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ANTI-GIARDIA ACTIVITY OF HEXANE EXTRACT OF *CITRUS AURANTIFOLIA* (CHRISTIM) SWINGLE AND SOME OF ITS CONSTITUENTS

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Abstract

Background: *Giardia lamblia* is a parasite that causes giardiasis in humans and other mammals. The common treatment includes different drugs, which were described to produce unpleasant side effects. *Citrus aurantifolia*, popularly known as "lima", is a plant used in traditional medicine to treat gastrointestinal symptoms. The aim of the present study was to evaluate the anti-Giardia activity of 10 pure compounds obtained from a hexanic extract of Mexican lime on the basis of trophozoite growth inhibition.
Materials and Methods*:* A hexanic extract obtained from fresh fruit peels of *Citrus aurantifolia* was tested on *G. lamblia* strain 0989:IMSS trophozoites cultured in TYI-S-33 medium. The concentration of all standard drugs, analyzed by gas chromatography, was adjusted at 10 mg/mL. Metronidazole was used as a positive control. Growth inhibition was determined by counting the number of trophozoites using a Neubauer chamber. The 50% inhibitory concentration (IC_{50}) of each drug was calculated by probit analysis and 95% confidence limits were calculated.
Results: 4-hexen-3-one, citral and geraniol showed IC_{50} values of 34.2, 64.5 and 229.49 µg/ml in axenic cultures after 24 hr of incubation, respectively. When these results were compared with a positive control of metronidazole; 4-hexen-3-one was 66 times; citral was 112 and geraniol was 441 times less active respectively. The other tested compounds did not inhibit the growth of cultured *G. lamblia* trophozoites.
Conclusion: The obtained results lead us to propose that these tested compounds from *C. aurantifolia* have potential for use as therapeutic agents against giardiasis.

Keywords: antigiardial ; *Citrus aurantifolia*; antiprotozoal activity; *Giardia lamblia*

Introduction

Giardia lamblia (syn. *Giardia intestinalis*, *Giardia duodenalis*) is a flagellated unicellular eukaryotic parasite that commonly causes diarrhea throughout the world (Adam, 2001). The parasite exists in two forms, the infectious cysts, which are resistant to many environmental factors, and the disease-causing trophozoites, which colonize the intestinal lumen but do not invade the mucosa (Carranza & Lujan 2010). Human giardiasis is a true cosmopolitan illness, with highest prevalence in developing countries. Giardiasis can present with a broad range of clinical manifestations, from asymptomatic, to acute or chronic diarrheal disease associated with abdominal pain and nausea, vomit, and weight loss. Most infections are self-limiting, although re-infection and chronic infection can occur (Halliez & Buret 2013).

On the other hand, Metronidazole is the most widely used drug for treatment against anaerobic protozoan parasitic infection caused by *G. intestinalis*. Although it is effective, there are treatment failures, relapses, and undesirable side effects (Solaymani-Mohammadi et al., 2010).Therefore, there is need to develop alternative drugs that do not have these unpleasant drawbacks (Escobedo et al., 2003). Currently, the search for more natural substances to treat giardiasis has increased in an effort to reduce resistance and minimize drug side effects (Said-Fernández et al., 2005; Vital & Rivera, 2011; Li et al., 2012; Zhang et al., 2012).

According to phylogenetic studies, Mexican lime, *Citrus aurantifolia* (Christim) Swingle (Rutaceae) is a hybrid between citron (a cluster of *C. medica* and *C. indica*) and *C. micrantha* and is considered as a native species from Indo-Malayan region in Southeast Asia (Nicolosi et al., 2000). *C. aurantifolia* is widespread in tropical and subtropical regions around the world such as North America (Florida, Texas, California, Mexico, etc.), India, Egypt, and Central America. Lime essential oil is used in traditional medicine as an antiseptic, anthelmintic, mosquito bite repellent, for stomach ailments, tonic, scorbutic, astringent, diuretic, headache, arthritis, digestive and appetite stimulant, and for colds, coughs and sore throats (Apraj et al., 2011). Previous investigations have reported flavonoids, coumarins, and terpenoids extracted from fruits of *C. aurantifolia* (Feger et al., 2000; Jiwajinda et al., 2000; Johann et al., 2007; Piccinelli et al., 2008). Peel oil of *C. auranifolia* has also been studied by gas chromatography mass spectrometry (GC-MS) analysis and established its chemical profiles (Chisholm et al., 2003; Afolayan & Asekun, 2008). Lime peel oil has shown antimicrobial (Jafari et al., 2011), radical scavenging and anti-cholinesterase (Tundis et al., 2012), anthelmintic (Taur et al., 2009), and anticancer (Gharagozloo et al., 2002) activities. Furthermore, extracts from leaves of lime have showed protective effect against osteoporosis (Shalaby et al., 2011), and induced platelet aggregation (Piccinelli et al., 2008). The hexane extract from fruit peels of *C. aurantifolia*, also exhibited activity against isoniazid, streptomycin or ethambutol mono-resistant *M. tuberculosis* strains, with minimum inhibitory concentrations (MIC) ranged 25 to 50 µg/mL (Camacho-Corona et al., 2008). Therefore, the oily extract subjected to further chemical and anti-mycobacterial studies in order to identify the active compounds (Sandoval-Montemayor et al., 2012).

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In view of this interesting issue involving lime extracts as an antimicrobial strategy, we have undertaken in the present, *in vitro* study to further evaluate the effect of *C.aurantifolia* hexane extract from peel fruits on *G. lamblia* trophozoite growth.

Material and methods

Plant Material

Fruits and flowering branches of *Citrus aurantifolia* were collected in Montemorelos, Nuevo León, México in May 2009. A voucher specimen (Number: 024769) was deposited at the Herbarium of the Facultad de Ciencias Biológicas, Universidad Autónoma de Nuevo León, México.

Preparation of hexane extract

Peels (1.4 kg) were removed from fresh fruits (7.8 kg) and macerated twice with *n*-hexane (6 L) for 72 hr at room temperature. Solvent was removed under reduced pressure to give a yellowish oily residue (16.05 g).

GC-MS Analysis

Chemical composition of volatile compounds from the active hexane extract was analyzed on a gas chromatograph (HP Agilent Technologies, 6890, USA) equipped with a quadruple mass detector in electron impact mode at 70 eV. Volatile compounds were separated on a HP 5MS capillary column (25 m long, 0.2 mm i.d., 0.3 µm film thickness). The oven temperature was set at 40 °C for 2 min and then programmed from 40 to 260 °C at 10 °C/min, and kept, 20 min at 260 °C. Mass detector conditions were as follows: interphase temperature 200 °C and mass acquisition range 20–550. Temperature of injector and detector were set to 250 °C and 280 °C, respectively. The split less injection mode was carried out with 1 µL of oily extract. The carrier gas was helium at a flow rate of 1 mL/min. Identification of volatiles was performed comparing their mass spectra with those of the National Institute of Standards and Technology NIST 1.7 library. In addition, a standard solution of C7-C40-alkanes was used to obtain the retention index of compounds and comparing them with literature data (Adams, 2007). Semi-quantitative data were calculated from the GC peak areas without using correction factors and were expressed as relative percentage (peak area %) of the total volatile constituents identified (Sandoval-Montemayor et al., 2012). A total of 10 pure compounds from hexane extract were considered for this study, as showed in table 1.

Parasite culture

G. lamblia strain 0989: IMSS was used. *G. lamblia* was cultured in TYI-S-33 supplemented with bile, as previously described (Keister, 1983). *G. lamblia* trophozoites were sub-cultured three times a week. Parasites used in the assays to determine drug susceptibility were harvested when cultures reached the middle of their logarithmic growth phase.

Growth inhibition assay

The concentration of all standard drugs was adjusted at 10 mg/mL. Metronidazole (used as a positive control), and pure compounds were dissolved in dimethyl sulfoxide (DMSO). All stock solutions were stored at –20 °C until used. Immediately before the assays, serial two-fold dilutions of the stock solutions were made in basal TYI medium (without serum). Fifty micro liters of each solution was put into 1 mL glass screw-capped cylindrical vials with a conical interior (Wheaton vial). All vials were filled with 950 µl of a freshly prepared parasite suspension in TYI-S-33 medium plus 10% bovine serum, *G. lamblia* was tested at concentrations of 2×10^5 trophozoites/mL respectively. All vials were incubated at 36 °C for 24 hr. The vials were then chilled in ice water for 20 min, and the number of trophozoites per milliliter in each tube was counted using a haemocytometer (Neubauer cell-counter chamber) (Mata-Cárdenas et al., 2008). The percentage of growth inhibition with respect to untreated controls was then determined.

Statistical Analysis

All experiments were performed in triplicate and in at least three independent bioassays (n=9 cultures for each analyzed compound). The data were analyzed using SPSS software version 20.0 (SPSS Inc., Chicago, IL, USA). The 50% inhibitory concentration (IC_{50}) of each drug was calculated by probit analysis and 95% confidence limits were calculated.

Results

For investigating the antiprotozoal activity of 10 pure compounds obtained from lime hexane extract, these compounds were tested for their inhibitory effects on the reproduction of trophozoites of *G. lamblia*. Of these compounds examined, only the compounds citral, geraniol and 4-hexen-3-one showed activity against protozoa. Table 1 shows the *in vitro* anti-giardial effect of these pure compounds obtained from a hexanic extract of *Citrus aurantifolia* fresh fruit peels, the IC_{50} values after probit analysis and confidence intervals are presented. 4-hexen-3-one exhibited the greatest inhibitory and lethal activity against *G. lamblia* with an IC_{50} of 34.72 µg/ml. The IC_{50} of citral was 58.42 and for geraniol was 229.49 µg/ml. When these results were compared with a positive control of metronidazole; 4-hexen-3-one was 66 times, citral was 112 and geraniol was 441 times less active respectively. The other tested compounds did not inhibit the growth of *G. lamblia* trophozoites.

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Discussion

The current treatments of giardiasis include nitroimidazoles (usually metronidazole), nitrofurans, quinacrine, or paromycin. However, all of these drugs are reported to have unpleasant side effects, and potential carcinogenic, teratogenic, and embryogenic questions have been addressed (Friedman, 1980; Davidson, 1990). In addition, some strains of *G. lamblia* have developed resistance to some of these remedies (Sawangjaroen et al., 2005). Nowadays, the effectiveness of alternative anti-parasitic treatments is extremely important, and by the way, there is resurgence in the use of natural alternative therapies instead of synthetic pharmaceuticals that often have severe side effects as mentioned.

Table 1: *In vitro* anti-giardial effect of 10 pure compounds obtained from a hexanic extract of *Citrus aurantifolia* fresh fruit peels. IC_{50} values were obtained after probit analysis from three assays with three replicates each, for a total of nine cultures analyzed for each compound. CI= confidence interval. *Positive control.

Pure compound	IC_{50} (µg/ml)	95 % CI
Geranil format	> 300	
Citral	58.42	43.51 – 73.33
Geraniol	229.49	180.45 – 278.44
Resorcinol	> 300	
Linalool oxide	> 300	
3-methyl-3-penten-2-one	> 300	
(+) - terpinen-4-ol	> 300	
Palmitic acid	> 300	
4-hexen-3-one	34.72	26.44 – 43.0
Methyl palmitate	> 300	
Metronidazole*	0.52	0.453 – 0.587

Literature exists on the antimicrobial activities of lime extracts (Rodríguez et al., 2000; Onyeagba et al., 2004; Aibinu et al., 2007). In addition, *C. aurantifolia* extracts have showed activity against drug resistant-tuberculosis strains (Camacho-Corona et al., 2008). Furthermore, pre-treatment of HIV with lime juice demonstrated direct virucidal activity, with promising results (Fletcher et al., 2008).

To our knowledge, there are no studies on the use of *C. aurantifolia* hexanic extract as anti-giardia approach. Therefore, the current study was conducted to evaluate the *in vitro* anti-giardia activity of hexanic extract of lima and some of its constituents. However, several plants have been used as anti-giardia remedy, with positive results, among others: *Rubus coriifolius*, *Cuphea pinetorum* and *Helianthemum glomeratum* (Calzada et al., 1998), *Zanthoxylum liebmannianun* (Arrieta et al., 2001), *Artemisia ludoviciana* (Said-Fernández et al.,2005), *Curcuma longa* (Pérez-Arriaga et al., 2006), *Senna racemosa* (Moo-Puc et al., 2007), *Ocimum basilicum* (De Almeida et al., 2007), *Syzygium aromaticum* (Machado et al., 2011), *Pulsatilla chinensis* (Li et al., 2012), Sambucus ebulus (Rahimi-Esboei et al., 2013).

On the other hand, it is known that the potency of lime extracts is enhanced by the type of solvent used, indicating that there are some active ingredients in lime which have high antimicrobial/antifungal effect but which would not be released except when lime fruit is used in conjunction with a particular solvent (Taylor, 2004). For the present work, we choose hexane in accordance with previous antimicrobial results (Camacho-Corona et al., 2008; Sandoval-Montemayor et al., 2012).

In the present study, we observed anti-giardia activity in three pure compounds extracted from lime: citral, geraniol and 4-hexen-3-one, in cultures after 24 h of incubation. With respect to citral, there are several reports indicating that this monoterpene and derivatives showed antimicrobial effects, for instance; antimalarial activity (Singh et al., 2014), anti-leishmania activity (Machado et al., 2012), anti-nematicidal (Echeverrigaray et al., 2010), as antiprotozoal agent, against *Trypanosoma cruzi* trypomastigotes and amastigotes (Santoro et al., 2007), and even with antifungal action, against the growth of *Candida albicans* (Lima et al., 2012).

Concerning geraniol, Nowotarska et al. (2014) reported that this terpen was active against both Gram-positive and Gram-negative pathogenic microorganisms. Singh et al., 2002; 2003 found that geraniol-derived compounds showed very promising antimalarial *in vivo* activity against multi-drug resistant *Plasmodium yoelii*. By the way, the essential oil of *Cymbopogon martinii* (palmrosa) and one of its main constituents geraniol showed potent anthelmintic activity against the nematode *Caenorhabditis elegans* (Kumaran et al., 2003).

With regard to 4-hexen-3-one compound, we found few reports to compare our results, however, a previous study suggests that 4-hexen-3-one possesses antimycobacterial activity (Sandoval-Montemayor et al., 2012). In the present study, 4-hexen-3-one was the most active anti-giardial pure compound with IC_{50} value of 34.72µg/ml. It has been suggested that low-molecular weight and highly lipophilic characteristics help this compound to diffuse across cell membrane to induce biological reactions (Orji et al., 2012).

The above results lead up to conclude that hexane extract of *C. aurantifolia* and its three constituents: citral, geraniol and 4-hexen-3-one could be used as prototypes to develop new anti-giardial agents.

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