The Cytotoxic and Antileishmanial Effects of *Satureja khuzestanica* Essential Oil

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Abstract

Background and Aim: Leishmaniasis is endemic in 98 countries including Iran. Pentavalent antimony compounds resistance as first-line therapy is increasing in some local areas. Also side effects of these drugs are limited at the beginning of treatment, but the toxicity increases with time. The aim of this study was to assess the effect of Satureja khuzestanica essential oil (SKEO) on promastigote and amastigote *Leishmania major* forms.

Materials and Methods: The components of *S. khuzestanica* oil were identified by gas chromatography/mass spectroscopy (GC/MS) analysis. To evaluate antipromastigote activity the different concentrations of extract and glucantime were added to the wells that contained *L. major*. The plates were incubated at 26±1°C for a week. On days 1, 3 and 5, the number of live promastigotes in each well was counted. For assessment of SKEO effect on intracellular amastigotes, mouse peritoneal macrophages were isolated and infected with promastigotes. Different concentrations of the extract and glucantime were added to the cultures. The cultures were incubated at 37°C and CO_2 5%. The number of infected macrophages and amastigotes within each macrophage were counted. Toxicity assessment of SKEO on macrophages was done by MTT method.

Results and Conclusions: The mean number of promastigotes, infected macrophages and amastigotes in a macrophage in the control and treated groups had significantly difference. So that their number in the treated groups was less as a dose-dependent response. The present research showed potent antileishmanial activity of, SKEO; additionally this plant had no toxic effect on mammalian cells.

Keywords: Satureja khuzestanica essential oil, Leishmania major, Macrophage, Amastigote, Promastigote

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Introduction

Western countries, due to the increase in simultaneous infection with HIV, the incidence is increasing (1, 2).

The most common form of leishmaniasis is cutaneous leishmaniasis (CL) with 1 to 1.5 million annual cases (3). CL is considered as one of the health problems in some provinces of Iran and more than 20000 cases of it are reported annually (3). In Iran, the Leishmania tropica and Leishmania major are the factors for urban leishmaniasis and rural leishmaniasis, respectively (3). The antimony pentavalent compounds such as stibogluconate sodium (Pentostam) and meglumine antimonate (Glucantime) are generally considered as first-line treatment of cutaneous leishmaniasis. The side effects were limited at the beginning of treatment, but the toxicity continues to rise.

In Iran, 10%-15% of infected patients with CL do not respond to treatment with glucantim, and one of the reasons is the drug resistance (4). For this reason, several studies have been done to treat the disease (5-9).

There is no drug prevention about this disease, the vaccines are still being studied and controlling the vectors is possible only in some epidemic certain conditions (10). Hence, in recent years the researchers have studied the use of medicinal plants to treat this disease. One of the interesting plants is genus of Satureja that belongs to the family Lamiaceae.

The essential oil of this plant, which is extracted from its leaves, includes two types of phenol isomer named Thymol and Carvacrol, and the medicinal effects of the plant is because of these two substances (11).

Carvacrol with chemical formula of (C10H14O) 2-methyl-5-[1-methylethyl] and molecular weight of 150.221 gr/mol is a phenolic compound, which is the most constituent of Satureja khuzestanica essence.

The Iranian specie is S. khuzestanica Jamzad ("Marzeh khuzestani" in Persian), which has been used in folk medicine to treat several illness and diseases. Review have reported some therapeutic properties of this plant such as analgestic, antiseptic, antibacterial, antiviral and antifungal (18-24). The presnt study aims to evaluate the antileishmanial effect of *S. khuzestanica* essential oil (SKEO) on promastigote and amastigote stages of *L. major* on invitro and also its cytotoxic effects on murine macrophage cells.

Materials and Methods

Preparation of SKEO

The extraction of *S. khuzistanica* was performed by hydro distillation and clevenger apparatus for 5 hours. Once extraction was completed, the resulted essential oil was dehydrated using sodium sulfate (Merck). The essential oil was stored at temperature of 4ºC until using (12). In order to provide different concentrations of the extract, Dimethyl sulfoxide (DMSO) was used.

Gas chromatography/mass spectrometry analysis of essential oil

Isolation and measurement of the sample was done by coupled gas chromatography device (GC/MS) SHIMADZU 17 A with SHIMADZU mass spectrometry of QP5050A model and isolation of components was conducted in Fused Silica capillary column of the DBX-5 95% (polydimethylsiloxane) with a length of 30 m with internal dimensions of 0.25 mm and film thickness of 0.25µm. The column temperature was increased from 40°C to 220°C with speed of 5°C/min. Then it reached to 280°C with speed of 30°C/min and was stored there for 2 minutes. The temperature of injection site was adjusted at 260°C and temperature of detector was adjusted at 260°C (transfer line). The Helium with a speed of 0.9 ml/min was used as carrier gas with a purity of 99.999%. The spectrometer condition was exactly in accordance with gas chromatography, and only the ionization energy of 70 eV was used, and also for identification of the spectrum with inhibition indices, the normal hydrocarbon injection (C8-C20) under the conditions of sample injection was used.

Parasite Culture

L. major (MRHO/IR/75/ER) promastigotes were cultured at 26±1ºC in RPMI 1640 (Gibco- BRL) plus 10% fetal bovine serum (FBS) (Gibco-BRL), 100 IU/mL penicillin and 100µg/mL streptomycin.

Antipromastigote activity of SKEO

L. major promastigotes (5×10^4 cells/well) were added to each of the 96 plate wells.

The wells containing promastigotes were classified based on the substances that were added to them as following:

1. Control group 1: The wells containing promastigotes and complete RPMI culture medium.
2. Control group 2: The wells containing promastigotes and DMSO.
3. Intervention groups: The wells containing promastigotes and different concentrations of SKEO (0.1, 0.3, 0.6, 1.2, 2.5, 5, 10 and 20 µg/ml).
4. Groups treated with glucantime: The wells containing promastigotes and different concentrations of glucantim (0.2, 1, 5, and 25 µg/ml).

The tests were done at three series. The plates were incubated at 26 ± 1°C for a week. In days 1, 3 and 5, the number of live promastigotes in each well was counted using hemacytometer cover glass and try pan blue staining. The dead parasites were blue and completely inactive and the live parasites were active and not dyed.

MTT assay

Antipromastigote effect of SKEO on promastigote stage of *L. major* by colorimetric cell viability MTT was assessed (13). Preparations plates were performed similar to those mentioned above and incubated at 25±1°C for 72h. After incubation, supernatants were thrown off by centrifuging plates in 2000 rpm for 5min. Then 100 µl RPMI-PR- which included 10µl MTT (5mg/ml) was added to each well. The plates were incubated for 4h at 22°C in the dark. After that plates were centrifuged again for 5 min in 2000 RPM and supernatants discarded. One hundred µl DMSO was added to each well.

Positive control was cultured promastigotes in the complete medium with no drug. Also blank was the complete medium with no promastigotes and drugs. Absorbance was measured at 492nm by an ELISA reader. The 50% inhibitory concentration (IC50) was determined as antipromastigote activity using linear regression (13).

The effect of SKEO on intracellular *L. major* amastigotes

BALB/c male mice were used for evaluation of SKEO effect on amastigote. The peritoneal macrophages were recovered, counted and the viability of cell percentage was determined (14). The protocol was approved by the Committee on the Ethics of Animal Experiments, Lorestan University of Medical Sciences. Then the cells were transferred to glass coverslips inserted in 6-well plates at 5×10^5 cell/well and incubated at 37°C in 5% CO2 for 5 h. Afterward non- adherent cells were removed by washing with 37°C phosphate buffered saline (PBS) and RPMI1640 medium containing FBS 10%, 100 IU/ml penicillin and 100µg/ml streptomycin was added to each well and incubated overnight. No parasite was added to a group of plates that were considered for viability evaluation of macrophage during the study. The next day different concentrations of SKEO (0.1, 0.3, 0.6, 1.2, 2.5, 5, 10 and 20 µg/ml) and glucantime (0.2, 1, 5 and 25 µg/ml) were added into separate wells and incubated at 37°C in 5% CO2. The extract was not added to the number of wells considered as control group. Tests were performed as triplicate. On days 1, 3 and 5 after adding SKEO the coverslips were washed, fixed in methanol, and stained with Giemsa. The number of infected macrophages and amastigotes was determined by counting at least 200 macrophages in triplicate cultures. The results were expressed as the mean of infected macrophages and amastigotes per infected macrophage. IC50 values of the extract and MA were calculated.

Cytotoxic effects of SKEO

To evaluate the cytotoxic activities on peritoneal macrophage, CC_{50} (cytotoxicity concentration for 50% of cells) of increasing concentrations SKEO and glucantime was determined. For this purpose, 1×10^5 macrophages in the presence of various concentrations of SKEO (0-160 µg/ml) and glucantime (0-250 µg/ml)were incubated in 96-well flat-bottomed culture plates for 72h at 37°C in 5% CO_2. Control group included cultured macrophage without extract and drug was considered. MTT assay was done as mentioned above. The absorption rate in wells was measured with ELISA reader at wavelength of 570nm (14). CC_{50} was determined using linear regression. Selectivity index (SI), was calculated based on the ratio of CC_{50} for peritoneal macrophage/IC_{50} for intra macrophage amastigotes, as described by Weniger e*t al.* (15).

Statistical analysis

In order to compare the number of promastigotes, infected macrophages and amastigotes per infected macrophages in different test groups, the one-way

ANOVA was used at a significant level of 0.05. For pairwise comparison of test groups, the Tukey post hoc test at significant level of 0.05 was used.

To measure the effect of time on the number of promastigotes, amastigotes and infected macrophages and also interaction effect of test group and time, the repeated measures analysis test at the significant level of 0.05 was used. Comparison of tests was conducted pairwise based on Sidak test.

Results

Characteristic of SKEO extracted

According to Table 1, the concentration of carvacrol, which is the active substance in SKEO, was equal to 77.51%. The GC chart of the cultured SKEO compounds was shown in Fig. 1. The extraction efficiency was reported equal to 2.9%. The essential oil density was 0.951 g/ml.

The effect of SKEO on promastigote growth

The one-way ANOVA results showed that in terms average number of promastigotes, there was a significant difference between test groups (P<0.001). The results of counting promastigotes and MTT assay showed a significant difference between the intervention groups and control groups (P<0.001). Comparing the effect of highest concentration of SKEO and glucantim on average number of promastigotes representing the faster effect of SKEO compared to glucantim (Fig. 2).

IC_{50} value of SKEO and Glucantime at 72h after treatment was 11.31±0.22 µg/ml and 14.98±0.93 µg/ml, respectively.

The effect of SKEO on amastigotes

According to Tukey test results, in 2% SKEO group the highest effect on the number of infected macrophages and amastigotes per infected macrophages was reported (Fig. 3, Fig. 4).

According to Fig. 3 and 4, the number of macrophages and amastigotes per infected macrophage had an absolute increasing trend in the test group, which showed a significant correlation between different times of counting and the number of infected macrophages.

The results showed a significant difference between the number of infected macrophages and amastigotes per infected macrophage in different groups (P<0.001). IC50 value of SKEO and Glucantime at 72h after treatment was 10.19±0.16 µg/ml and 12.65±0.74 µg/ml, respectively.

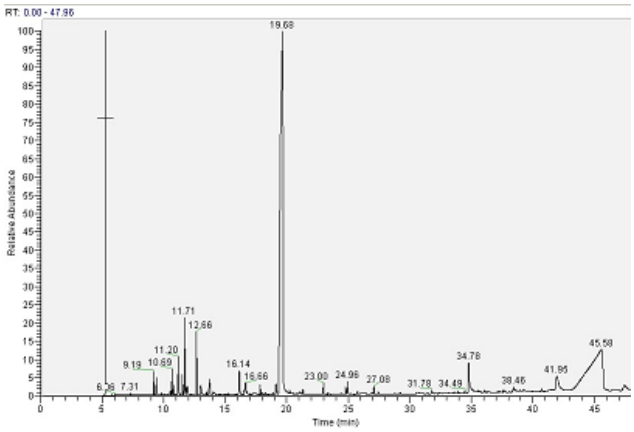

Fig. 1. Chromatogram of SKEO compounds by using GC/MS method.

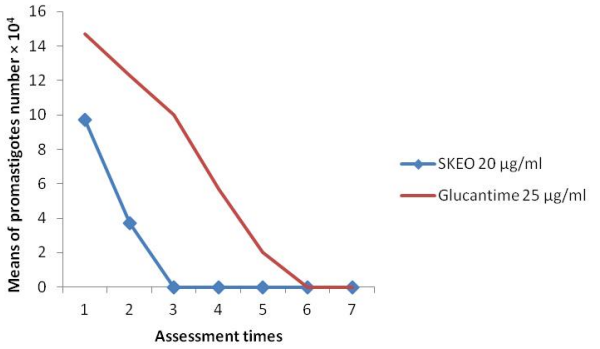

Fig. 2. Comparison of the highest concentration effect of SKEO and glucantime on the average number promastigotes.

The cytotoxic effect of SKEO on macrophage

The cytotoxic effect of SKEO on peritoneal macrophages with MTT assay demonstrated that applied concentrations had no toxic effect on macrophages. CC50 values for SKEO and Glucantime were 161.42±4.35 µg/ml and 169.87±6.82 µg/ml, respectively. SI values were 15.84 and 13.42 for SKEO and Glucantime, respectively. SI of greater than 10 for SKEO extract indicates their safety to the macrophages and specificity to the parasite according to Weniger *et al*. (2001) (16).

Discussion

The obtained findings revealed that the essential oil of *S. khuzestanica*, which is obtained from its leaves contain phenol isomer named Carvacrol and the

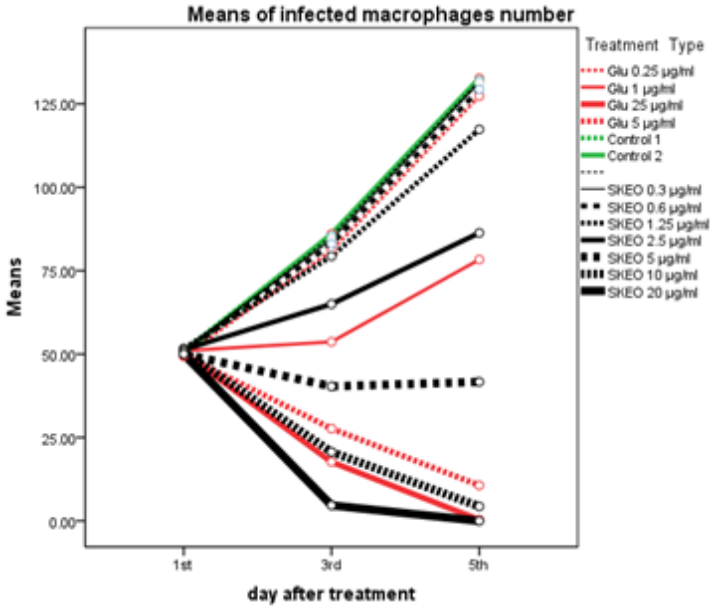

Fig. 3. Effect of different concentrations of SKEO on mean of infected macrophage in each group over the study days.

medicinal effects of the plant are related to it (11). Several studies have been conducted on the effects of medicinal plants on *Leishmania*.

El-On *et al*. conducted a study in 2009 on anti-*Leishmania* property of *Nuphar lutea*. The results showed that the plant reduced the growth of promastigote and amastigote (17). In 2009, Yousefi et al. examined the effect of Harmal seeds and *Alkanna tincturia* root and stem on the promastigotes and amastigotes of *Leishmania* in vitro culture. The extract could reduce the growth of the parasite factors (18). In several studies, different properties of *S. khuzestanica* were examined. In 1384, Sepahvand *et al*. showed the antifungal effect of essential oil of *S. khuzestanica* on several genera of fungi (19). In 1380, Delfan and Lorzadeh examined the effect of *S.*

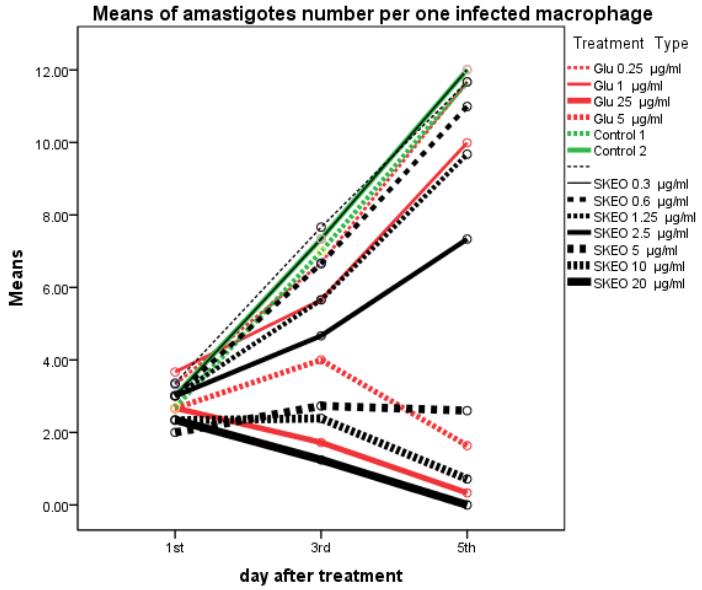

Fig. 4. Effect of different concentrations of SKEO on mean of amastigotes in a infected macrophage in each group over the study days.

khuzestanica on the treatment of dysmenorrhea and achieved its positive effect on pain reduction (20). In a study, the effect of SKEO was examined on cutaneous lesion of *L. major* in BALB/c mice, which resulted in the decreased mortality in infected mice (21). For accurate identification of anti-Leishmania effect of research extract in vitro culture, it was required to design the complementary research based on its results.

Table 1: Chemical compounds of SKEO.

RI	Area%	*Satureja khuzestanica essential oil*
841	0.04	2-methyl ethyl ester butanoic acid
928	0.63	α-thujene
938	0.49	α-pinene
954	0.05	Camphene
965	0.04	2-methylbuthyl propionate
982	0.15	β-pinene
988	0.72	β-Myrcene
993	0.27	dehydro-1,8-cineole
1008	1.16	α-phellandrene
1014	0.04	(MS)
1019	0.6	α-Terpinene
1026	2.62	P-cymene
1031	0.2	limonene
1033	0.28	β-phellandrene
1035	0.27	1,8-cineole
1060	2.04	γ-Terpinene
1073	0.89	cis-sabinen hydrate
1091	0.12	α-Terpinolene
1099	1.03	Linalool
1110	0.31	trans-sabinen hydrate
1184	1.53	4-Terpineol
1202	1.04	α-Terpineol
1207	0.12	cis dihydro carvone
1244	0.31	Carvacrol methyl ether
1251	0.05	Z-Citral
1260	0.08	D-Carvone
1283	0.1	E-Citral
1291	0.77	Thymol
1311	**77.51**	**Carvacrol**
1333	0.08	(MS)
1362	0.11	Eugenol
1373	0.14	Carvacrol acetate
1438	0.37	trans caryophyllene
1452	0.05	Geranyl aceone
1508	0.22	(E,E)-α-Farnesene
1516	0.41	β-Bisabolene

In the present study, the effect of different concentrations of SKEO was examined the number of promastigotes, the number of infected macrophages and amastigotes per infected macrophage.

According to the results of the present study, the mean number of promastigotes was decreased by increasing the concentration of the extract, and there was a significant difference in comparison with control groups ($P<0.001$), so that the decreased light absorption in the MTT assay was also demonstrated this subject. It should be noted that the effect of 25µg/ml glucantim and 20µg/ml SKEO have significant difference ($P<0.001$), so that the SKEO extract has a greater effect.

The results of examining the extract effect on the number of infected macrophages and amastigotes per infected macrophage showed that in vicinity of the extract with high concentration over the time, they had an absolute reduction and significant difference from control groups ($P <0.001$). According to the results of this research, it seems that not only SKEO is antifungal, antiviral, antibacterial but also antiparasitical.

The results showed that SKEO can reduce the number of promastigotes, infected macrophages and the amastigotes per infected macrophage in vitro culture. The effect was obvious in higher concentrations of extract (20 and 10 µg/ml) and their effect can be competed with the highest glucantim concentration (25µg/ml).

Conclusion

According to SKEO plant origin, and its significant effect on parasite proliferation inhibition, and also the higher effect of extract compared to glucantim, which is now considered as first-line treatment, it is suggested that by having further studies on the mechanism of the extract effect, good results can be achieved in the treatment of cutaneous leishmaniasis.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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