Antileishmanial, cytotoxicity and antioxidant activities of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* against *Leishmania major* and *Leishmania tropica* promastigotes

Abstract

**Aims:** This study was aimed to use three plant extracts against *Leishmania major* and *L. tropica* promastigotes, the two primary causative agents of cutaneous leishmaniasis (CL) in the Old World, zoonotic and anthroponotic types (ZCL and ACL, respectively), as a model to compare various concentrations of these plant extracts.

**Study design:** This study was performed as experimental using various concentrations of three medicinal plant extracts compared to untreated control and the first-line drug, Glucantime as comparative positive control.

**Methodology:** Fresh seeds of *C. Arabica* and *C. carvi* and leaves of *S. officinalis* were gathered from Kerman province, southeast of Iran. Each powdered plant material was extracted by maceration. Complete medium was used to prepare nine final concentrations (1-1000 µg/mL) for experiments. The cytotoxicity and antioxidant activities of the extracts were carried out by using colorimetric cell viability WST1 and DPPH assays. All experiments were repeated in triplicate and analyzed by t-test. Antileishmanial, cytotoxicity and antioxidant effects of these extracts were explored.

**Results:** The results indicated that the *Carum carvi* extract had potent antileishmanial activity against the promastigotes of both *Leishmania* species based on a dose-dependent response (*P* = 0.001) followed by *Coffea arabica* and *Salvia officinalis* extracts which showed antileishmanial effects to lower extent. These extracts were safe for mammalian cells as also determined by safety index (selectivity index; SI). Results also confirmed the great antioxidant activities for these extracts.

**Conclusion:** The present findings indicated that the extracts possess potential antileishmanial activity and remarkable antioxidant levels with an excellent safety index.
**Keywords:** *Coffea arabica* ; *Salvia officinalis*; *Carum carvi* ; Antileishmanial; Cytotoxicity, Antioxidant

1. INTRODUCTION

Leishmaniasis is an import 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 reached to 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, these drugs are no longer effective and lost their activities to some extents due high toxicity index, long duration of use and development of resistances [4]. At present, 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 such as microbial diseases [6]. Medicinal products have been traditionally used throughout histories worldwide. These natural products [7-9] due to high safety index, availability in leishmaniasis-endemic countries and low price are valuable sources of active compounds which are traditionally used to treat a wide spectrum of disease conditions [10].

*Salvia officinalis* L. (Sage) is a perennial round shrub in the family of Labiatae/Lamiaceae. *Salvia* is the main genus of this family and comprises near 900 species. Plants of this genus cultivate throughout the world and *S. officinalis* is natural to Middle East and Asian countries. Nowadays, the aerial parts of *S. officinalis* shrub has a long history of use in cookery and...
traditional medicine. As of its flavor and seasoning properties, this plant has been widely used in foods preparations. In folk medicine of Asia and Latin America, it has been used for the treatment of various kinds of disease syndroms comprising seizure, ulcers, gout, rheumatism, inflammation, dizziness, tremor, paralysis, diarrhea, and hyperglycemia [11]. In traditional medicine of Europe, S. officinalis has been used to treat mild dyspepsia (such as heartburn and bloating), extreme sweating, age-related cognitive complaints, and inflammations in the throat and skin [12].

Persian cumin, (Carum carvi) is a biennial plant in the family of Apiaceae which is native to western Asia, Europe, and North Africa. Carum carvi L. (C. carvi, Caraway) as an important medicinal plant has been long cultivated in many countries notably in the north and center of Europe, Egypt, Australia, China and Iran. It is traditionally used as a spice in foods and beverages. It has also been utilized as an alternative herbal medicine for gastrointestinal (GI) disorders including dyspepsia and various spasmodic conditions, bloating, diarrhea and in flatulent colic [13]. It is diuretic and expectorant and used for increasing maternal milk and dysmenorrhea. Caraway has both anti-hyperglycemic and anti-hyperlipidemic activities in STZ-induced diabetic rats. Its volatile oil probably has a protective role in kidney tissue against oxidative stress in advanced stages of sepsis and it is hepatoprotective against carbon tetrachloride-induced liver toxicity in rats [14].

Caraway has also been used in bronchopulmonary disorders as cough remedy and demulcent [15]. Vapors from caraway seeds have been reported to be effective in releasing pain and inflammation in patients suffering from lumbar pains and rheumatism. Moreover there are numerous studies about the treatment of GI disorders like Helicobacter pylori - induced gastritis and relaxant effect of ethanolic extract of caraway on isolated intestinal smooth muscle cells of the guinea pig. This response may explain, in part, the beneficial effects of caraway in relieving GI symptoms associated with dyspepsia. Two important natural compounds of caraway including, carvone and limonene are mucoprotective on duodenal and peptic ulcer and gastroduodenitis and also antiulcerogenic effect. Due to the antiulcerogenic, antioxidative, antispasmodic and immunomodulatory properties of caraway, it is suggested that it has a high therapeutic potential for inflammatory bowel disease (IBD)[16].
In fact, such materials because of having variety of bioactive complexes and lead molecules including alkaloids, terpenoids, antioxidants and flavonoids could make available important preliminary screening to help select plant extracts with potential antileishmanial properties for planning future investigations [17]. 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 anthropoontic 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 shows the seeds of *Coffea arabica* (a) *Carum carvi* (c) and the leaves of *Salvia officinalis*.

![Fig.1. a: Coffea arabica, b: Salvia officinalis and c: Carum carvi](image)

2. MATERIALS AND METHODS

2.1 Chemicals and plant materials

2.1.1 Chemicals

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

2.1.2 Preparation of extracts
Fresh seedss of *C. arabica* and *C. carvi* and leaves of *S. officinalis* 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 plants as reference was provided in the Herbarium of Faculty of Pharmacy, Kerman University of Medical Sciences. Air-dried plant materials (100 g) were separately extracted by percolation method using 70% aqueous ethanol successively for 72 h at ambient temperature. The extracts were passed through a Whatman filter paper (no. 3, Sigma, Germany) to remove excessive materials. 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 [18].

2.2 Preparation of the crude extracts

Aqueous extractions were performed for each plant; each powdered plant material was extracted by maceration of plant powder in absolute ethyl alcohol and dimethyl sulfoxide (DMSO) separately for 72 h at room temperature with gentle shaking. Proportion of solvent to plant materials was 10:1, respectively. The filtrate obtained through Whatman No. 1 filter paper was concentrated under reduced pressure in a rotary evaporator at 30°C. The extraction yields were calculated and the plant crude materials were dissolved in their respective solvents to a concentration of 160 mg/ml. All crudes were kept at room temperature and protected from light until further processing.

2.3 Parasite Culture

Standard strains of *Leishmania tropica* (MHOM/IR/2002/Mash2) and *L. major* strain (MRHO/IR/75/ER) were provided from 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 till 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.
2.4 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.

2.5 Anti-promastigotes assay

*Coffea arabica, Salvia officinalis* and *Carum carvi* on promastigotes was evaluated by colorimetric cell viability WST1 assay using the method described elsewhere[19]. Briefly, 100 µl of the promastigotes (10⁶ cells/ml) harvested from logarithmic growth phase were added into a 96-well microtiter plate. Then 100 µl of various concentrations (1, 5, 10, 25, 50, 100, 250, 500 and 1000 µg/ml) of each extract was added to each well and incubated at 24°C ± 1°C for 72 h. After incubation, 10µl of WST1 solution (10 µg/ml) was added into each well and they were then 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 thrice. Finally, absorbance was measured by an enzyme-linked immunosorbent assay (ELISA) reader (BioTekELX800) at 490 nm. 50% inhibitory concentrations (IC₅₀ values) were also calculated by Probit test in SPSS software.

2.6 Cytotoxicity effects on macrophages J774 cell line

For evaluation of cytotoxicity activities on macrophages J774 cell line, we determined the CC₅₀ (cytotoxicity concentration for 50% of cells) for various concentrations of extracts of *Coffea arabica, Salvia officinalis* and *Carum carvi* on macrophages. Macrophage cells were plated at 10⁶ cells/mL in 96-well Lab-Tek (Nunc, USA) and left to adhere for 24 h at 37 °C and 5% CO₂. After removing the non-adherent cells by washing with DMEM medium, they were incubated at similar conditions as mentioned. After that, 190 µl of complete DMEM medium was added in each well, and then 10 µl of each extract dilution, as previously prepared in medium, was added. Macrophages were preserved with the extracts from 1 to 1000µg/ml for 72 h. The cytotoxicity rate was evaluated using the WST1 colorimetric cell viability assay as previously defined in the promastigote sensitivity assay [20, 21]. All experiments were carried out thrice similar to previous stages.
2.7 Antioxidant activity

2.7.1 DPPH assay

Antioxidant assay was performed by the method introduced by Benzie and Strain (1999). This method measured the ability of the plant extract to scavenge DPPH free radicals [22]. 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 the concentrations of 0-400 µg/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. Decreasing the DPPH solution absorbance shows an increase of the DPPH radical scavenging activity [23]. This activity is given as % DPPH radical scavenging which is calculated by the following equation:

\[
\text{%DPPH radical scavenging} = \left( \frac{\text{control absorbance} - \text{sample absorbance}}{\text{control absorbance}} \right) \times 100
\]

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

2.8 Statistical Analysis

Student’s t-test was used to analyze intergroup differences. Experiments were repeated in triplicates and data are represented as the mean ± 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 \textit{Carum carvi} extract had significant antileishmanial activity against the promastigotes of both Leishmania species based on a dose-dependent response \((P=0.001)\) followed by \textit{Coffea arabica} and \textit{Salvia officinalis} extracts which showed antileishmanial effects against promastigotes of \textit{L. tropica} and \textit{L. major}, respectively (Fig.1). The IC\(_{50}\) values for the extracts and Glucantime against promastigotes of \textit{L. major} were 49.4µg/ml and 79.2 µg/ml, 98.7µg/ml and 28.07µg/ml, respectively and against promastigotes of \textit{L. tropica} were 23.9µg/ml and 40.8µg/ml, 45.5µg/ml and 14.5µg/ml as shown in Table 1. Optical density and inhibitory
effect of three extracts and untreated control compared to Glucantime have shown in Fig.2 and Fig.3.

Table 1. IC₅₀ values of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* and Glucantime against the growth rate of promastigotes of *Leishmania major* and *L. tropica*. Data are expressed as the mean ± SD

<table>
<thead>
<tr>
<th></th>
<th>IC₅₀ (µg/ml)</th>
<th>P value</th>
<th>IC₅₀ (µg/ml)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>L. major</em></td>
<td></td>
<td></td>
<td><em>L. tropica</em></td>
<td></td>
</tr>
<tr>
<td>Glucantime</td>
<td>28.07</td>
<td>NA</td>
<td>14.5</td>
<td>NA</td>
</tr>
<tr>
<td><em>Coffea arabica</em></td>
<td>79.2</td>
<td>0.061</td>
<td>40.8</td>
<td>0.053</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>98.7</td>
<td>0.073</td>
<td>45.5</td>
<td>0.056</td>
</tr>
<tr>
<td><em>Carum carvi</em></td>
<td>49.4</td>
<td>0.001*</td>
<td>23.9</td>
<td>0.001*</td>
</tr>
</tbody>
</table>

*Concentration of drug that caused 50% of growth inhibition of promastigotes

*Glucantime defined as first choice comparative positive control

* NA; Not applicable

* Not significant

**Fig.2.** Optical density of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* on promastigotes of *L. major* (a) and *L. tropica* (b) compared to Glucantime as positive control. Data are mean ± SD of triplicate experiments
Fig. 3. Inhibition percent of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* on promastigotes of *L. major* (a) and *L. tropica* (b) compared to Glucantime as positive control. Data are mean ± SD of triplicate experiments.

### 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 *Coffea arabica*, *Salvia officinalis*, *Carum carvi* and Glucantime were 957.5µg/ml, 892.6µg/ml, 691.9µg/ml and 287.1µg/ml, respectively (Table 2) that showed these extracts were safe for mammalian cells as also 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.

**Table 2. The CC$_{50}$ values for DPPH radical scavenging activity (%) of crude extracts (*Coffea arabica*, *Salvia officinalis* and *Carum carvi*) and BHA**

<table>
<thead>
<tr>
<th></th>
<th>CC$_{50}$ $^b$ (µg/ml)</th>
<th>SI$^c$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>L. major</em></td>
</tr>
<tr>
<td>Glucantime$^a$</td>
<td>287.1</td>
<td>10.25</td>
</tr>
<tr>
<td><em>Coffea arabica</em></td>
<td>957.5</td>
<td>12.1</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>892.6</td>
<td>9.04</td>
</tr>
<tr>
<td><em>Carum carvi</em></td>
<td>691.9</td>
<td>14</td>
</tr>
</tbody>
</table>

$^a$Glucantime defined as first choice positive control

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

$^c$Selectivity index (CC$_{50}$/IC$_{50}$)
On the other hand, Fig. 4 represents the cell viability of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* on macrophage J774 cell line compared to Glucantime as 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.

![Figure 4: Cell viability of Coffea arabica, Salvia officinalis and Carum carvi on macrophage J774 cell line compared to Glucantime as positive control. Data are mean ± SD of triplicate experiments.]

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 demonstrated notable activities (Table 3). Butylated hydroxyanisole (BHA) was used as standard. The IC$_{50}$ values for the extracts (*Coffea arabica, Salvia officinalis* and *Carum carvi*) were 29.2 ± 0.042 µg/ml, 40.3 ± 0.045 µg/ml and
54.91 ± 0.11 µg/ml, respectively as compared to the standard control (22.5 ± 0.021 µg/ml). The antioxidant level of the first extract was not significantly difference relative to the control group, while this difference for *Salvia officinalis* was marginally significant (*P* = 0.059). Fig.5 presented 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 triplicates.

Table 3. The IC\(_{50}\) values for DPPH radical scavenging activity (%) of crude extracts (*Coffea arabica*, *Salvia officinalis* and *Carum carvi*) and BHA

<table>
<thead>
<tr>
<th>Compounds</th>
<th>IC(_{50}) ± SD (µg/ml)</th>
<th><em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylated hydroxyanisole</td>
<td>22.5 ± 0.021</td>
<td>NA</td>
</tr>
<tr>
<td>(BHA)</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Coffea arabica</em></td>
<td>29.2 ± 0.042</td>
<td>0.001(^*)</td>
</tr>
<tr>
<td><em>Salvia officinalis</em></td>
<td>40.3 ± 0.045</td>
<td>0.059</td>
</tr>
<tr>
<td><em>Carum carvi</em></td>
<td>54.91 ± 0.11</td>
<td>0.068</td>
</tr>
</tbody>
</table>

\(^*\) NA: Not applicable
\(*\) : Not significant
Fig. 5. Scavenging effects of *Coffea arabica*, *Salvia officinalis* and *Carum carvi* on DPPH free radicals compared to BHA as a standard control. Data are mean ± SD of triplicate experiments.

4. DISCUSSION

Leishmaniasis represents a complex disease with major veterinary and medical health problems at global scale. Unfortunately, at present no effective drug nor efficacious vaccine to cure the disease is available. Medicinal plants could provide a potential source of new alternative medicines against infectious diseases, as they are safe, accessible and low costs [24]. Numerous plant derivatives consist of varying degrees of antioxidant levels [25]. 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 [8, 9]. 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 [26]. The three plant extracts exhibited a significant inhibitory activity against the promastigotes as displayed by the low OD and also IC$_{50}$ values. The beneficial aspects of these plants against various diseases have already been reported [11, 13].

The aforementioned plant extracts demonstrated 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 experimental model. As far as the cytotoxicity level of these extracts are considered, they were all safe as the SI was considerably higher than the cut-off point (SI$\geq$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 [27]. Although, the mechanism of action of these products 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 [28, 29]. To 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.

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.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

References


