



ISSN 2320-3862

JMPS 2014; 2(5): 12-18

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Received: 18-07-2014

Accepted: 01-08-2014

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Antileishmanial activity of *Hyssopus officinalis*, *Tussilago farfara*, *Carum copticum* extracts in comparison with Glucantime in Iran

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ABSTRACT

Leishmania is transmitted by sandflies that ingest the parasite in the amastigote phase resident within macrophages, then inoculate the promastigote phase into body hosts. The present study was conducted to evaluate the *in vitro* effects of alcoholic extracts of plants on *L. major*. The extract of aerial parts of plants were obtained by maceration. The *in vitro* experiments were performed on promastigotes to assess anti Leishmanial activity of the extracts using glucantime as a reference. The extract of plants and glucantime solutions for biological testing were prepared in PBS at several concentration, respectively. All experiments were repeated at least three times in duplicate. For the extract of plants and glucantime, the concentration-response curve was plotted, from which IC50 values were determined also MTT assay was done. The different concentrations resulted in different optical densities or inhibitory percentages ($P < 0.05$) so that extract of plants were effective against *L. major in vitro*. The Findings of this study indicates that these plants are effective against *L. major in vitro*.

Keywords: Anti Leishmanial activity, *Leishmania major*, Glucantime, Promastigote.

1. Introduction

Cutaneous *Leishmaniasis* is an endemic disease in many tropical and subtropical areas. The disease usually self-limiting but may result in severe disfigurement. The manifestations can be greatly variable depending on the strain of parasite, the host's immunological status or secondary infection. Glucantime has been the mainstay therapy in the endemic regions because of its efficacy and cost effectiveness. The disadvantages of the antimonials are their requirement for intramuscular or intravenous injection each day for 20-28 days, their toxicity and the growing incidence of resistance in endemic and non-endemic regions.

Recent investigations focused on plants have shown an alternative way to get a potentially rich source of drug candidates against *Leishmaniasis*, in which effective alkaloid, phenol, tannin, flavonoid, thymol have been found. Moreover, topical treatment of CL is attractive compared with the systemic treatment because of the easy application, particularly in remote areas [1-4].

1.1 Medicinal uses of plants used in this research

Hyssopus officinalis or Hyssop (Family: Lamiaceae) has soothing, expectorant, and cough suppressant properties. The plant also includes the chemicals thujone and phenol, which give it antiseptic properties. Its high concentrations of thujone and chemicals that stimulate the central nervous system can provoke epileptic reactions when taken in high enough doses. The oil of hyssop can cause seizures and even low doses (2-3 drops) can cause convulsions in children. It has been also used in the formulation of eye drops and mouthwash. Herb hyssop has also been observed to stimulate the gastrointestinal system. Antimicrobial, antifungal, antiprotozoal and anticancer effects of Hyssop extract have been reported [5, 6].

Tussilago farfara or coltsfoot (Family: Asteraceae) has been used in herbal medicine and has been consumed as a food product with some confectionery products, such as Coltsfoot Rock.

Tussilago farfara leaves have been used in the traditional Austrian medicine internally (as tea or syrup) or externally (directly applied) for treatment of disorders of the respiratory tract, skin, locomotor system, viral infections, flu, colds, fever, rheumatism and gout [7, 8].

Carum copticum or *Trachyspermum ammi* (Family: Apiaceae) is administered in flatulence, atonic dyspepsia and diarrhea, and often recommended for cholera. In the Unani system, the herb is used as a drug to enhance the body's resistance, and is prescribed in amoebiasis, a parasitic infection of the intestines. It is a potent antimicrobial agent. The principal constituents of Bishop's Weed oil are the phenols, mainly thymol and some carvacrol. Thymol is a powerful antiseptic and antifungal agent. It is an ingredient in deodorant, mouthwashes and toothpastes. The aqueous portion, left after the separation of the essential oil, is known as omum-water and is prescribed in flatulence and gripe, especially in children. The herb is administered in gastrointestinal disorders [9-11]. The objective of the present study was to determine the effect of plant extract compared with Meglumine antimoniate (Glucantime) drug on the *in vitro* growth and viability of *Leishmania major* promastigotes.

2. Methods

The *Carum copticum* seeds and *Hyssopus officinalis* and *Tussilago farfara* leaves were air dried at room temperature and kept in a dark amber-colored bottle until processed. The plants were verified by taxonomists and botanists in the Department of Pharmacognosy, Faculty of Pharmacy, Tehran University of Medical Sciences and Faculty of Pharmacy, Iran University of Medical Sciences, Tehran, Iran.

2.1 Preparation of plants extract

The crude extracts were obtained by maceration of 100 g dried leaves and seeds in 200 ml solvent (55 ml double distilled water, 45 ml ethyl acetate and 100 ml 95% ethanol) in a dark place at room temperature. After one week, the pure extracts were filtered. The solvents were evaporated in vacuo. Dried extracts were stored in a dark amber-colored bottle. All the concentrations of the extracts were based on dry weight of the extracts.

2.2 Antileishmanial drug

Glucantime was supplied by Sigma (Sigma Chemical Co., St Louis, Mo.).

2.3 Parasite preparation

The *L. major* used in this study was the standard strain MRHO/IR/75/ER. The infectivity of the parasites was maintained by regular passage in susceptible Balb/c mice. The parasites were cultured in the RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS), 292 µg/ml l-glutamine and 4.5 mg/ml glucose (all supplied by Sigma). Under these culture conditions, the stationary phase of parasite growth was obtained in 6 days. The culture was incubated at 25 °C and used within 2 weeks of cultivation.

2.4 Various concentrations of compounds

The extract of plant solutions for biological testing and the drug were prepared in PBS at 0.05-0.1-0.2-0.4 µg/mL and 1 µg/mL, respectively.

2.5 In vitro experiments

Leishmania major promastigotes in late log phase were incubated in RPMI medium supplemented with 10% fetal calf serum, at an average of 106 parasites/ml. Parasites were incubated, in duplicate cultures, with ascending concentrations of the extract solubilized in PBS. After 24 hours incubation period at 25 °C, the surviving promastigotes were counted in a Neubauer's chamber. Half maximal (50%) inhibitory concentration (IC50) was determined as the concentration of the extract necessary to inhibit 50% of parasite growth. Negative controls treated by solvent (PBS) and positive controls containing Glucantime were added to each set of experiments.

2.6 Antileishmanial activity assays (MTT assay)

The antileishmanial activity of extracts in comparison to potassium antimonyl tartrate were evaluated *in vitro* against the promastigote forms of *Leishmania major* using a MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide)-based microassay as a marker of cell viability. The MTT assay used was based on that originally described by Mosmann (1983) modified by Niks and Otto (1990). A stock solution of MTT (Sigma Chemical Co., St. Louis, Mo.) was prepared by dissolving MIT in phosphate-buffered saline (PBS) at 5 mg/mL and storing in the dark at 4 °C for up to 2 weeks before use. For the antileishmanial activity assays, 100 µL/well of the culture which contained 2.5×10⁶ cells/mL promastigotes was seeded in 96-well flat-bottom plates. Then, 10 µL/well from various concentrations of potassium antimonyl tartrate and extracts were added to duplicate wells and plates were incubated for 72 hours at 25 ± 1 °C. The first well of 96 wells was as a blank well which only contained of 100 µL culture medium without any extracts, drug or parasite. At the end of incubation, 10 µL of MTT was added to each well and plates were incubated for 3 hours at 25 ± 1 °C. Enzyme reaction was then stopped by the addition of 100 µL of 50% isopropanol and 10% sodium dodecyl sulfate. The plates were incubated for an additional 30 min. Under agitation at room temperature. Relative optical density (OD) was then measured at a wavelength of 570 nm using a multiwell scanning spectrophotometer (ELISA reader). The background absorbance of multiwell plates was measured at 690 nm and subtract from 570 nm measurement. The absorbance of the formazan produced by the action of mitochondrial dehydrogenases of metabolically active cells is shown to correlate with the number of viable cells [12, 13, 14].

2.7 Data analysis

All experiments were repeated at least three times in duplicate. Mean values were analyzed with a two way analysis of variance (ANOVA) and the Student's *t*-test, with significance at P values of <0.05, were used to compare the antileishmanial activity of the extracts with drugs. For extracts and drug, the concentration-response curve was plotted, from which IC50 values (50% inhibitory concentrations) were determined. Differences between mean values were accepted as significant when *p* < 0.05. For calculation of IC50, they used the following mathematical formula:

$$\log(\text{IC}_{50}) = \log(x_1) + [(y_1 - y_0/2)/(y_1 - y_2)] [\log(x_2) - \log(x_1)].$$

The percentage of non-viable organisms which failed to metabolize MTT and therefore did not produce the formazan product was determined by applying the following formula (Bansal *et al.*, 2004): Percentage of non-viable organisms or inhibitory percentage at each compound concentration = $100 - ((\text{Test OD} - \text{Blank OD}) / (\text{Control OD} - \text{Blank OD})) \times 100$. All statistical analyses were done using SPSS software, version 11.5 [12, 15, 16].

3. Results

3.1 Antileishmanial assays

The extract of plants and drug inhibited the growth of promastigote forms of *L. major* *in vitro* after 24 h, 48 h, 72 h of incubation, and had a 50% inhibitory concentration (IC₅₀)

that were shown in figures respectively. Details of the *in vitro* inhibitory effect of different concentrations of extracts and drug against *Leishmania major* promastigotes are presented in Figures. 1, 2 and 3. Comparison IC₅₀ of the extracts and drug was shown in figure.4.

Also, details of reducing optical density, caused by the antileishmanial activity of different concentrations of extracts and drug on the *in vitro* growth of *Leishmania major* promastigotes, are presented in Figures. 5, 6 and 7.

Using univariate ANOVA statistical analysis and the Student's *t*-test on the results (optical density and inhibitory percentage) of the different concentrations of extracts and drug. Significance of differences ($P < 0.05$) was not determined by an unpaired Student's *t*-test and ANOVA using.

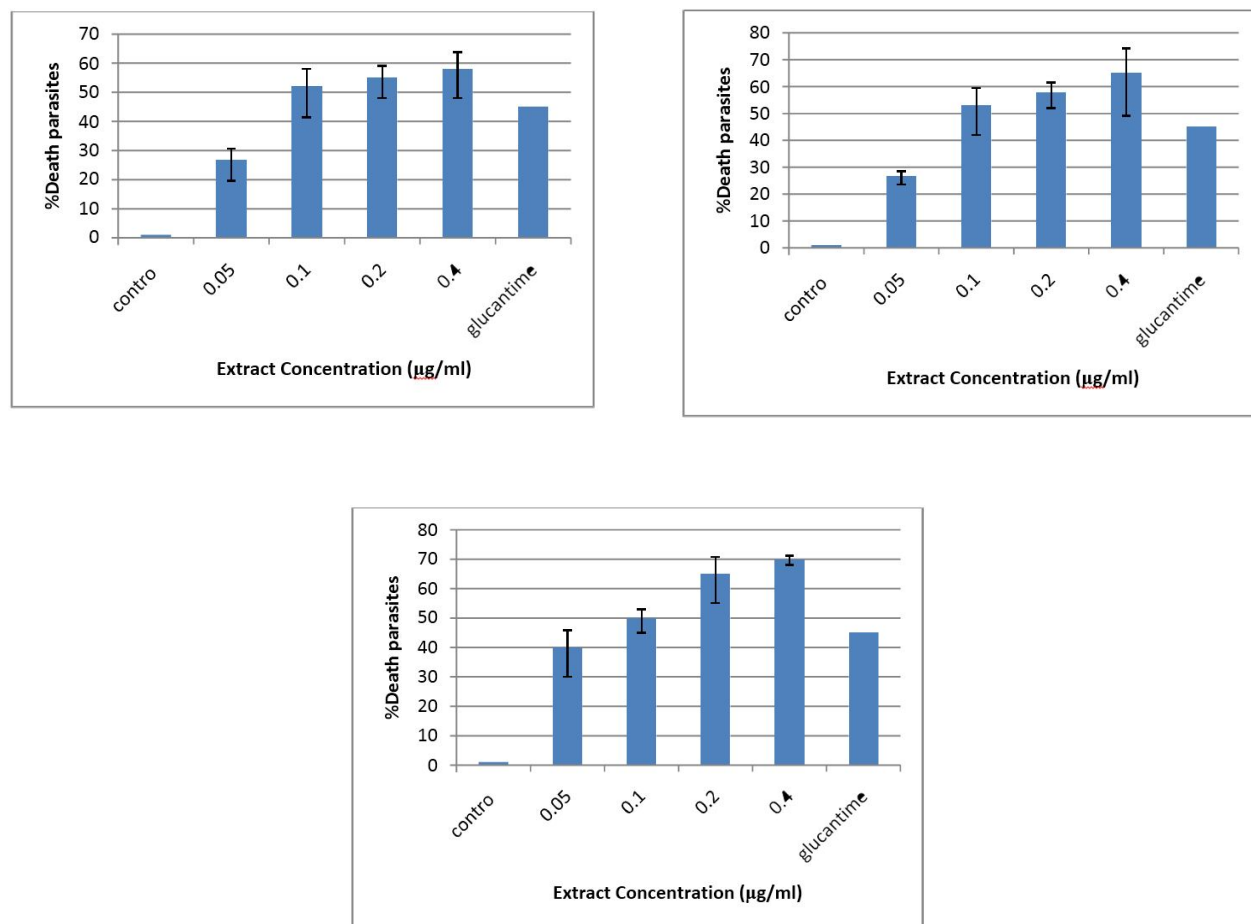


Fig 1: Inhibitory effects of different concentrations of Tussilage farfara extract on the *in vitro* growth of *L. major* promastigotes after 24, 48, 72 h respectively.

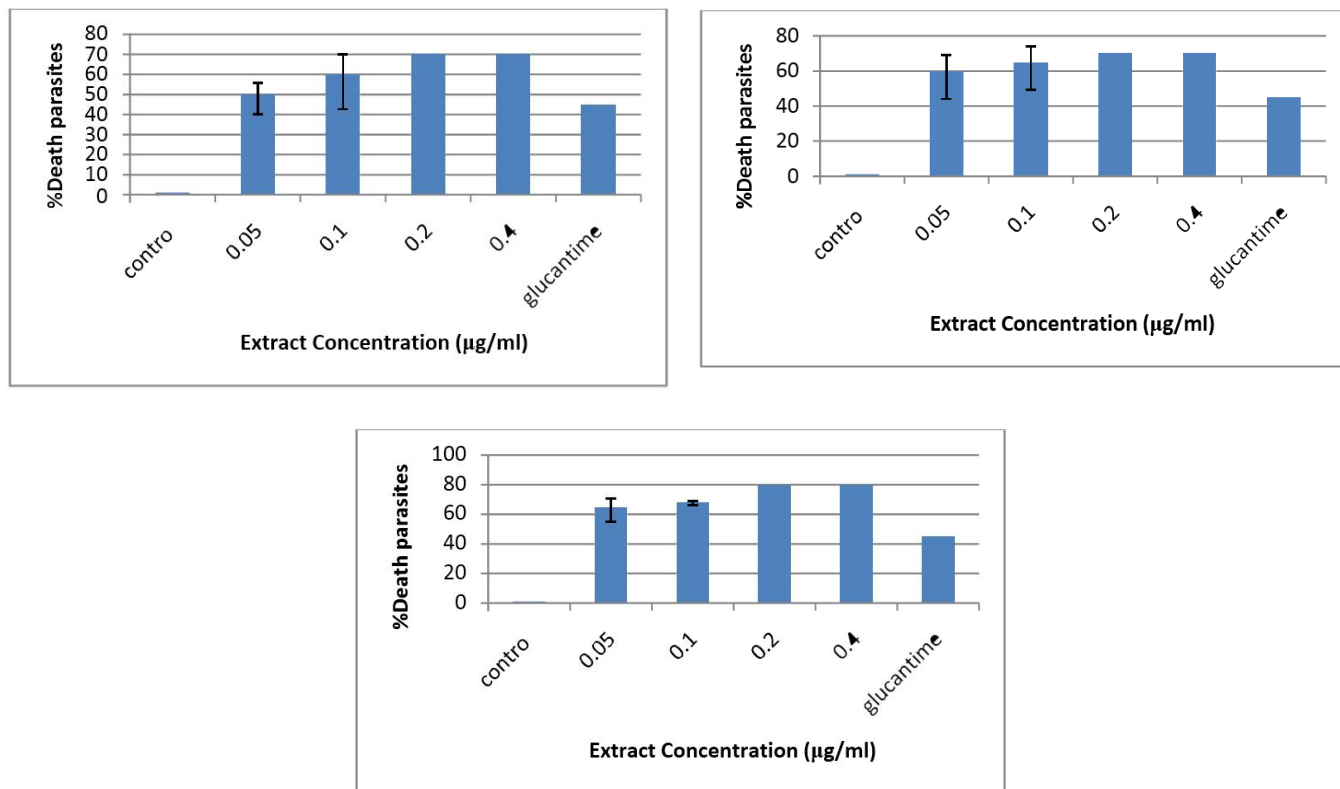


Fig 2: Inhibitory effects of different concentrations of *Hyssopus officinalis* extract on the *in vitro* growth of *L. major* promastigotes after 24, 48, 72 h respectively.

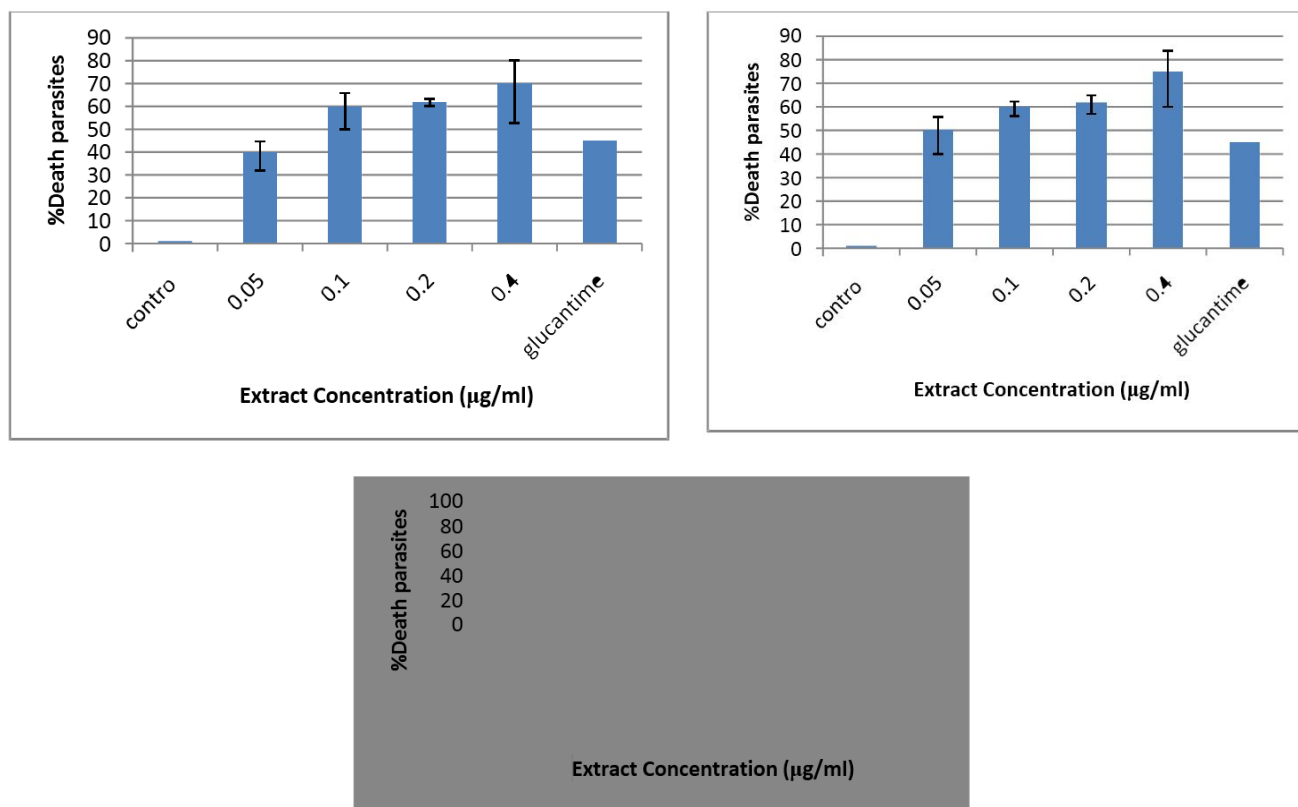


Fig 3: Inhibitory effects of different concentrations of *Carum copticum* extract on the *in vitro* Growth of *L. major* promastigotes after 24, 48, 72 h respectively.

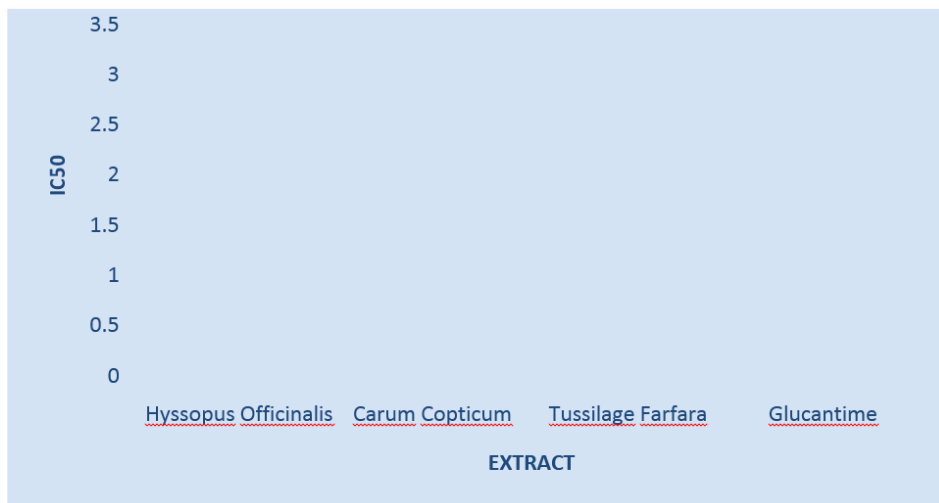


Fig 4: Comparison IC50 of the extracts and drug.

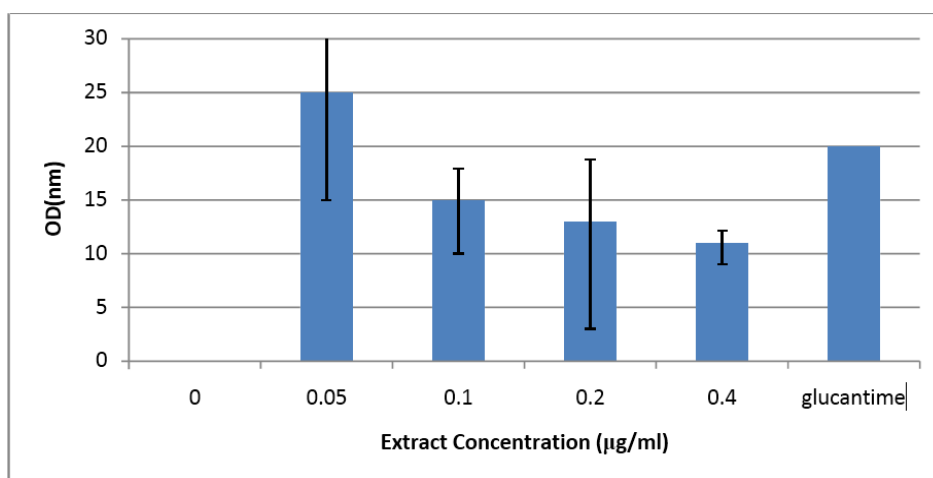


Fig 5: Reducing optical density caused by antileishmanial activity of different concentrations of Drug and Tussilage farfara on the *in vitro* growth of *L. major* promastigotes.

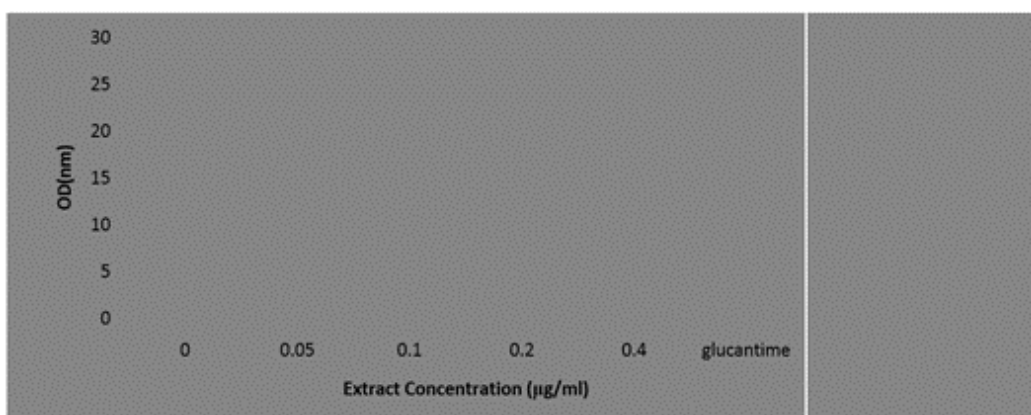


Fig 6: Reducing optical density caused by antileishmanial activity of different concentrations of Drug and *Hyssopus officinalis* on the *in vitro* growth of *L. major* promastigotes.

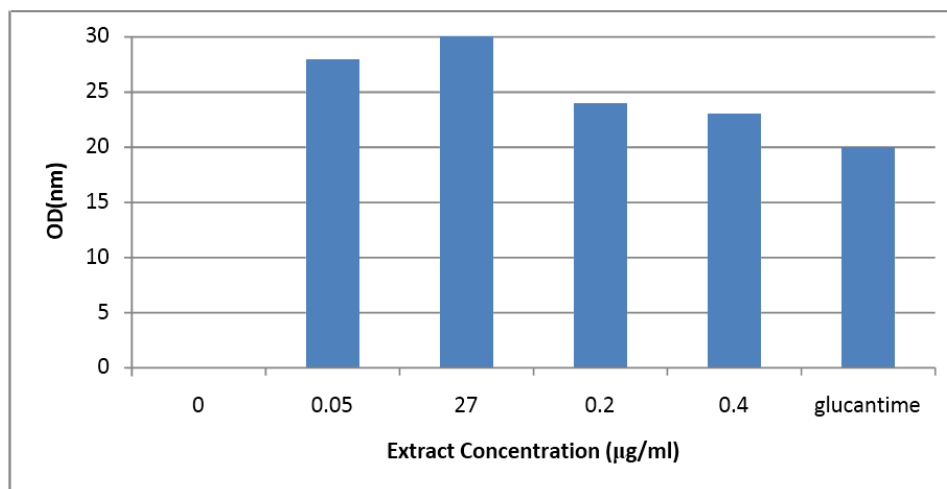


Fig 7: Reducing optical density caused by antileishmanial activity of different concentrations of Drug and Carum copticum on the *in vitro* growth of *L. major* promastigotes.

4. Discussion

There is an inclusive lack of effective and inexpensive chemotherapeutic agents for treatment of *Leishmaniasis*. Although trivalent antimonial [Sb(III)] like potassium antimony tartrate and pentavalent antimonial drugs are the first-line treatment for leishmaniasis, with amphotericin B and pentamidine being used as alternative drugs, all of these have serious side effects and resistance has become a severe problem. Therefore, new drugs are urgently required. Natural products have potential in the search for new and selective agents for the treatment of important tropical diseases caused by protozoans [17]. The results of this research show the antileishmanial activity of plants extract against *Leishmania major in vitro*. To our knowledge, based on a search of the literature, no studies have been conducted on the effects of (Carum copticum - *Hyssopus officinalis* - Tussilage farfara) plants extract on the *in vitro* growth of *Leishmania major* promastigotes. The *in vivo* efficiencies of drugs have been reported to be under the control of different parameters, such as pharmacokinetic parameters, so that for different reasons, including simplicity *in vitro* culture maintenance, routine screenings of antileishmanial chemotherapeutic agents are often based on promastigote susceptibility assays [18, 19].

In this report, a relevant viability test (MTT) was used to investigate the inhibitory effect of plant extract on the *in vitro* growth of *Leishmania major* promastigotes. Previous *in vitro* experiments with *L. mexicana* promastigotes demonstrated that antimony sodium gluconate (Triostam), a trivalent analog of sodium stibogluconate, had a 50% lethal dose of 20 µg of Sb (III)/mL. Other investigators have shown that trivalent antimonial compounds were high toxic to different *Leishmania* species in the promastigote form at concentrations ranging from 1.58 to 35.00 µg of Sb (III)/mL. Potassium antimony (III) tartrate was shown to be substantially more potent than sodium stibogluconate against promastigotes (20-22) several studies have shown that different protozoan infections have been susceptible to *P. harmala* extract in varying degrees, including *Theileria annulata*, *Theileria hirci*, *Theileria sergenti*, *Babesia bigemina*, *Anaplasma marginale*, *Babesia equi*, *Babesia cabali* and *L. major* [23, 24]. The used traditional plants in this research, medicinal herbs, have been used for antibacterial, antifungal, and antiprotozoal effects for centuries. The our results showed that there was no significant difference between plants extract

and glucantime ($P > 0.05$) in MTT and IC50 test. Consequently, plant extract is very effective against *Leishmania major* promastigotes *in vitro*. In this study, the results showed that with a concentration increase of plants extract or drug, while the inhibitory effect on the growth of *Leishmania major* promastigotes will be increased, relative optical density will be decreased. The reason for OD decrease is a decrease of formazan, which is produced by the action of mitochondrial dehydrogenases of metabolically active cells and is shown to correlate with the number of viable cells [19, 25, 26]. Therapeutic evaluations of medicinal plants are essential because of the growing interest in alternative therapies and the therapeutic use of natural products. Natural products can be lead compounds, allowing the design and rational planning of new drugs, biomimetic synthesis development, and the discovery of new therapeutic properties not yet attributed to known compounds [27]. Plant extracts *Artemisia aucheri*, *Ferula asafoetida* and *Gossypium hirsutum* inhibited the growth of promastigote forms of *L. major in vitro* after 72 hours of incubation and drug control. The other research results showed a significant decrease in the number of *Leishmania* parasites over time was due to the effect of the Abulkhalsa extract. The effect of different concentrations of Abulkhalsa extract on *Leishmania* in comparison with control medium was determined for all concentrations of the extract. All extract concentrations of extract could reduce the number of *Leishmania* parasites. Abulkhalsa extract can be applied on anti-parasite *Leishmania* researches and development of herbal medicine [28, 29].

According to other results, the concentration of 100 mg/ml of *A. annua* after 24 hour has the highest cytotoxicity effect on *G. lamblia* cysts. The other researchers in Brazil showed effective antileishmanial activity of the extract of plants (30-32). The researchers in 2010 showed Antimicrobial, Antifungal and Antioxidant Activities essential oil of Carum copticum and Hyssop essential oil [9, 33].

Natural products have made, and are continuing to make, an important contribution to this area of therapeutics. Perhaps their future potential will be even greater. This activity represents an exciting advance in the search for novel antileishmanial agents from natural sources, since an important effect against the promastigote form of the protozoan was demonstrated [33, 34]. Although these plants

showed significant activity against *Leishmania major* promastigotes *in vitro*, further synthesis and *in vivo* studies in animal models is indicated to validate these results.

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