J Parasit Dis
DOI 10.1007/s12639-015-0650-8

ORIGINAL ARTICLE

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Effect of olive leaf, *Satureja khuzestanica*, and *Allium sativum* extracts on *Giardia lamblia* cysts compared with metronidazole in vitro

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Received: 9 December 2014 / Accepted: 20 January 2015

Abstract *Giardia lamblia* is one of the common causes of worldwide diarrhea in children. Appropriate medicinal treatment for giardiasis is available but there are some evidences of drug resistance, insufficient efficacy, and unpleasant side effects. In order to reach a more natural drug with suitable efficacy and the lowest side effects, the effects of the hydroalcoholic extracts of olive leaf, *Satureja khuzestanica*, and *Allium sativum* on *G. lamblia* cysts were evaluated in vitro, as well as antigiardial effect of the extracts was compared with metronidazole as the drug of choice. 2 and 5 mg of the plants extracts and powder of metronidazole 250 mg pills were added to 1 ml of *G. lamblia* cysts suspension (containing 5,000 cyst/ml normal saline), and the percentages of bioavailability of *G. lamblia* cysts were examined at the 2nd and 4th h after exposure and in 4 and 37 °C temperatures using eosin 0.1 % and a haemocytometer. The data were analyzed by multiway ANOVA test, Tukey's test, and the SPSS software, version 18. The examinations demonstrated that olive leaf extract had the most fatality rate on *G. lamblia* cysts in vitro (37.90 ± 7.01 %), followed by the extract of *S. khuzestanica* (32.52 ± 9.07 %). Metronidazole 250 mg pills had relatively effective fatality rate on *G. lamblia* cysts in vitro (28.75 ± 10.30 %), whereas *A. sativum* (garlic) had the lowest fatality effect on *G. lamblia* cysts in vitro (22.65 ± 10.47 %). With respect to higher fatality effect of olive leaf and *S. khuzestanica* extracts compared with metronidazole in vitro, these plants can be used as suitable candidates to make new antigiardial drugs with low side effects and without drug resistance in the treatment of giardiasis in children.

Keywords Olive leaf · *Satureja khuzestanica* · *Allium sativum* · Metronidazole · *Giardia lamblia* · In vitro

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Introduction

Despite great scientific advances of humans, giardiasis is still one of the most common parasitic diseases producing diarrhea in children worldwide. In 1998, the World Health Organization (WHO) reported that only in Asia, Africa and America over 200 million *Giardia lamblia* infections are reported yearly (Macotela et al. 2004). Contamination with this pathogenic protozoan leads to malnutrition, diarrhea, nausea, cramps, jaundice, avitaminosis, and weight loss in

Published online: 14 February 2015

children (Gadelha et al. 2005). The drug of choice in the treatment of giardiasis is metronidazole (Flagyl), which is among the nitroimidazole derivatives and effective on anaerobic bacteria and protozoa. Other drugs used to treat giardiasis are furazolidone and quinacrine (Harris et al. 2001a; Calzada and Alanis 2007). Metronidazole has been used for several decades in the treatment of protozoan, bacterial and fungal infections; nonetheless, there have been some reports regarding the carcinogenic and teratogenic effects on laboratory rats and drug resistance to some isolates of *Giardia*. Unpleasant side effects including headache, nausea, dizziness, and metallic taste in the mouth have been reported in patients treated with metronidazole (Harris et al. 2000, 2001a). In order to invent appropriate alternative drugs with low side effects and high efficacy, studies on various plants have been started.

Satureja khuzestanica, an indigenous plant widely found in southern parts of Iran, is known for its antiseptic and painkilling health benefits in traditional medicine. In recent years, its antiviral, antibacterial, antifungal, anti-inflammatory, antispasmodic, anti-diarrheal, and vascular dilatation effects of various species of this plant have been reported in different regions of the world (Haeri et al. 2006). Chemical analyses carried out on *S. khuzestanica* have shown that its effective substance is Carvacrol that constitutes most of the compound (Shahsavari et al. 2009). Garlic (*Allium sativum*) has been used by humans for many years due to its numerous health benefits. According to old documents, central Asia and China are said to be the origin of this plant due to its properties. The plant has a wide range of medicinal properties. It not only is antibacterial, antiviral, antifungal and anti-protozoan, but also it has beneficial effects on the cardiovascular and human immune systems (Harris et al. 2000, 2001b). Most of these properties of garlic are attributed to Allicin, its effective substance (Harris et al. 2000; Ankri and Mirelman 1999). In addition to its fatality effect on various pathogens, the extract prepared from olive tree leaves can reduce blood pressure and cholesterol as well. Olive leaf extract properties are due to the effectiveness of its component called Oleuropein. The extract of the leaf of this plant has antibacterial, anti-viral, anti-fungal and anti-protozoan effects. Some common pathogenic parasites destroyed by the plant in vitro include amoeba, *Cryptosporidium* Spp., *Giardia* Spp., *Enterobius vermicularis*, tapeworms, nematodes and spiral worms (Paliva-Martines 2001).

Considering the disadvantages of metronidazole as the first-line treatment of giardiasis and in order to access natural medicines with limited side effects and high efficacy, the effects of the hydroalcoholic extracts of *S. khuzestanica*, *A. sativum*, and olive leaf were evaluated on *G. lamblia* cysts in vitro and their effects were compared with the effect of metronidazole.

Materials and methods

Preparation of plant extracts and concentrations needed

Fresh leaves of olive and *S. khuzestanica* collected from Khersdar region in Poldokhtar (south of Lorestan Province, Iran) were washed with distilled water and dried in the shade. The dried leaves were powered using a mill, and the extracts were taken using a Soxhlet extractor and 80° ethanol for olive leaf and 70° ethanol for *S. khuzestanica*. After concentrating the extracts obtained with a rotary device (vacuum distillation), they were kept and flattened in glass containers and dried at room temperature. Then, the extracts were powdered and kept in completely closed dark glass containers at 4 °C until they were used. The operational efficiency for olive leaf and *S. khuzestanica* extracts were 25 and 17.5 % respectively. The commercial hydroalcoholic extract of *A. sativum* (EBN-E-MASOUYEH, Iran) was bought and used.

Evaluation of *Giardia* cysts sensitivity to the plant extracts

Giardia cysts suspension used in this study was obtained from samples of patients with giardiasis which had been concentrated using the sedimentation technique with normal saline and centrifuged 5 min at $500\times g$ several times. A hemocytometer $5 \times 1{,}000$ cyst/ml was applied to determine the number of the cysts. 2 and 5 mg of each extract were added to tubes containing 1 ml of *Giardia* cysts suspension. Thus, two concentrations (2 and 5 mg/ml) were prepared for each plant extract and were evaluated along with two similar concentrations of metronidazole at two temperatures of 4 and 37 °C and at the 2nd and 4th h after exposure. The bioavailability of *G. lamblia* cysts were examined using 0.1 % eosin vital staining and a hemocytometer with 40× magnification of an optical microscope. Eosin stain solution accompanied by *Giardia* cysts suspension, and *Giardia* cysts suspension alone were considered as the control groups of the study. All the examinations were repeated three times and the percentage of the dead cysts was calculated via the following formula:

$$(D/(L + D)) \times 100$$

L is the number of living cysts, D is the number of dead cysts.

Statistical analysis of the data

After completing the tests, the data were analyzed using the statistical test of multiway analysis of variance (ANOVA), Tukey's test and the SPSS software, version 18.

Incidentally, all the tests were performed considering the significance level of 0.05.

Results

The results of multiway analysis of variance statistical test indicated that there was a significant relationship between the variables of type of plant extract/drug ($P < 0.001$), concentration of plant extract/drug ($P = 0.002$), exposure time ($P = 0.012$), and temperature ($P = 0.011$), on the one hand, and the percentage of dead cysts, on the other hand. The examinations showed that that olive leaf extract had the greatest fatality rate on *Giardia* cysts in vitro (37.90 %), followed by *S. khuzestanica* (32.52 %). 250 mg metronidazole pills had relatively effective effect on *Giardia* cysts in vitro (28.75 ± 10.30 %), while the lowest effect was observed for *A. sativum* with a fatality rate of 22.65 ± 10.47 %. However, Tukey's multiple comparison test revealed that the fatality rate of olive leaf extract was clearly and solely more than the effects of the other extracts/drug. The extracts of *S. khuzestanica* and metronidazole fell in a similar group in terms of the percentage of fatality rate on cysts, and *A. sativum* extract fell in a separate group alone. Moreover, the results of multiway ANOVA showed an interaction between type of extract/drug and concentration of extract/drug ($P = 0.007$), so that in all the extracts/drugs, except olive leaf extract, the fatality rates increased with an increase in concentration. No significant interactions were found between the other factors of the experiment. The details of the multiway ANOVA test are presented in Table 1.

Olive leaf extract with a concentration of 2 mg/ml, had the most fatality rate on *Giardia* cysts in vitro 4 h after exposure at a temperature of 37 °C (45.34 ± 0.42 %). *S. khuzestanica* with a concentration of 5 mg/ml, had an effective fatality rate on *Giardia* cysts in vitro 4 h after exposure at 4 °C (38.05 ± 5.66 %). The highest fatality rate on *Giardia* cysts for *A. sativum* was found with a concentration of 5 mg/ml in vitro 4 h after exposure at 37 °C (36.69 ± 6.99 %). Also, the highest fatality rate for 250 mg metronidazole pills in vitro was found with a concentration of 5 mg/ml 4 h after exposure at 4 °C (34.86 ± 4.57 %). The details of the experimental groups in terms of concentration, exposure time and temperature are presented in Figs. 1, 2, 3 and 4.

Table 1 Factors affecting the percentage of fatality rates on cysts based on multiway ANOVA test

Variable	P value
Type of extract/drug	$P < 0.001$
Concentration of extract/drug	$P = 0.002$
Exposure time	$P = 0.011$
Temperature	$P = 0.012$
Interaction of type of extract/drug and concentration	$P = 0.007$
Interaction of type of extract/drug and exposure time	$P = 0.433$
Interaction of type of extract/drug and temperature	$P = 0.436$
Interaction of concentration and exposure time	$P = 0.309$
Interaction of concentration and temperature	$P = 0.741$
Interaction of exposure time and temperature	$P = 0.203$
Interaction of type of extract/drug, concentration, and exposure time	$P = 0.979$
Interaction of type of extract/drug, concentration, and temperature	$P = 0.779$
Interaction of type of extract/drug, exposure time, and temperature	$P = 0.451$
Interaction of concentration, exposure time, and temperature	$P = 0.050$
Interaction of type of extract/drug, concentration, exposure time, and temperature	$P = 0.477$

Discussion

Many studies have been conducted to evaluate the anti-parasitic properties of various plants as follows:

In 2008, Shahabi et al. evaluated the effect of the extract and essence of Zenian on *Giardia* cysts and showed that the minimum inhibitory concentrations (MIC) of the hydroalcoholic extract and essence of Zenian were 100 and 8 mg/ml respectively (Shahabi et al. 2009). The results of a study by Sadjadi et al. in 2006 revealed that the vinegar from grapes had fatality rate on *Giardia* cysts (Sadjadi et al. 2006). Saffarharandi and his colleagues in a study conducted in 2006 showed that the chloroform extract of garlic on *Giardia* cysts was completely effective (Saffarharandi et al. 2006). Nazari et al.'s study showed that the hydroalcoholic extract of *S. khuzestanica* could dramatically reduce glucose and triglycerines levels in rats (Nazari et al. 2005). The results of Soffar et al.'s study in 1991 revealed that garlic extract in children with *Giardia* and *Hymenolepiasis nana* was effective and had short treatment periods (Soffar and Mokhtar 1991). Lun et al. attributed the anti-parasitic effect of *A. sativum* extract with a 14 ug/ml IC50 to diallyl trisulphate, the final stable product resulting from deformation of Allicin (Lun et al. 1994). In Malek et al.'s study carried out in 2006, similar results were reported for metronidazole and furazolidone in the treatment of giardiasis in children (Malek et al. 2006). In our study, after the tests were completed, it was found that olive leaf had the highest effect on *Giardia* cysts in vitro, followed by *S. khuzestanica* and metronidazole. In this study, unlike other previous studies, *A. sativum* extract had relatively a weak effect on *G. lamblia* cysts (Azadbakht

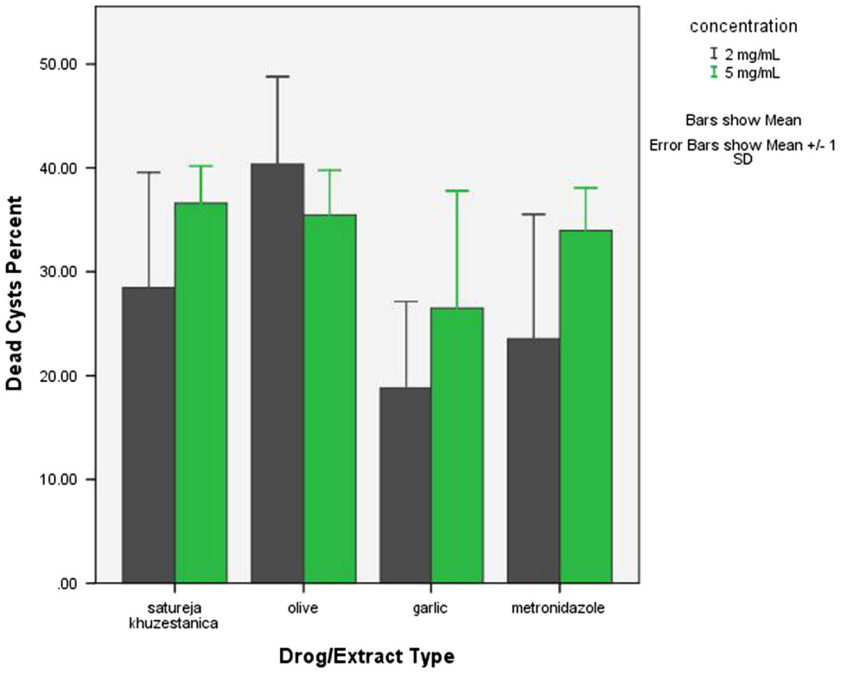

Fig. 1 Percentage of dead cysts in terms of type of extract/drug and concentration

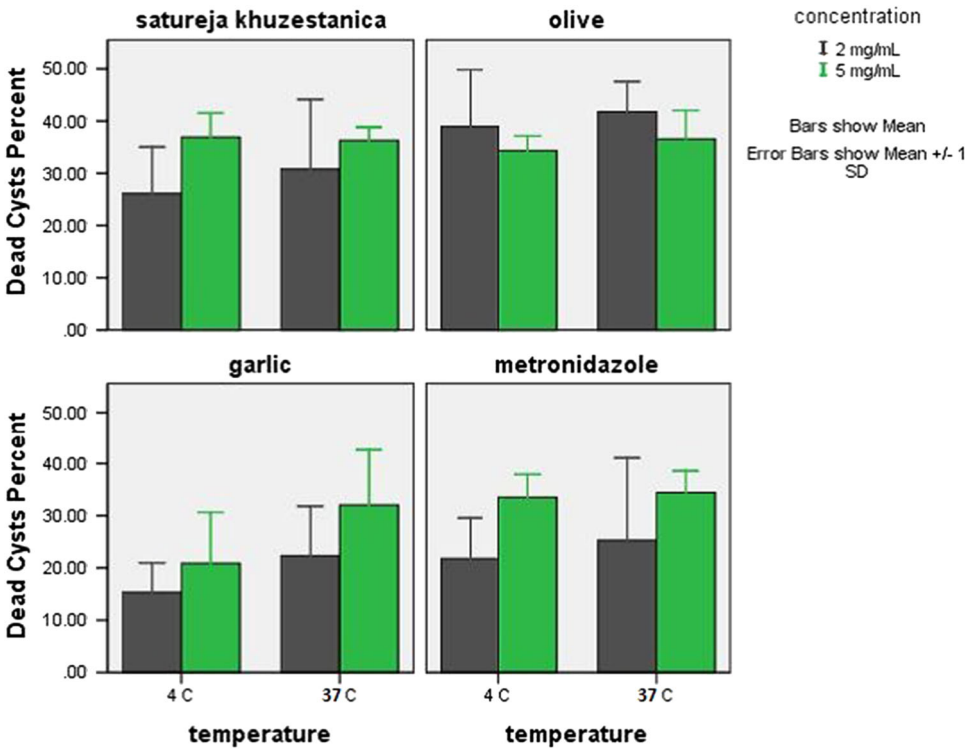

Fig. 2 Percentage of dead cysts in terms of type of extract/drug, temperature, and concentration

et al. 2003; Behnia et al. 2008). This weak effect can be attributed to the commercial preparation of the extract from drug companies, different methods used by the companies for preparing and processing the extract, the effect of environmental conditions on *A. sativum* extract compound until consuming time, and diverse species of *A. sativum* in other studies. In Azadbakht et al.'s study, the IC50 of the extract *A. sativum* on *Giardia* cysts was 0.2 mg/ml (Azadbakht et al. 2003). Behnia et al. showed in their study that the original extract of *A. sativum* had a high anti-amebic effect in a low minimum inhibitory concentration (MIC) (Behnia et al. 2008). Increasing concentrations of plant extract/drug can not generally increase the fatality rate on cysts unless the type of extract/drug is taken into

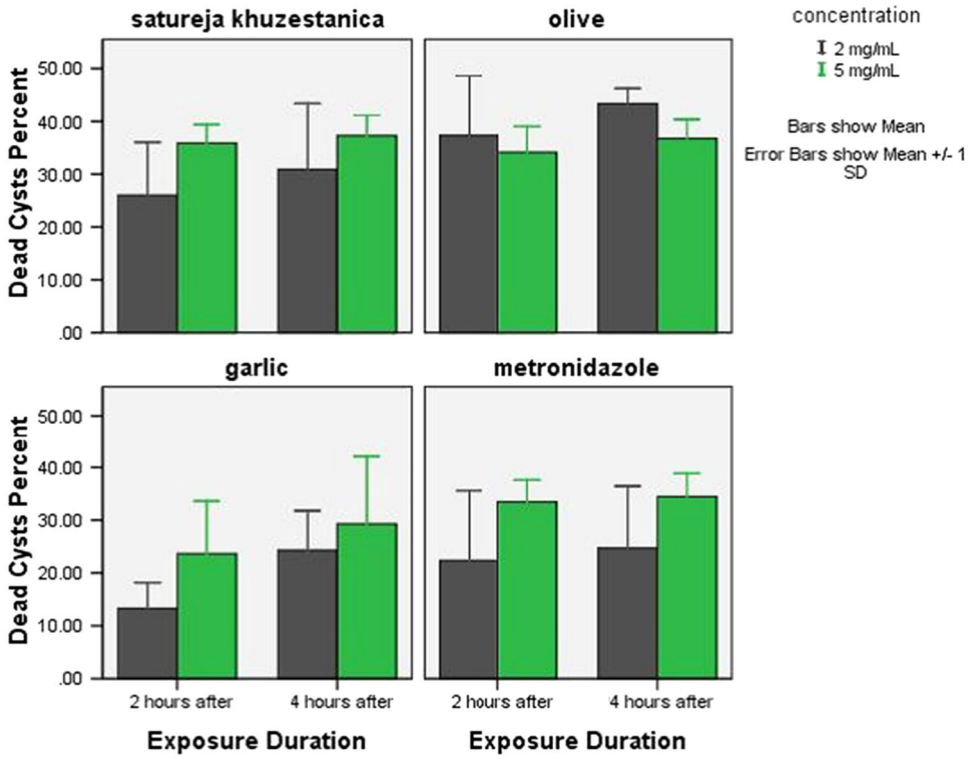

Fig. 3 Percentage of dead cysts in terms of type of extract/drug, exposure duration, and concentration

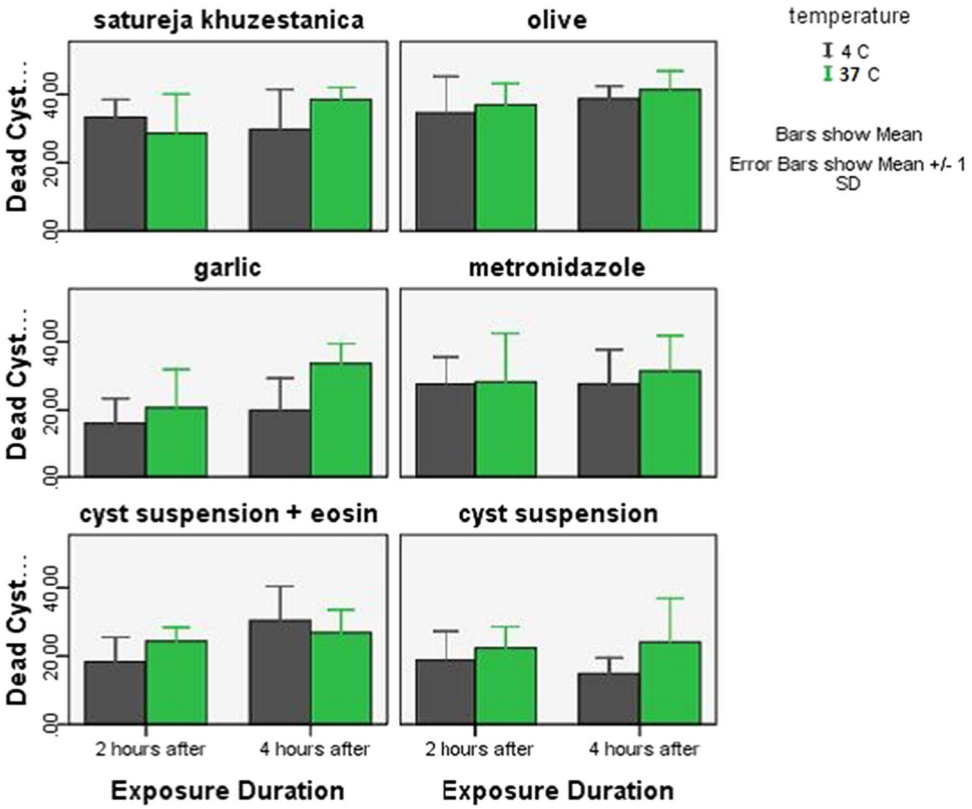

Fig. 4 Percentage of dead cysts in terms of type of extract/drug, exposure duration, and temperature

consideration. For example, olive leaf extract with a concentration of 2 mg/ml had the greatest effect on the fatality rate on *Giardia* cysts. Olive leaf extract probably has a fatality threshold on *Giardia* cysts, so that if the concentration of the extract is more than the threshold, the fatality rate on the cysts will not increase any more. In other words,

the fatality rate of olive leaf extract on *Giardia* cysts can be increased only to certain concentrations. Increasing exposure duration increases the fatality rates on the cysts, regardless of the type of extract/drug. Generally, except for *S. khuzestanica*, an increase in temperature increases the fatality rates on the cysts because, contrary to our expectations, in *S. khuzestanica* extract the fatality rate decreased with increasing temperature at the exposure time of 2 h. It can be due to the negative effect of temperature on the effective components of *S. khuzestanica* extract. Since *S. khuzestanica* is a plant native to Iran found in specific regions, very few studies has been conducted on the anti-parasitic properties of this plant. However, other researches have confirmed the fatality property of other species of this plant on culicidae mosquito larvae (Michaelakis et al. 2007). Generally, in eosin solution and *Giardia* cysts suspension it can not be claimed that the increasing rate of temperature increases the fatality rate on cysts. Contrary to our expectations, at exposure duration of 4 h the fatality rates decreased with an increase in temperature, and the reason is unknown. Some recommendations for subsequent studies are as follows:

1. Lower concentrations of olive leaf extract on *Giardia* cysts can be compared.
2. The differences in some of the variables of the study including the effect of concentration of extract/drug, temperature, and exposure time on fatality rate can be reevaluated.
3. The effect of the extracts of olive leaf, *S. khuzestanica*, and *A. sativum* can be studied on *G. lamblia* in vivo.

Acknowledgments This research was financially supported by Vice Chancellors for Education of Lorestan University of Medical Sciences, Khorramabad, Iran. The authors wish to thank the Razi Herbal Medicine Center staffs in Khorramabad city, for their great assistance.

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