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Full Length Research Paper

In vitro study of antiamoebic activity of methanol extract of fruit of *Pimpinella anisum* on trophozoites of *Entamoeba histolytica* HM1-IMSS

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The aniseed plant *Pimpinella anisum* (Saunf-Hindi) is one of the most ancient medicinal plants used by man. Currently, this plant has several uses in the food industry as spice, whereas in the pharmacopoeia, it is used as an expectorant in digestive disturbances, as mild diuretic, and as insect repellent in external use. In this paper, we evaluated the biological activity of methanolic extract of *P. anisum* on *in vitro* growth of *Entamoeba histolytica* HM1-IMSS under axenic conditions. We observed that the growth inhibition of *E. histolytica* was at CI_{50} = 0.034 µg/mL. Results confirm the antiamoebic activity of the methanolic extract of *P. anisum.*

Key words: *Pimpinella anisum*, *Entamoeba histolytica,* antiamoebic activity, medicinal plants.

INTRODUCTION

Among parasitic infections, amoebiasis ranks third worldwide among lethal infection, after malaria and schistosomiasis (Walsh, 1988; Petri and Mann, 1993). Amoebiasis is caused by *Entamoeba histolytica,* a protozoan parasite of humans and the causative agent of intestinal amoebiasis. This disease is a major health problem in developing countries (Stanley, 2003). Amoebiasis is acquired by ingestion of the *E. histolytica* cyst in contaminated food or water (Botero and Restrepo, 2003). Although it is asymptomatic in 90% of cases, about 50 million people are estimated to suffer from symptoms associated with amoebiasis, such as hemorrhagic colitis and amoebic liver abscess (Ravdin, 1995). These infections result in 50,000 to 100,000 deaths annually. Amoebiasis contributes to the prevalence of gastrointestinal disease and is defined by the World Health Organization and the Pan American Health Organization as the presence of the parasite with or without clinical manifestations (WHO, 1997).

The Dauphin de France was the first known patient with amoebiasis treated by an extract of the root of ipecacuana plant, and little progress was made during the next 200 years (Laserre, 1966). Many drugs have been used for the treatment of amoebiasis, mainly nitroimidazole derivatives of such, as emetine, metronidazole, and ornidazole. Powell et al. (1966) reported the success of metronidazole in the treatment of amoebic dysentery and liver abscess. Metronidazole and imidazole derivatives are the drugs of choice for the treatment of amoebiasis however, *E. histolytica* has developed resistance mechanisms to these drugs (Samarawickream et al., 1997; Wassmann et al., 1999; Orozco et al., 2002). In addition, there have been reports that these drugs could induce mutagenic (Legator et al., 1975), carcinogenic (Chacko and Bhide, 1986) and neurotoxic activity (Olson et al., 2005). In addition to nutrients, vegetables and fruits may contain several phytochemicals that prevent certain diseases. The classic concept of nutrition has been expanded to include functional nutrition or nutraceuticals, which refers to the potential of certain foods to promote and improve health by reducing the risk for disease (Vaclavik, 2008).

Pimpinella anisum is one of the oldest plants used in food industry, perfumery and medicine. *P. anisum* has

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been used as digestive stimulant, antiparasitic and antifungal (Soliman and Badea, 2002) and antipyretic agent (Afifi et al., 1994). In addition, the plant and especially the essential oil of fruit have been used for the treatment of some diseases including epilepsy and seizures (Avicenna, 1988; Abdul-Ghani et al., 1987); as well for constipation (Chicouri and Chicouri, 2000), and has proven activity as a muscle relaxant (Albuquerque et al., 1995).

Recently, it has been reported that this oil has been used as a substitute for antibiotics in chickens (Mehmet et al., 2005). Nevertheless, there are few reports on antibacterial activity studies of *P. anisum* (Singh et al., 2002; Tabanca et al., 2003). Recently, Akhtar et al. (2008) demonstrated the *P. anisum* bactericidal activity after testing the methanolic extract from seeds against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli* and *Klebsiella pneumoniae*.

In developing countries, medicinal plants are popular because their products are safe and widely available at low cost. Some compounds extracted from medicinal plants already play an important role against infectious disease for example, the quinine extracted from *Cinchona* sp., and the artemisinin from *Artemisia annua*. Both compounds are effective against malaria. Considering their therapeutic potential, it is important to obtain information from the exact doses to become effective. Therefore, the aim of this study was to evaluate the antiamoebic activity of methanolic extract from *P. anisum in vitro* under axenic conditions on *E. histolytica* HM1-IMSS and identified antiamoebic components present in methanolic extract of *P. anisum.*

MATERIALS AND METHODS

Vegetal materials and preparation of methanol extracts

Fruits collection

The fruits of *P. anisum* were purchased from the local market. The material was identified as *P. anisum* by the Department of Botany, Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León.

Extraction

The methanolic extract of *P. anisum* was obtained by taking 100 g samples of fruit and individually processed in a Soxhlet unit with methanol (MeOH) at 60°C for 7 h. The extract was then filtered and concentrated in vacuum at 45°C. Methanol extract was dried under room temperature and weighed to calculate the extractability percentage. The extract was stored at 40°C until further use.

Antiamoebic activity of the P. anisum methanolic extract

Trophozoites of *E. histolytica* strain HM1:IMSS at a density of 1 × 10^4 trophozoites per mL were added to TYI-S-33 medium (Diamond et al., 1978), containing 1% penicillin/streptomycin and 10% heat inactivated bovine serum. *E. histolytica* trophozoites were then incubated in 13 × 100 mm screw-cap tubes (PYREX®) at 37°C for 72 h. Tubes were then placed in iced water for 10 min to detach cells adhering to the base of the tube and were inverted 15 times and then cell density was determined by using a hemocytometer (Neubauer, Hausser Scientific). When the culture of *E. histolytica* was grown logarithmically, trophozoites were harvested and suspended in 50 µL phosphate-buffered saline, pH 7.4 (phosphate buffered saline, PBS) at 1 × 10^4 trophozoites per mL. These suspensions were inoculated separately in 1 mL screw-capped borosilicate vials (Bellco, Vineland, NJ, USA) containing 1 mL of TYI-S-33 medium (Diamond et al., 1978), vials were added containing 50 µL of various concentrations of the *P. anisum* methanolic extract (0.1, 1.0, 3.0 and 5.0 mg per mL) and incubated for six days at 37°C. The solutions were sterilized by filtration through a 0.22 µm pore–size membrane (MercK Millipore).

A control containing 50 µL of DMSO was included in each assay. Preliminary test with dimethyl sulfoxide (DMSO) were performed to ensure that no trophozoite inhibition occurred at the concentrations used. After that, cell density was determined by using a hemocytometer (Barrón-González et al., 2008). The positive controls contained metronidazole (0.124 µg/mL) instead of methanolic extract. Bioassays were done by triplicate. Fifty percent inhibitory concentration was determined by using the Probit test.

The inhibitory effect of the *P. anisum* methanolic extract was estimated as the decreased percentage of trophozoite number with respect to non treated cultures. Corresponding averages and standard deviations (SD) were plotted. The fifty percent inhibitory concentration (IC_{50}) of the *P. anisum* methanolic extract was expressed as the concentration that produced a 50% decrease of trophozoite concentration in each of the three protozoa species. Each determination was performed three times, in triplicate. The effect of metronidazole was expressed as a diminution percentage with respect to non treated controls.

Statistical analysis

SPSS software, version 10.0 (SPSS, Inc.) was used for statistical analysis.

Phytochemical tests

Conventional chemical tests were used to identify functional groups in the methanolic extract (Domínguez, 1973).

RESULTS AND DISCUSSION

The yield of the methanolic extraction process for each 100 g of *P. anisum* fruit was 12.13% wt/wt. Methanolic extract from *P. anisum* showed *E. histolytica* trophozoites growth inhibition when tested by *in vitro* culture under axenic conditions. Results showed a dose-reponse behavior, where inhibition rate were significantly different extract doses. At a rate of 0.1 mg/mL, the inhibition ranged from 68 to 74% and it inhibited the dose of 1 mg/mL, at the dose of 3 mg/mL it was 87% and at 5 mg/mL, it was 99.18% (Figure 1).

The inhibition concentration analysis showed that methanolic extract had an IC_{50} of 0.0345 mg/mL on *in*

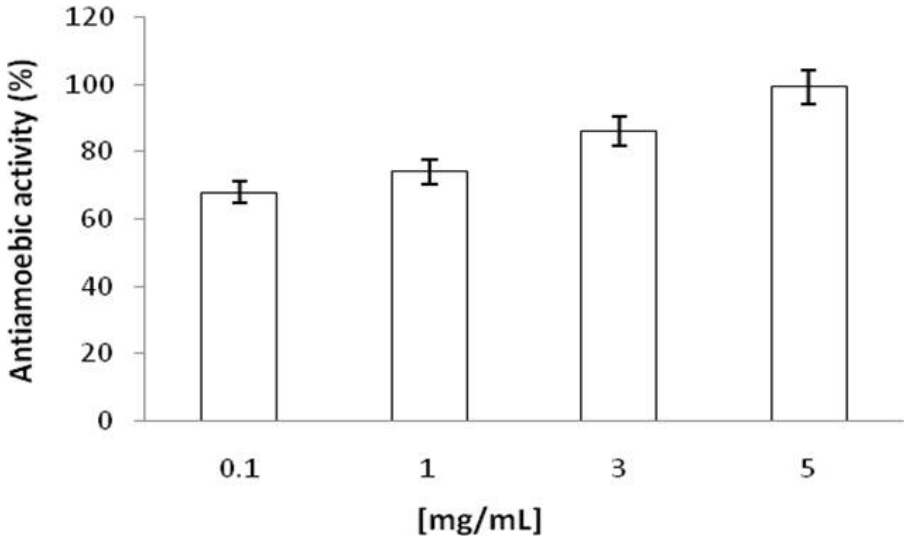

Figure 1. Inhibition of growth *in vitro* axenic of *E. histolytica* in the presence of methanol extract of fruit of *P. anisum*.

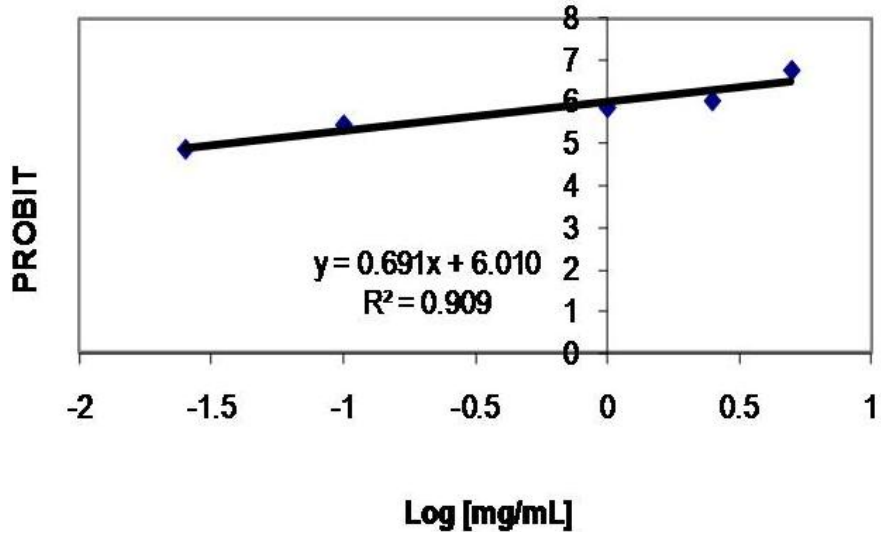

Figure 2. Probit graph of the activity of methanol extract of *P. anisum* against *E. histolytica* HM1-IMSS (50% inhibitory concentration [IC_{50} = 0.0345 mg/mL]).

vitro growth of *E. histolytica* trophozoites (Figure 2), whereas metronidazole had an IC_{50} of 0.1442 µg/mL. Values were expressed as the mean ± standard error (n = 9). The Probit value was expressed relative to the metronidazole effect.

Analysis of the *P. anisum* methanolic extract using silica gel chromatography showed three fractions. The inhibition rate of each fraction was separately evaluated. Results show that regardless of the fraction tested, all presented the highest inhibition growth at 0.47 mg/mL (74% inhibition). In order to identify the compound(s) present in each fraction, the third fraction was analyzed by mass spectrometry, showing a lead compound with a molecular weight of 175, an aromatic ring, double bonds and a methyl group (methoxy), obtained as the structure of 1-benzopyrylium which is responsible for the antiamoebic activity. 1-benzopyrylium is a widely distributed molecule within plants, and it is partially present as anthocyanins flavones. All together, results provide evidence of *E. histolytica* trophozoites growth inhibition by methanol extract of *P. anisum* fruits. In addition, one out of three fractions obtained from the methanolic extract was positively identified as 1-benzopyrylium. We believe that this support new evidence of metabolites derived from plants with antiamoebic activity. The methanolic extract of *P. anisum*

has a great popularity worldwide, as it is used as food which is an advantage in terms of popular acceptance for use.

Other species, such as *Illicium anisatum*, is similar in morphology and chemical composition to *Illicium verum*. In *I. verum*, the veranisatinas A, B and C has been identified, which present low-power neurotoxic, while in *Illicium anisatum*, the presence of anisatin and neoanisatin has been detected, the most toxic therapeutic compounds against pathogenic microorganisms to man, with anti-inflammatory and antifungal activities. It has also been reported that the aqueous extract of the fruit of *I. verum* has antibacterial activity against *Bacillus subtilis*, as well as antiviral activity against herpes virus-type 2, influenza virus, smallpox virus and poliovirus II. The essential oil obtained from the fruit is active against *B. subtilis, E. coli* and *Pseudomonas aeruginosa*, as well as antifungal activity against *Candida albicans* and seed essential oil which has activity against *C. lipolytica* (Argueta, 1994).

Methanolic extract of *P. anisum*, as well as three of their fractions, could be evaluated on the process of encystment of *Entamoeba histolytica* and biological activity on other pathogens of man.

Conclusion

The methanolic extract of the fruit *P. anisum* shows growth inhibition activity against *E. histolytica* trophozoites strain HM1:IMSS under axenic conditions *in vitro.*

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