Coffea arabica</i>	79.2	0.061	40.8	0.053
<i>Salvia rhytidea</i>	98.7	0.073	45.5	0.056
<i>Bunium persicum</i>	49.4	0.001*	23.9	0.001*

^a Concentration of drug that caused 50% of growth inhibition of promastigotes

^b Glucantime defined as first choice comparative positive control

^c NA; Not applicable

* Not significant

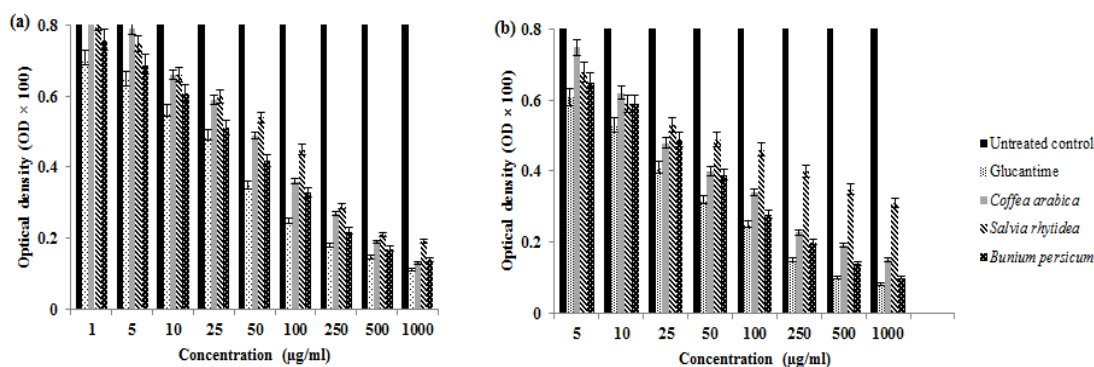


Fig. 2. Optical density of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* on promastigotes of *L. major* (a) and *L. tropica* (b) compared to Glucantime as a positive control
Data are mean \pm SD of triplicate experiments

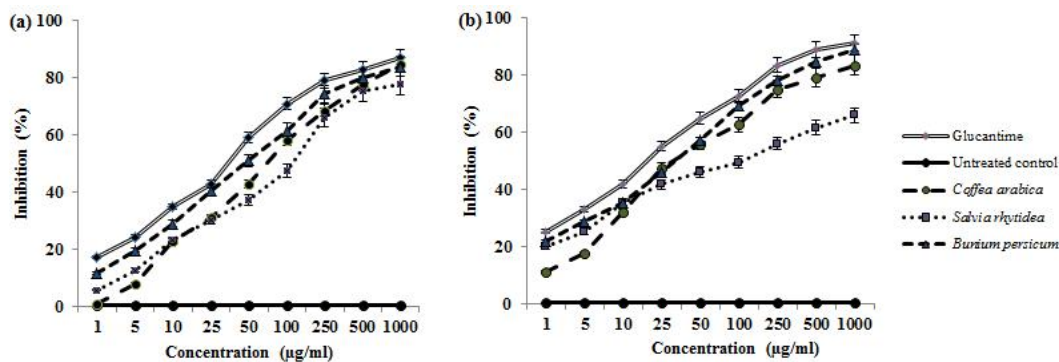


Fig. 3. Inhibition percent of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* on promastigotes of *L. major* (a) and *L. tropica* (b) compared to Glucantime as a positive control
Data are mean \pm SD of triplicate experiments

Table 2. The CC₅₀ values for DPPH radical scavenging activity (%) of crude extracts (*Coffea arabica*, *Salvia rhytidea* and *Bunium persicum*) and BHA

	CC ₅₀ ^b (µg/ml)	SI ^c	
		<i>L. major</i>	<i>L. tropica</i>
Glucantime ^a	287.1	10.25	19.8
<i>Coffea arabica</i>	957.5	12.1	23.5
<i>Salvia rhytidea</i>	892.6	9.04	19.6
<i>Bunium persicum</i>	691.9	14	28.9

^a Glucantime defined as first choice positive control

^b Concentration of extracts that caused 50% of growth inhibition in macrophages

^c Selectivity index (CC₅₀/IC₅₀)

On the other hand, Fig. 4 represents the cell viability of *C. arabica*, *S. rhytidea* and *B. persicum* on macrophage J774 cell line in comparison to Glucantime as a positive control. Although the extracts showed a dose-response linear effect, the viability of the harboring macrophages was relatively high, a further evidence for safety level of plants extracts.

3.3 Antioxidant Activity

3.3.1 DPPH assay

These extracts were examined for DPPH radical scavenging effects at different concentrations of 1, 5, 10, 25, 50, 75, 100 and 200 µg/ml crude extracts and exhibited notable activities (Table 3). Butylated hydroxyanisole (BHA) was used as a standard. The IC₅₀ values for the extracts (*C. arabica*, *S. rhytidea* and *B. persicum*) were 29.2 ± 0.042µg/ml, 40.3 ± 0.045µg/ml and 54.91 ± 0.11 µg/ml, respectively in comparison to the

standard control (22.5 ± 0.021 µg/ml). The antioxidant level of the first extract was not significantly different relative to the control group, while this difference for *S. rhytidea* was statistically significant (*P*= 0.059). Fig. 5 presents the scavenging effects of the extract on DPPH free radicals compared to BHA. Both components; BHA and the extracts displayed a dose-dependent response with similar antioxidant activities. Data are expressed as mean ± SD of triplicate.

4. DISCUSSION

Leishmaniasis represents a complex disease with major veterinary and medical health problems at global scale. Unfortunately, at present, neither effective drug nor efficacious vaccine is available to cure this disease. Medicinal plants could provide a potential source of new alternative medicines against infectious

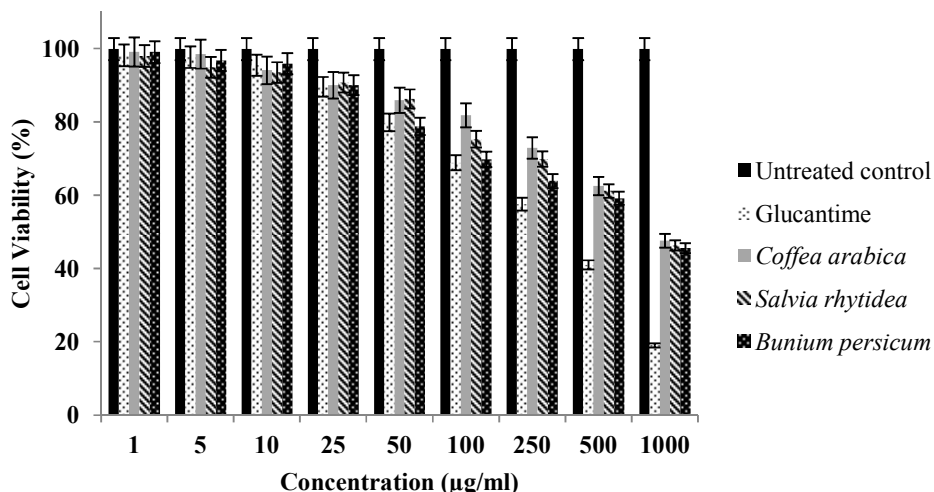


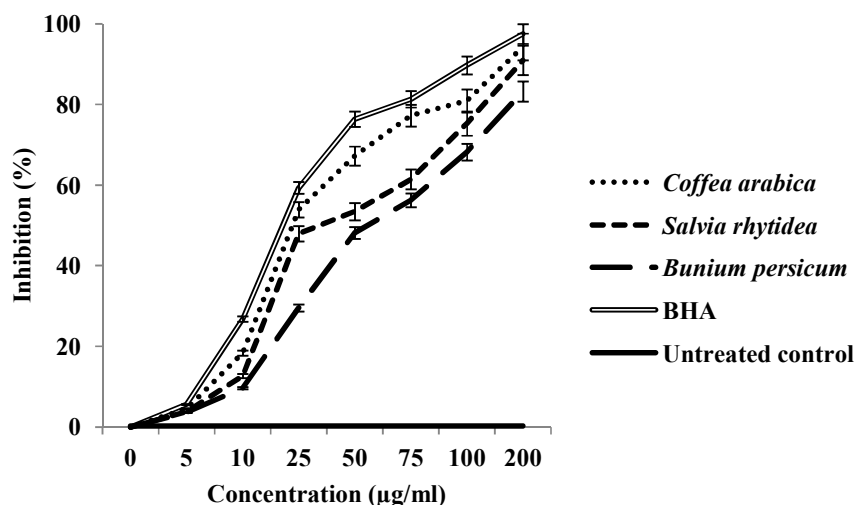
Fig. 4. Cell viability of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* on macrophage J774 cell line compared to Glucantime as positive control
Data are mean ± SD of triplicate experiments

Table 3. The IC₅₀ values for DPPH radical scavenging activity (%) of crude extracts (*Coffea arabica*, *Salvia rhytidea* and *Bunium persicum*) and BHA

Compounds	IC ₅₀ ^a ± SD (µg/ml)	P value
Butylated hydroxyanisole (BHA)	22.5 ± 0.021	NA
<i>Coffea arabica</i>	29.2 ± 0.042	0.001 [*]
<i>Salvia rhytidea</i>	40.3 ± 0.045	0.059
<i>Bunium persicum</i>	54.91 ± 0.11	0.068

^c NA: Not applicable

* : Not significant

**Fig. 5. Scavenging effects of *Coffea arabica*, *Salvia rhytidea* and *Bunium persicum* on DPPH free radicals compared to BHA as a standard control**

Data are mean ± SD of triplicate experiments

diseases, as they are safe, accessible and low costs [37]. Many plant derivatives consist of varying degrees of antioxidant levels [38]. These natural products play a major role as they are beneficial to the body and exert their deleterious effects on infectious agents, cancer and degenerative diseases [10,11]. Various naturally produced components (exogenous and endogenous) act as free radical scavengers by preventing and repairing damages caused by reactive oxygen species (ROS) and also reactive nitrogen species (RNS), as well. Such activities are mainly provided through enhancement of the immune defense mechanism of the body cells to prevent and inhibit the risk of infectious and chronic diseases [39]. All three plant extracts exhibited a significant inhibitory activity against the promastigotes as displayed by the low OD and also IC₅₀ values. The beneficial aspects of these plants against various diseases have already been reported [40].

The aforementioned plant extracts exhibited a remarkable index of antioxidant levels which was significantly higher than the standard control (BHA). This property could contribute to the inhibitory composition of the extracts against the two *Leishmania* species in an experimental model. As far as the cytotoxicity level of these extracts is concerned, they were all safe as the SI was considerably higher than the cut-off point (SI ≥ 10 = safety level). In fact, antioxidant property and safety index are the two major characteristics for preliminary evaluation of a product and ultimate decision to further advance the investigation. Therefore, knowledge of these indices would help in selecting appropriate effective components for future planning [7].

A wide variety of natural products such as medicinal plants, fruits, vegetables, vitamins, phenolic compounds consist of varying degrees of antioxidant activities [41]. Although the mechanism of action of these plants is not clear,

certainly they provide a deleterious condition to inhibit the growth of microorganisms such as *L. major* and *L. tropica* promastigotes. Recent epidemiological data indicated that there is a positive association between the intake of such materials and a reduced rate of microbial infections [42,43]. To the best of our knowledge, this investigation is the first work to show a high safety index coupled with a significant level of inhibitory action against the causative agents of ZCL and ACL and remarkable antioxidant activity. Further investigations are crucial to isolate, analyze and identify phytochemical compositions of these medicinal plants for their future prophylactic and therapeutic effects [44, 45].

5. CONCLUSION

The present findings indicated that the extracts obtained from Kerman province possess an extraordinary antioxidant with an excellent safety level and remarkable leishmanicidal activities. Further investigations are crucial to isolate, analyse and identify the plant extracts compositions for future planning on intramacrophage amastigotes.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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