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Original Article

In Vitro Antiamoebic Activity of Iranian *Allium sativum* in Comparison With Metronidazole against *Entamoeba histolytica*

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Abstract

Background: Amoebiasis is due to infection with the protozoan parasite *Entamoeba histolytica.* The patients infected with *E. histolytica* must be treated right after definite diagnosis and no need to treat infected individuals with *E. dispar* isolates. Metronidazole is used as a drug of choice against amoebiasis. However, like a lot of other chemical agents, this drug has its own side effects. This prompted us to carry out, an in vitro research into antiamoebic effect of Iranian *Allium sativum* (garlic), which has been used for centuries, as an herbal medicine, without harmful side effects.

Methods: Hydro-alcoholic, hexanic extracts and essential oil of 100 gram of crushed *A. sativum* was isolated and the minimal inhibitory concentration (MIC) of the extracts and essential oil in comparison with metronidazole were obtained on trophozoite of *E. histolytica,* HM-1: IMSS strain in TYI-S-33 medium.

Results: The MIC for *A. sativum* hydroalcoholic, hexanic extracts and essential oil after 24 hours was 60mg mL^{-1}, 4mg mL^{-1} and 0.4mg mL^{-1}, respectively. After 48 hours the MIC for *A. sativum* hexanic extract and essential oil was 3mg mL^{-1} and 0.3mg mL^{-1}, respectively. MIC for metronidazole was obtained 2μg mL^{-1} and 1.5μg mL^{-1} after 24 hours and 48 hours, in that order.

Conclusion: Iranian *A. sativum* is effective on the trophozoites of *E. histolytica* species and the essential oil exhibited the greatest antiamoebic activity, at the lowest MIC.

Keywords: *Entamoeba histolytica*, *Allium sativum*, Antiamoebic

Introduction

Among parasitic infections amoebiasis caused by *Entamoeba histolytica* ranks third worldwide in lethal infection, after malaria and schistosomiasis (1, 2). Although it is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from the symptoms of amoebiasis such as hemorrhagic colitis and amoebic liver abscess. These infections result in 50000–100000 deaths annually (3). Data from some parts of Iran showed that 7.9% of the *E. histolytica/E. dispar* isolates were *E. histolytica* or mix infection and 92.15% were *E. dispar* (4). The current treatments of choice are either one of a family of nitroimidazoles (usually metronidazole), nitrofurans, quinacrine or paromomycin (5). However, metronidazole has been reported to cause mutagenicity in bacteria (6) and is

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carcinogenic in rodents (7, 8). It has been reported that the human pathogenic bacterium, *Helicobacter pylori*, becomes resistant to metronidazole in vitro (9). Moreover, it seems to act as an immunosuppressive agent in experimental rats, both in cell-mediated and humoral immune responses (10). However, like other chemical agents, metronidazole has some side effects including metallic taste, nausea, transient neutropenia, interaction with warfarin, and peripheral neuropathy (11).

These are the main reasons why it is essential to develop a safe and effective alternative antiamoebic agent. Garlic is one of the edible plants which have generated a lot of interest throughout human history as a medicinal panacea. A wide range of microorganisms have been shown to be sensitive to crushed garlic preparations. Garlic has been shown to be antibacterial (12), antiviral (13) and antifungal (14), as well as possessing both antitumor (15) and antithrombotic (16) properties. Moreover, garlic has been reported to reduce blood lipids (17). Chemical analyses of garlic have revealed an unusual concentration of sulfur-containing compounds (1–3%) (17, 18). Garlic is a very complex compound consisting not only of allyl components but many other components, some of which, kaempferol and quercetin, have been shown to have antigiardial activity in vitro (19). The use of garlic and some of its components as antiprotozoals has already been investigated by some authors (20-22).

To our knowledge antiamoebic of Iranian *A. sativum* have not been studied so far. This prompted us to study the possible antiamoebic properties of hydroalcoholic, hexanic extract and essential oil of Iranian *A. sativum* (garlic).

Materials and Methods

Plant extract preparation

A. sativum was collected in June 2006 from Hamedan, Iran, identified by the Tehran University Institute of Medicinal Plants (IMP) and the experimental study was carried out in Shahid Beheshti University of Medical Sciences. One hundred gram of crushed *A. sativum* was subjected to maceration at 25 ºC for 14 days using n-hexane and a mixture of ethanol and water (60:40 ratio). The extracts obtained were then filtered using Whatman No. 1 filter paper. The filtrates were concentrated with a rotary evaporator at 40º C to facilitate their further freeze-drying process. The concentrated extracts were finally freeze-dried at -50 ºC for 24h and stored at -20 ºC. Extracts were redissolved in their solvents before each individual experiment (23). The yield of *A. sativum* using ethanol/ water (60:40) as solvent was 13.82% dry extract and with n-hexane was 0.24 % dry extract.

Isolation of essential oil

One hundred gram of crushed garlic were subjected to hydrodistillation using a clevenger-type apparatus for 3 h. The essential oil obtained was dried with anhydrous sodium sulfate and kept at 4 ºC (24).

The hydrodistillation of the crushed plant yielded amber-like oil, in a 0.22% yield with a characteristic odor.

Parasite culture

E. histolytica, HM-1: IMSS strain was used in all experiments. The trophozoites were cultured axenically in screw-capped tubes at 35.5 °C on Diamond's TYI-S-33 medium, supplemented with 10% (v/v) heat-inactivated bovine serum (25). Subculturing was performed routinely at 48 h intervals by replacing the medium without detaching the monolayer. Cells were harvested by replacing the medium with a fresh one, chilling on ice for 20 min, and inverting gently to detach the monolayer.

Preparation of stock solutions

One hundred gram $mL^{-1}$of each extract was dissolved in its solvent, sterilized through a 0.22 µm Millipore filter and kept as stock solution. The essential oil was sterilized through a 0.22 µm Millipore filter and was then emulsified by 5% Tween 80.

Exposure and evaluation

A concentration range of 0-100mg $mL^{-1}$of plant extract and the essential oil was prepared in test tubes by serial dilution and air-dried under sterile conditions. To the test tubes was added Diamond's TYI-S-33 medium. The contents of the test tubes were then turned into a suspension by using the ultrasound bath for 30 min and were kept at rest for 24 h at 4° C. To the test tubes were subsequently added 1.0×10^3 trophozoites of *E. histolytica* followed by incubation at 35.5° C for 24 h and 48 h.

The tests were also repeated using the standard amoebicidal drug metronidazole (26). The experimental controls were assigned in the following manner: I) Diamond's TYI-S-33 medium+trophozoites II) Diamond's TYI-S-33 medium+trophozoites+5% Tween 80 and III) Diamond's TYI-S-33 medium+trophozoites+ metronidazole (0.5, 1, 1.5 and 2µg mL^{-1}).

After the 24 h and 48 h periods, the tubes were chilled for 20 min and the attached trophozoites were detached by gentle inversion. The number of viable cells was determined using eosin 0.01% (27) and the microscope (the Neubauer chamber), the criteria for viability being motility and dye exclusion.

The lowest concentration of each plant extract and essential oil which completely inhibited the growth of trophozoites of *E. histolytica* was considered the MIC (26). After determining the MIC, concentrations of ¼, ½ and ¾ of MIC were used. Growth rate (GR) is defined as the difference between the number of viable protozoa counted at 0h and after 24 h and 48 h. The percentage of growth inhibition (% GI) was calculated using the following formula (28):

$$\%GI = (1 - \frac{GR_{extract}}{GR_{control}}) \times 100$$

The experiments were performed in duplicate and repeated three times.

Statistical analysis

Data were analyzed using two-way analysis of variance (ANOVA) (29).

Results

The inhibitory capacity of the extracts and essential oil of *A. sativum* were assayed using eosin, as a result of which viable trophozoites of *E. histolytica* remained unstained whereas dead cells were light red in color (27). The effect of various concentrations of *A. sativum* extracts and essential oil against trophozoites of *E. histolytica* and the calculated %GI (%growth inhibition) at 24 h and 48 h are shown in the table of results (Table 1).

Both the extracts and the essential oil of *A. sativum* exhibited antiamoebic activity. However, the essential oil proved to be more effective. The MIC of the essential oil after 24 h was 0.4mg mL^{-1} and after 48h was 0.3mg mL^{-1}. As shown in Table 1, 60mg $mL^{-1}$of hydroalcoholic extract after 24 h exhibited inhibition and therefore had little effect on the trophozoites of *E. histolytica*, whereas 4mg mL^{-1}after 24 h (Fig. 1) and 3mg mL^{-1} after 48 h (Fig. 2) of the hexanic extract caused complete growth inhibition. On the other hand, metronidazole, the current drug of choice, had a MIC of 2µg mL^{-1} after 24 h and 1.5µg mL^{-1} after 48 h.

Experiments showed that as the concentration increased, the number of viable trophozoites decreased. Moreover, concentration being kept constant, the results in Fig. 1 and Fig. 2 indicate that longer periods of exposure to the extracts and the essential oil, decreased the number of viable trophozoites.

Table 1: Effect of metronidazole, the hydroalcoholic, hexanic extracts and essential oil of *A. sativum* and on trophozoites of *E. histolytica* after 24 h and 48 h incubation

Antiamoebic agent	Concentration mg mL^{-1}	Growth Inhibition (%)* Mean ± SD	
		24 h	48 h
Hydroalcoholic extract of *A. sativum*	15	65.68 ± 2.67	77.37 ± 1.68
	30	80.25 ± 1.93	97.93 ± 0.76
	45	96.54 ± 1.15	99.48 ± 0.76
	60**	100	100
Hexanic extract of *A. sativum*	1	35.1 ± 3.08	87.69 ± 1.31
	2	88.2 ± 2.51	99.74 ± 0.6
	3	99.26 ± 1.33	100
	4**	100	100
Essential oil of *A. sativum*	0.1	54.19 ± 4.88	79.17 ± 1.38
	0.2	85.38 ± 2.16	99.62 ± 0.69
	0.3	95.13 ± 1.44	100
	0.4**	100	100
Metronidazole	0.5 µg mL^{-1}	79.09 ± 3.2	97.67 ± 0.8
	1 µg mL^{-1}	90.64 ± 2.47	99.74 ± 0.6
	1.5 µg mL^{-1}	95.32 ± 1.99	100
	2 µg mL^{-1}**	100	100

* The values are mean values obtained in three assays done in duplicate.

** The lowest concentration of each plant extracts, essential oil and metronidazole which completely inhibited the growth of trophozoites of *E. histolytica* (MIC)

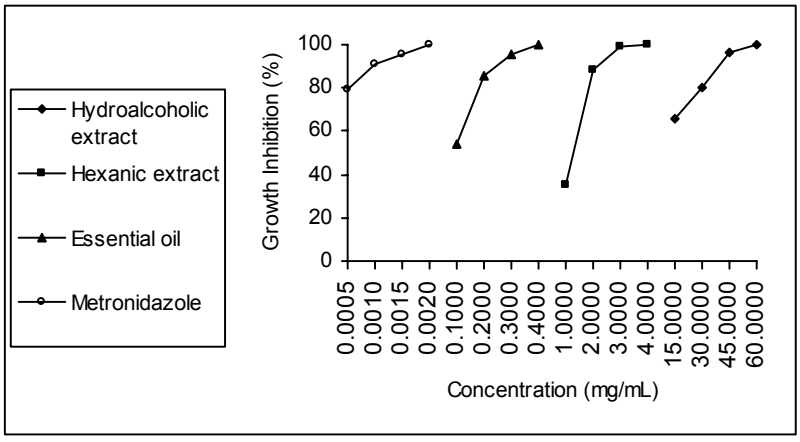

Fig. 1: Effects of metronidazole, the hydroalcoholic and hexanic extracts and the essential oil of *A. sativum* on the growth inhibition of *E. histolytica* HM-1: IMSS trophozoites after 24 h

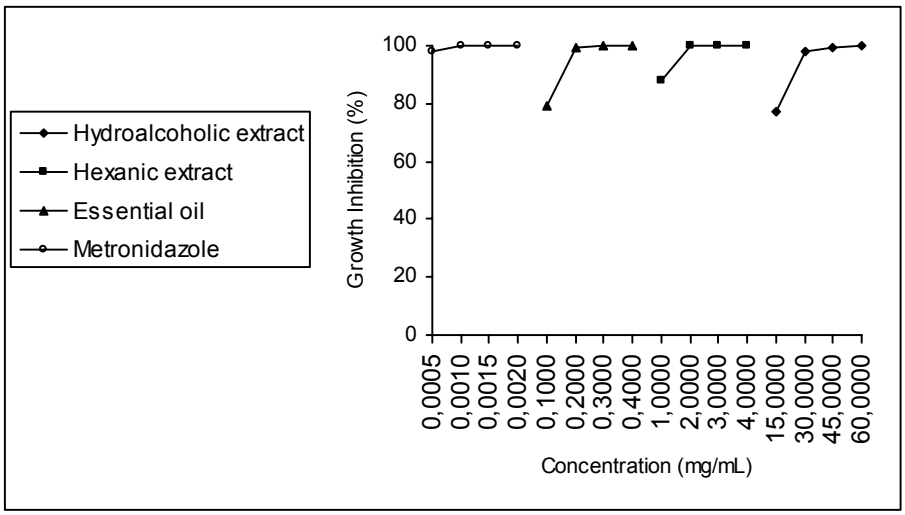

Fig. 2: Effects of metronidazole, the hydroalcoholic and hexanic extracts and the essential oil of *A. sativum* on the growth inhibition of *E. histolytica* HM-1: IMSS trophozoites after 48 h

Discussion

For many years, garlic has been used as an effective therapeutic agent having antibacterial, antifungal, antiviral, antiprotozoal and antihelmintic properties (30).

This study investigated the antiamoebic activity of *A. sativum* extracts and essential oil and compared the MICs of these agents with metronidazole.

Parasite growth depends on the primary number of active parasites, temperature and culture medium. In our study, in similar experimental conditions, the results of the experiments in study group were significantly different from those of the control group ($P<0.01$).

Mirelman *et al*. investigated the use of allicin, a sulfur compound on *E. histolytica* (20); Soffar & Mohktar assessed its use as an antigiardial alongside its use as an antihelminthic in a selection of patients (21), whilst Lun *et al.* looked at the effect of diallyl trisulphide on *E. histolytica*, *G. intestinalis* and *Trypanosomes* (22). Harris *et al*. showed that the efficacy of garlic extract as an antigiardial (23).

In this study, the essential oil, which is composed of allicin, allylpropyl disulphide (31), exhibited the greatest antiamoebic activity, at the lowest MIC, which the hexanic extract showed 100% lysis of trophozoites of *E. histolytica* at a concentration of 4 mg mL^{-1} in comparison to the hydroalcoholic extract which exhibited almost no antiamoebic activity.

From our findings as well as those of other researchers concerning the antiprotozoal property of garlic, it can be concluded that the essential oil and the hexanic extract of garlic seems to be a good antiamoebic candidate for amoebiasis treatment and inhibition of growth of *E. histolytica* by garlic is dose-dependent. However some in vivo studies regarding to its effects on animals and human should be investigated.

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