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Comparison of *Thymus vulgaris* (Thyme), *Achillea millefolium* (Yarrow) and propolis hydroalcoholic extracts *versus* systemic glucantime in the treatment of cutaneous leishmaniasis in balb/c mice

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Abstract

Background & objectives: Leishmaniasis is a parasitic disease transmitted by sand flies. Many investigations are performed to find an effective and safe treatment for leishmaniasis. In this study, we evaluated the efficacy of herbal extracts of *Thymus vulgaris* (Thyme) and *Achillea millefolium* (Yarrow), propolis hydroalcoholic extract and systemic glucantime against cutaneous leishmaniasis in Balb/c mice.

Methods: A total of 45 mice were randomised into five groups each including nine mice. They were treated with pure ethanol 70°, systemic glucantime, *Achillea millefolium* hydroalcoholic extract, *Thymus vulgaris* hydroalcoholic extract and propolis hydroalcoholic extract for six weeks. The statistical tests including student *t*-test were used for analysis. Data were analyzed by SPSS software, *ver* 13.00.

Results: Mean of ulcer size reduction were –17.66, –22.57, 43.29, 36.09 and 43.77% for the alcohol, glucantime, yarrow, thyme and propolis groups, respectively. The results were suggestive that *Thymus vulgaris*, *Achillea millefolium* and propolis hydroalcoholic extracts were significantly more effective in reduction of ulcer size as compared with glucantime ($p = 0.006$, 0.002 and 0.008, respectively).

Interpretation & conclusion: Our results are suggestive that *Thymus vulgaris*, *Achillea millefolium* and propolis extracts are effective for treatment of cutaneous leishmaniasis in mice. Regarding these results, we suggest that efficacy of these extracts alone or in combination are evaluated against human cutaneous leishmaniasis as a randomized clinical trial.

Key words Balb/c mice – glucantime – leishmaniasis – propolis – thyme – treatment – yarrow

Introduction

Leishmaniasis is a parasitic disease transmitted by sand flies. It is characterized by a spectrum of cutaneous, mucocutaneous and visceral manifestations. The clinical presentation of this infection depends largely on the species of parasite involved and the host immune response[1].

According to recent estimates, 1.5 million new cases of cutaneous leishmaniasis (CL) occur each year. More than 90% of cases occur in five countries in the Old World (Afghanistan, Algeria, Iran, Iraq and Saudi Arabia) and two countries in the New World (Brazil and Peru)[1].

CL in the Old World is caused by *Leishmania infantum, L. major*, *L. tropica* and *L. aethiopica,* which are found in southern Europe, the mediterranean basin, the middle-east and Africa. CL in the New World is mainly caused by members of the *L. braziliensis* complex *(L. braziliensis* and *L. peruviana), L. mexicana, L. amazonensis* and *L. guyanensis* complex *(L. guyanensis* and *L. panamensis)*[1,2]. Though many therapeutic modalities have been suggested but still no definite treatment for this infection is available.

Many investigations have been performed to find an effective and safe treatment for leishmaniasis. Pentavalent antimonials are still the mainstay of treating all forms of leishmaniasis. The most commonly used organic compounds of antimony are sodium antimony gluconate (SAG) and meglumine antimoniate (MA). Although the precise mechanism of action is not fully known, the antimonials are known to inhibit glycotic enzymes and fatty acid oxidation in leishmania amastigotes, and there is a dose dependent inhibition in net formation of adenosine triphosphate (ATP) and guanosine triphosphate (GTP)[3].

Many compounds, including alkaloid, quinones, iridoids, terpenes, indole analogues have been documented to have antileishmania activity *in vitro*[4,5]. On the other hand, the aforementioned compounds can be found in some of the herbs including *Thymus vulgaris* (Thyme) and *Achillea millefolium* (Yarrow)[4,5].

Propolis is a brown colour substance that is collected by honey bee from plants. Antibacterial, antiprotozoal, antimycotic and antiviral activities have been attributed to propolis. This inhibitory effect on 21 species of bacteria including methicillin-resistant *Staphylococcus aureus* (MRSA), nine species of fungi (including *Giardia*) and wide spectrum of viruses (such as herpes and influenza) has been documented[6]. The efficacy of propolis against Trichomonas, Toxoplasmos, amebiasis, giardiasis, leishmaniasis and some other protozoal diseases has been documented[6–15].

Regarding the lack of data about the possible efficacy of these compounds against leishmaniasis, the efficacy of herbal extracts of *Thymus vulgaris* and *Achillea millefolium*, propolis and systemic glucantime against cutaneous leishmaniasis in Balb/c mice was evaluated in this study.

Material & Methods

Preparation of herbal extracts: Thymus vulgaris and *Achillea millefolium* were collected from the Isfahan Research Farms and then mended and filtered. Plant extracts were prepared by cold percolation method. The plant materials were dried under shade, ground into fine powder using electric blender and 50 g of dried powder was soaked in 300 ml 80° ethanol for 48 h with intermittent shaking. The plant extracts were filtered through Whatman No. 1 filter paper into pill vials. The filtrates were dried until a constant dry weight of each extract was obtained. The residues were stored at 4°C for further use. The remaining plant residue was dried and soaked in 300 ml of 80° ethanol as above and the extract was collected as described earlier[16].

Subjects: In this study, we used inbred; female Balb/c mice aged 4–6 weeks and weighted 30–40 grams. The mice provided by Iran Pasteur Institute were randomized into five groups each including nine mice. Groups 1 to 5 were treated with pure ethanol 70°, systemic glucantime, *Achillea millefolium*, *Thymus vulgaris* and propolis hydroalcoholic extracts.

Inoculation of parasites: Leishmania major (MRHO/

Table 1. The mean of ulcer size (mm) at the start and at the end of study and the mean of ulcer size reduction in different treatment groups

Treatment type	Mean of ulcer size (mm) before treatment (mean ± SD)	Mean of ulcer size (mm) at the end of study (mean ± SD)	Mean of ulcer size (mm) percent reduction	Standard deviation	Significance (p-value) as compared with glucantime group
Alcohol	4.27 ± 0.96	4.80 ± 2.08	–17.66	56.33	0.23
Glucantime	4.04 ± 2.29	4.74 ± 2.40	–22.57	26.40	NA
Yarrow	4.35 ± 1.20	2.7 ± 2.05	43.29	35.15	0.006
Thyme	5.39 ± 0.92	3.5 ± 1.88	36.09	26.96	0.008
Propolis	4.28 ± 0.67	2.42 ± 1.25	43.77	35.15	0.002

NA—Not available.

IR/75/ER) was the strain used. Promastigotes were cultured in Novy-Nicolle-McNeal (NNN) culture medium (10% of rabbit blood in 4% of Bacto-blood agar base) and incubated at 24°C. About 6–8 week-old female Balb/c mice were then infected with 1.6×10^6 viable stationary-phase promastigotes through intradermal injection of parasites at the base of tail[17]. The progression of the infection was monitored biweekly by the measurement of the diameters of the resulting cutaneous lesion. After 35 days of inoculation of the leishmania promastigotes, the treatment was started at the nodule site.

Treatment: The vertical and horizontal diameters of the lesions were measured by Kulis Vernier at the start of the study and weekly intervals[17]. The photography was performed using 5 Megapixel Sony digital camera and magnification of 5x at the baseline and at the end of study (eight weeks after initiation of the treatment). Hydroalcoholic extracts and pure ethanol were applied twice daily to the nodule site using cotton applicator for a period of six weeks.

Group 2 was treated with intraperitoneal injection of glucantime (0.02 ml/g) once daily for 20 days. Direct smear for leishman body was prepared at the start of treatment and also at Week 8.

Statistical tests: The descriptive statistical tests including student *t*-test were used for analysis. Data were analyzed by SPSS software, *ver* 13.00.

Results

Overall, 45 mice were evaluated. Mean of ulcer size reduction were –17.66, –22.57 , 43.29, 36.09 and 43.77% for the alcohol, glucantime, yarrow, thyme and propolis groups, respectively. The results of treatment are summarized in Table 1.

The results were suggestive that *Thymus vulgaris, Achillea millefolium* and propolis hydroalcoholic extracts were significantly more effective in reduction of ulcer size as compared with glucantime ($p = 0.008$, 0.006 and 0.002, respectively) (Figs. 1 & 2).

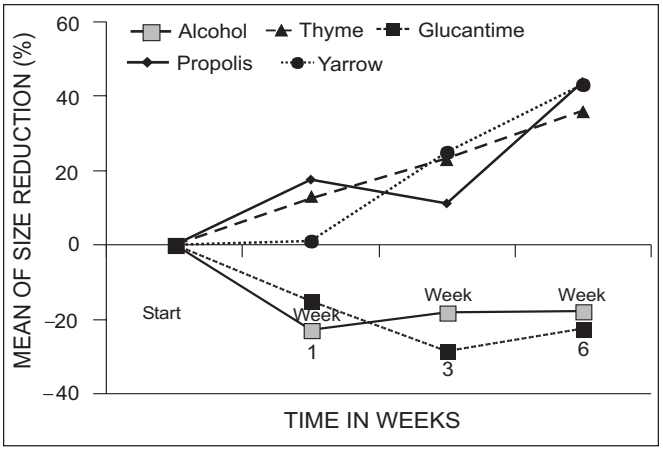

Fig. 1: Mean of leishmaniasis ulcer size reduction with alcohol, glucantime, yarrow, thyme and propolis groups throughout the study

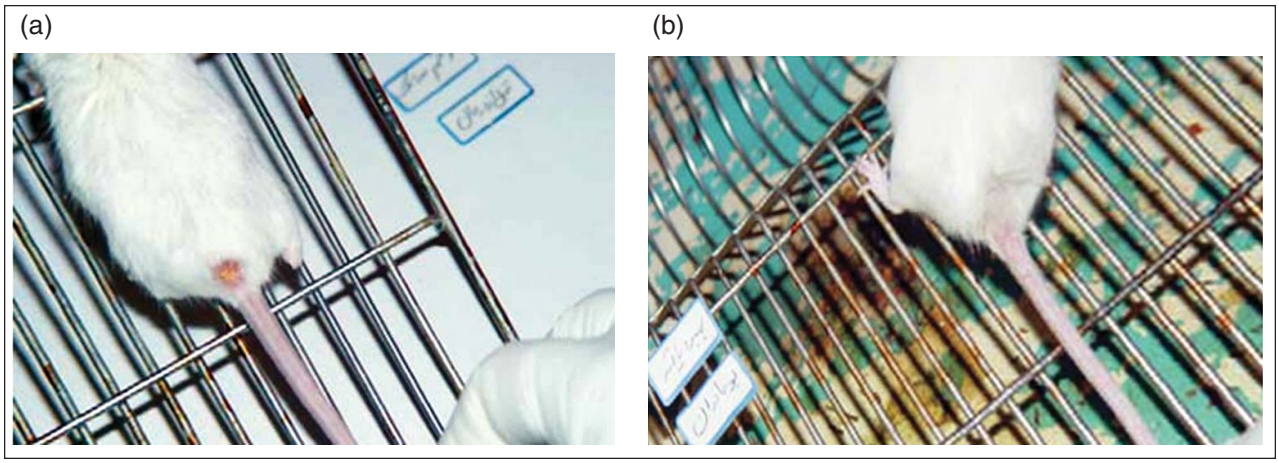

Fig. 2 (a & b): Leishmaniasis ulcer before and after treatment with yarrow hydroalcoholic extract (notice the significant reduction of ulcer size)

Discussion

Leishmaniasis is a group of tropical diseases caused by a number of species of protozoan parasites belonging to the genus *Leishmania*. This ailment affects around 12 million people in 80 countries and it is estimated that there are about two to three million new cases each year. It is also considered that presently there exists a population of 350 million of people under risk of infection[18].

Many therapeutic modalities have been used for the treatment of cutaneous leishmaniasis including physical and surgical methods. Physical methods that are commonly used for treatment of cutaneous leishmaniasis include cryotherapy, heat therapy, curettage and CO_2 laser[19–22].

Medical treatments include systemic and topical treatment. Pentavalent antimony compounds (glucantime and pentostam), chloroquine, pentamidine, metronidazole, ketoconazole, dapsone, itraconazole, terbinafine and rifampicin are common systemic drugs that are prescribed for CL[23]. Topical treatments of leishmaniasis are numerous and include quinacrine, miconazole, clotrimazole, chlorpromazine, amphotericin, garlic cream and ZHE cream[22–27]. Moheb Ali and his colleagues evaluated the efficacy of 2–5 and 5% hydroalcoholic extract of *Cassia fistula* against CL ulcers in small, white mice[28]. According to their results, 2–5 and 5% hydroalcoholic extract of *Cassia fistula* had no significant effect on healing of CL lesions. However, when higher concentration of this herbal extract was used (i.e. 25 and 40%), significant reduction of CL ulcers was observed as compared with control group. Combination of 75% *Cassia fistula* plus 2% dimethyl-sulfamide also significantly reduced diameter of ulcers[28]. The results showed that glucantime was not effective for the treatment of CL in mice. This result is consistent with the results of a previous study[29].

Due to the limited availability of effective pharmaceutical products, most people in areas where leishmaniasis is endemic depend largely on popular treatments and traditional medicines to alleviate the symptoms[4]. In addition to the various methods already mentioned, the treatment of leishmaniasis following the traditional medical practices of different cultures depends heavily on the use of native plants. In traditional medicines, the treatment of leishmaniasis usually consists of the oral administration of crude plant extracts for the systemic form of the disease and as topical preparations of the corresponding extracts for the treatment of skin infections[18]. With this knowledge, and as part of its search for new and bet-

ter pharmaceuticals of high availability and low toxicity, the Tropical Diseases Programme of the World Health Organization has considered the investigation of plants used in traditional medicine practices for the treatment of leishmaniasis as essential and of high priority[4]. Most of the studies directed towards the detection of plant secondary metabolites with leishmanicidal activity, have been done using the promastigote form of the parasite because it is easier to maintain these under *in vitro* conditions. However, since the promastigote is not the infective form of the parasite in vertebrate hosts, evaluations done with promastigotes have only an indicative value of the possible leishmanicidal activity of the metabolite tested. As a result of this, a preliminary evaluation using promastigotes must be complemented with an evaluation using intracellular amastigotes in macrophages as an *in vivo* study[30].

The results of present study showed that hydroalcoholic extracts of *Thymus vulgaris*, *Achillea millefolium* and propolis were significantly more effective than systemic glucantime or alcohol for the treatment of leishmaniais in Balb/c mice. The highest efficacy was observed for propolis, followed by *Achillea millefolium* and then *Thymus vulgaris*.

Thymus vulgaris and *Achillea millefolium* contain different compounds including alkaloid, quinones and iridoids. The exact mechanisms of action of these compounds should be investigated although it has been shown that alkaloids are able to intercalate DNA or interfere with the metabolism of aromatic amino acids in the parasite[31]. Application of mixture of thyme and yarrow compounds may even yield better results. We suggest that further studies on efficacy of this mixture *versus* each extract alone against CL in Balb/c mice are carried out.

The antibacterial, antiprotozoal, antimycotic and antiviral activities of propolis have been documented previously. The *in vitro* antileishmania activity of propolis has been suggested by Savoia[9].The results of our study showed high efficacy of this compound against leishmaniasis *in vivo*. This effect is possibly due to stimulation of natural killer cells activity[12]. Other possible mechanism for this effect is through release of nitric acid and tumor necrosis factor from macrophages[15].

The most effective concentration along with best vehicle for this compound should be evaluated in a more extensive study. Our results are suggestive that *Thymus vulgaris*, *Achillea millefolium* and propolis extracts are effective for the treatment of cutaneous leishmaniasis in mice. Regarding these results, we suggest that efficacy of these extracts alone or in combination are evaluated against human cutaneous leishmaniasis as a randomized clinical trial.

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