ORIGINAL ARTICLE

Anti-giardial activity of the aqueous extracts of Cymbopogon citratus leaves (Lemongrass) and Pulicaria undulata herb in comparison with Metronidazole, in vitro and in vivo

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ABSTRACT

Background: Metronidazole (MTZ) is the drug of choice for treatment of giardiasis, but it has many drawbacks and there is a need to find out medicinal plants having anti-giardial efficacies to be alternative to MTZ. Objectives: This work aimed to study the in vitro and in vivo anti-giardial effectiveness of the aqueous extracts of two plants; Cymbopogon (C.)citratus and Pulicaria (P.)undulata in comparison with MTZ. Methodology: For in vitro study, one mL from different concentrations of plant extracts and MTZ were added to one mL of Giardia cysts suspension for 5, 15, 30 and 60 min. The viability was distinguished by 0.1 % eosin. Also, morphological alterations of Giardia cysts were examined by scanning electron microscope (SEM). For in vivo study, 50 male Albino mice were divided into: Group I: non-infected control; Group II: infected-untreated (IU); Group III: infected-treated with 15 mg/kg of MTZ; Group IV: infected treated with 500 mg/kg of C. citratus extract and Group V: infected- treated with 200 mg/kg of P. undulata extract. The effectiveness of the extracts was evaluated by cyst count per gram of feces, histopathological and transmission electron microscopic (TEM) examination of the small intestine. Results: in vitro mortality percentages of Giardia cysts showed a significant dose and time dependent effect regarding each extract. The aqueous extract of C. citratus extract at 500 μg/mL revealed the highest significant mortality percentages. P. undulata at 400μg/mL, MTZ and C. citratus at 250 μg/mL showed high mortality percentages with significant differences on comparison with C. citratus at 500 μg/mL. The ultrastructure morphological alterations of G. lamblia cysts were observed mainly with C.citratus. In the in vivo study, C. citratus revealed significant early complete absence of the parasite from fecal samples at 5th days post treatment, while P. undulata and MTZ achieved complete cure at 10th days post treatment. Progressive improvement of intestinal mucosa pathological changes and the mucosal ultrastructure were observed in the treated mice. Conclusion: C. citratus and P. undulata aqueous extracts were effective against G. lamblia both in vitro and in vivo and they could be natural therapeutic alternative agents to MTZ.

INTRODUCTION

Giardiasis is one of common infectious disease caused by the flagellated protozoan G. lamblia. It affects about 280 million people annually worldwide. G. lamblia is the most common parasite that affects children and the most frequent protozoan isolated from water samples as it is highly resistant to chlorination. The prevalence rates of giardiasis are 2-5% in industrialized countries and 20-30% in the developing ones. In Egypt, giardiasis affects over 48% of the population. Human infection happens on ingestion of the cysts in contaminated water and food, and in small intestine, they develop into mature trophozoites which attach themselves to the epithelial microvillus and colonize. Clinical picture of giardiasis in human may be asymptomatic or causes nausea, vomiting, diarrhea and abdominal cramps in the acute phase. With chronicity, giardiasis can cause host IgA-deficiencies, malnutrition and immunosuppression. Also, giardiasis may cause growth retraction, cognitive impairment, irritable bowel syndrome, arthritis, pulmonary infiltrate and urticaria. Metronidazole (MTZ) is the first choice drug for treatment of giardiasis, others such as furazolidone, albendazole and tinidazole are also used. MTZ is associated with many drawbacks including resistance, low efficacy and occurrence of side effects as diarrhea, anemia and severe inflammation of liver, pancreas and...
also identified and compared with well medicinal plants of pharmacological anti-giardiasis efficacy to be safe alternative to MTZ.

Plants used in traditional medicine have been proved to be sources for novel anti-protozoal drugs and several medicinal plants show significant anti-giardiasis efficacy with neglected side effects. C. citratus, also known as lemongrass, is belonging to the Poaceae family, and it is found mostly in tropical and subtropical countries. The aqueous extracts of lemongrass are used for relieving of the gastrointestinal disturbance, hypertension, fever and inflammation and it is the most commonly used antidiarrheal preparation by native Egyptians. Also, C. citratus possesses anti-bacterial, anti-fungal and anti-viral properties. Experimental studies revealed its toxic effects against Plasmodium falciparum, Leishmania amazonensis, Crithidia deanei and Trypanosoma cruzi.

Pulicaria undulata, belongs to the Asteraceae family and it is an annual herb or sometimes a perennial subshrub. In the central Sahara, it is used to treat abscesses and skin diseases. In addition, its oil extract has sedative and antibacterial activities. In Egypt, it is used as herbal tea, insects repellant and relieving of inflammation. P. undulata revealed also anti-inflammatory, antioxidant and anti-cancer properties.

This research aims to study the potential giardicidal effectivness of aqueous extracts of two natural plants; C. citratus and P. undulata in comparison with metronidazole in vitro and in vivo.

METHODOLOGY

The study was approved by the Scientific Research Ethical Committee, Faculty of Medicine, Menoufia University. Also, animal handling and all procedures were done in accordance with the international ethical guidelines. This study was performed from August 2018 to January 2019.

Plant materials:
The dried C. citratus leaves were purchased from the market for the sale of medicinal herbs in Ghoriyah, Cairo, Egypt. The aerial parts of P. undulata herb were kindly supplied from Environmental Studies and Research Institute, University of Sadat City where both plants were also identified and compared with well identified herbarium specimens.

Preparation of the plants aqueous extracts:
Each of the two plants was milted separately into powder. 500 g of each powder was put in 1000 ml distilled water and boiled for 120 minutes with intermittent shaking. They were left in the containers overnight. They were filtered through clean gauze and later through Whatman filter paper. The resulting filtrate was evaporated by a rotary evaporator, the dry residue was weighed and stored at 4 °C away from direct light until use. On demand, each extract was further diluted in distilled water into various concentrations for both in vitro and in vivo studies.

Metronidazole
Metronidazole (Amriya Pharm. Ind. Alexandria, Egypt) was used as the control drug. The tablets were dissolved in distilled water and diluted to give concentrations of 50 μg/mL for in vitro study and 15 mg/kg/day for in vivo experiment.

Collection of G. lamblia cysts:
G. lamblia cysts were collected from the stools of patients attending Laboratories of Department of Clinical Pathology, Menoufia University Hospitals. All the specimens were put in normal saline and transferred to the Laboratory of Department of Parasitology, Faculty of Medicine, Menoufia University. They were concentrated with sedimentation technique and centrifuged for 5 min at 2,000 rpm several times. The number of the cysts was counted with a hemocytometer and adjusted to be about 10,000 cysts/mL of phosphate-buffered saline (PBS).

Assessment of plants extracts effects against G. lamblia cysts in vitro:

For the in vitro study, concentrations of 250 and 500 μg/mL of aqueous lemongrass extract and 150 and 400 μg/mL for P. undulata extract were used. Six sterile labeled test tubes were loaded with 1 mL of suspension containing about 10,000 Giardia cysts. One mL of each plant extract concentrations and MTZ were added into the corresponding tube, while Giardia cyst suspension alone was considered as the negative control. The contents of each tube were gently mixed and left at 37 °C, then examined at 5, 15, 30 and 60 min. The cyst viability was assessed by 0.1% eosin stain and the dead ones took light red color. The percentage of mortality was calculated according to this formula: (D / (D+L)) × 100, where D is the number of dead cysts and L is the number of living cysts. Each experiment was repeated three times and the means were calculated.

SEM of Giardia cysts:
Exposed Giardia cysts to the higher concentration of each plant extract and other controls for one hour were collected by centrifugation, washed in PBS and fixed with 2.5% buffered glutaraldehyde in 0.1 M PBS, pH 7.4, at 4°C for 2 hour. After washing with PBS, they were post fixed for 30 min at room temperature in 1% osmic acid and dehydrated with ascending series of ethyl alcohol. After infiltration with acetone, they were dried and coated with gold in a SPI-Module, TM VAC/Sputter. Images were obtained using a JEOL JSM-5200LV SEM, Japan, at Electron Microscopic Unit - Tanta University.

Experimental animals:
Pathogen free male Albino mice (6-7 weeks old, 20±2 g) were purchased from the animal house, Theodore Bilharz Research Institute, Egypt.
were kept in a standard environment with well-ventilated cages. They were fed on a standard diet with free access to water.

**Experimental design:**

In this study, 50 mice were used; ten non-infected mice were assigned to the negative control (NC) group (Group I) and the remaining 40 *G. lamblia*-infected mice were divided randomly into the following four groups each of 10 mice: Group II was untreated group (IU); Group III was treated with 15 mg/kg of MTZ. Group IV was treated orally with 500 mg/kg aqueous extracts of *C. citratus* 14, 20 and Group V was treated orally with 200 mg/kg of aqueous extracts of *P. undulata* 23.

Each mouse in all groups, except group I, was inoculated, using oral catheter, with 10,000/mL *G. lamblia* cysts suspension. All the treatment regimens were given on the 3rd day post-infection (p.i.) and continued for 7 successive days. After the 2nd day p.i., stool samples of the mice were examined daily to detect the protozoan cysts/or trophozoites until the end of the experiment. Finally, all the mice were sacrificed 3 days after the end of treatments.

**Assessment of the treatment efficacy in vivo**

- **Parasitological examination:**

One gram of stool of each mouse was thoroughly mixed with 10 mL of formaline saline. Then, the specimen was concentrated by formol ether sedimentation method. Ten high power fields were examined for each sample for counting the cysts and the mean number was calculated.

- **Histopathology:**

Small pieces of duodenum of each sacrificed mouse were collected in 10% formalin and then embedded in paraffin wax. Thin sections (5 microns) were stained with haematoxylin and eosin (H&E) and the prepared slides were examined by light microscopy.

**TEM of the intestinal tissues:**

Small pieces from the duodenum and proximal jejunum of the sacrificed mice were processed as previously mentioned in SEM. After dehydration, the samples were embedded in Araldite 502 resin. The plastic molds were cut in the LEICA Ultra-Cut, ultramicrotome, and stained with 1% toluidine blue. Then, ultra-thin sections were cut, stained with uranyl acetate, counter stained with lead citrate and examined. Images were obtained using a JEOL JEM-100SX, EM. Japan at Electron Microscopic Unit, Faculty of medicine, Tanta University, Egypt.

**Statistical Analysis**

The results were expressed as mean ± standard deviation (SD) and statistically calculated using SPSS version 20 software (SPSS Inc., Illinois, USA). Kruskal-Wallis test was done to compare between different groups. Friedman test was performed to differentiate changes in follow up within the same group. These tests were followed by Post Hoc Value test to analyze the significant difference between the two means of two individual groups. *P* values of ≤ 0.05 indicates statistical significance.

**RESULTS**

### In vitro effects of the plant extracts:

In *in vitro* effects of *C. citratus* and *P. undulata* on *Giardia* cysts mortality in comparison with MTZ and control negative, at different times of exposure, were summarized in table 1.

### Table 1: *In vitro* percentages of *Giardia* cysts mortality caused by different concentrations of *C. citratus* and *P. undulata* on comparison with MTZ and negative controls at different times of exposure:

<table>
<thead>
<tr>
<th>Exposure time</th>
<th><em>C. citratus</em></th>
<th><em>P. undulata</em></th>
<th>MTZ</th>
<th>Negative</th>
</tr>
</thead>
<tbody>
<tr>
<td>250 µg/ml</td>
<td>31.0±4.0#</td>
<td>46.3±2.08###</td>
<td>24.3±3.2#</td>
<td>40.3±3.5#</td>
</tr>
<tr>
<td>500 µg/ml</td>
<td>46.3±2.08###</td>
<td>32.7±5.7#</td>
<td>68.0±7.2#</td>
<td>3.3±1.5</td>
</tr>
<tr>
<td>15 min</td>
<td>51.0±4.6#</td>
<td>76.3±6.5###</td>
<td>57.3±8.1#</td>
<td>3.3±0.6</td>
</tr>
<tr>
<td>30 min</td>
<td>72.0±5.6#</td>
<td>89.3±5.0###</td>
<td>79.3±4.9#</td>
<td>4.3±1.5</td>
</tr>
<tr>
<td>60 min</td>
<td>89.3±1.5###</td>
<td>97.3±1.5###</td>
<td>90.0±1.4###</td>
<td>5.3±1.5</td>
</tr>
<tr>
<td>Friedman</td>
<td>0.001*</td>
<td>0.0001*</td>
<td>0.003*</td>
<td>0.002*</td>
</tr>
</tbody>
</table>

*#: P- value: estimated to compare between different groups
*#: P- value: estimated to compare within the same group

Significant at *P* ≤ 0.05. Highly significant at *P* ≤ 0.01
The results showed that the fatality rates with both plants, increased significantly ($P<0.01$) in correlation with the time of exposure and the used concentrations. After finishing the experiment, at 60 min of exposure, all the tested treatments increased the mortality in high significant ($P<0.01$) value when compared to the negative control. The aqueous extract of lemongrass at 500 µg/mL gave the highest mortality percentage achieving 97.3±1.5%, followed by $P. undulata$ at 400 µg/mL (90.0±1.4%) with a statistically ($P<0.05$) significant difference between both of them. Lemongrass at 250 µg/mL, $P. undulata$ at 400 µg/mL and MTZ achieved nearly similar result with non-significant difference among them (89.3±1.5, 90.0±1.4 and 89.5±2.1). While, $P. undulata$ of 150 µg/mL achieved significantly the lowest mortality percentage (66.3±2.3) when compared to other treatment regimens ($P<0.01$).

**G. lamblia** cysts SEM results

Normal cyst shows flattened ovoid cysts with a convex smooth dorsal surface. Treated cysts were either swollen or shrunken. Membrane blebs, sloughing and erosions in many cysts were observed. Marked deformation with complete destruction of the cyst was detected with $C. citratus$ (Fig.1).

![Fig. (1): SEM of G. lamblia cysts: (A and inset photo); negative control cysts showing normal flattened ovoid cysts with a convex intact and smooth dorsal surface (arrows), (B-C-D); treated cysts ($C. citratus$, $P. undulata$ or MTZ ), some are swollen and other are shrunken showing membrane blebs (b), sloughing and erosions(e). Complete destruction of the cyst was detected with $C. citratus$ as in the inset photo in Fig 1B](image)

**In vivo results**

**Parasitological examination:**

At the 5th days post treatment, $C. citratus$ treated mice achieved complete reduction of the cysts in their stool while both $P. undulata$ and MTZ decreased significantly ($P<0.001$) the mean number of cysts/g stool (7.7±4.2 and 6.9±2.4, respectively) compared to IU mice (15.3±1.2) with non-significant difference between them. This pattern continued for the 7th day post treatment when it downed to 3.1±1.2 with $P. undulata$ and 3.3±2.1 with MTZ and both achieved complete cession of the cysts excretion at the end of the experiment (Fig.2).
Fig. 2: The efficacy of *C. citratus* and *P. undulata* extracts on reduction of faecal *Giardia* cyst counts in treated mice in comparison with MTZ and positive controls at different days post-treatment.

*P* values estimated the relationship between different groups

Highly significant at *P* ≤ 0.001

**Histopathological results:**

Sections in the small intestinal mucosa of uninfected mice showed the normal histological appearance where there were intact villi and crypt and regular brush border with normal columnar enterocytes (Fig. 3A). *Giardia* infection in group II caused prominent pathological changes such as shortening and blunting of the villi with desquamation of epithelial lining. Some specimens revealed atrophy and even complete loss of the villi and disorganization of crypts. Also, there was necrosis of the enterocytes with pyknotic nuclei. The lamina propria was heavy infiltrated by inflammatory cells (Fig. 3B-C). Also, there were many *Giardia* trophozoites (Fig. 3D).

*P. undulata* and MTZ treatments caused some improvement of the intestinal mucosa with restoration of the brush border regularity and decrease in the cellular infiltration of the lamina propria. Also, few villi showed sloughed apical surfaces (Fig.3F). *C. citratus* caused more obvious improvement as villous architecture and the lining brush border were nearly normal with nearly disappearance of the cellular infiltration from lamina propria (Fig. 3E).
Fig. 3: Photomicrograph of a section in the mucosa of the small intestine stained with H & E (x 200) from: (A); control negative mice showing slender long villi(v), normal basal crypts, regular brush border with columnar enterocytes (arrows) and goblet cells in between (stars), (B); infected mice showing complete loss of the villi, disorganized crypts (c) and necrosis of the enterocytes which having pyknotic nuclei (pn). Heavy cellular infiltration of the lamina propria (CI) is also seen, (C); infected mice showing blunted and shorten villi (arrow), atrophy and erosion in others (arrowhead), few goblet cells (stars) and cellular infiltration of the lamina propria (CI), (D); many Giardia trophozoites can be seen in IU mice (x 1000), (E&F); treated mice showing normal brush border of the villi with tall columnar enterocytes and goblet cells in between. Some villi showing sloughing of their apical surfaces (arrowheads) and decrease in the cellular infiltration of the lamina propria (CI) can be seen.

TEM examination:

TEM of small intestinal mucosa of control negative group showed normal architecture of enterocytes with normal typical appearance of microvilli, they were regular in arrangement and equal in their length and diameter. Tight junction was present between the adjacent cells with intact terminal bar (Fig.4A).

In GI (infected un-treated), the microvilli were few, short, blunted and disrupted (Fig.4C). Destructed microvilli with tufts formation were also present and Giardia organism was seen embedded among them (Fig.4B). Beside, destructed intercellular space, the nuclei were faint with irregular peripheral chromatin and mitochondria were vacuolated (Fig.4B-C).

The treated mice groups showed nearly normal microvilli and enterocytes with normal nuclei (Fig 4D-E), however, intracytoplasmic and mitochondrial vacuolization in some specimens of mice treated with P. undulata and MTZ were found (Fig.4E).
DISCUSSION

Medicinal plants used in traditional medicine play an important role in promotion of health services in both developed and developing countries due to their pharmacological significance.27 C. citratus and P. undulata are used usually, as flavoring agents, herbal tea and medicinal plants in traditional folk medicine.10,28 In this study we evaluated the plant's anti-giardial effects in vitro and in vivo. We preferred the aqueous extracts of both plants as they usually prepare and use at home in traditional folk medicine. This study is considered to be the first one investigating the giardicidal effectiveness of P. undulata.

In our study, in vitro mortality percentages of Giardia cysts showed a significant dose and time dependent effect regarding each extract. The aqueous extract of C. citratus extract at 500 ug/mL revealed a significant high killing effect on Giardia cysts, proved by the highest mortality percentages. Also P.undulata at 400 ug/mL and MTZ showed high mortality. Similar result was achieved by the lower concentration of
Lemongrass at 250 μg/mL without significant difference between each of them but the significant difference was recorded on comparison with C. citratus at 500 μg/mL (P<0.05). On other side, the other lowest anti-giardial effect was revealed by P. undulata of 150 μg/mL, recording significant difference when compared with other treatment regimens (P<0.01).

Regarding C. citratus, our results coincide with Méabed et al. who reported a higher significant in vitro activity of C. citratus aqueous extract against Giardia trophozoites growth in comparison with MTZ. Effective in vitro anti-protozoa activity of essential oils of C. citratus was also reported, against Trypanosoma cruzi. Also, anthelmintic activity of the aqueous extract of C. citratus was observed.

The result of P. undulata in our study was supported by other research that reported leishmanicidal effect of P. inuloides. Generally, it was found that essential oils containing phenolic are strong anti-giardiasis and according to the phytochemicals structure of C. citratus and P. undulata, they are rich of phenols.

Our high in vitro mortality percentages caused by the used plant extracts were supported by the drastic electron microscopic morphological alternations of G. lamblia cysts that were observed, such as swelling, erosions or even fragmentation. These changes happened with both plants but they were more severe with C. citratus. These observations are consistent with the results obtained with phenolic-rich essential oils as they induced ultrastructural alterations in Giardia trophozoite appearance and plasmatic membrane alterations with loss of its osmoregulation. Other plants were reported causing similar structural changes like ginger and cinnamon extracts and Myrtus Communis and Olibanum. Also, Commiphora molmol induced membrane erosions of Giardia trophozoite with increased cytoplasmatic vaculation.

The effects of C. citratus extracts could be related to the disturbance occurring in the parasite membrane caused by antioxidants effects of phenolic and flavonoids contents. In parallel, the cytotoxic potential of P. undulata aqueous extract caused by its high contents of flavonoids and terpenoids could be had a role in the induced morphological damages.

Here-in, in vivo study showed that C. citratus and P. undulata aqueous extracts reduced significantly the fecal cyst counts in the treated mice comparing with IU mice. It was noticed that C. citratus revealed significant early complete absence of the parasite from fecal samples at 5th days post treatment, while P. undulata and MTZ achieved complete cure at 10th days post treatment.

Our results agree with Méabed et al. who reported 100 and 65.7% reduction in Giardia cyst in stool of mice treated with 500 and 250 mg/kg of aqueous extracts of C. citratus. Also, it was found that the essential oil of C. citratus reduced parasitemia in Plasmodium (P)berghei infected mice and in P. falciparum infected rats.

Considering this work is the first to study the anti-giardial effect of P. undulata, our attempt to explain the significant reductions of fecal cyst pass in the treated mice could be attributed to the phenolic-rich essential oils which affect negatively Giardia growth, viability, morphology and its adherence. Also, their anti-oxidant action affects the trophozoites attachment causing their washout and disintegration. On other side, the alkaloids in aqueous extract of C. citratus can inhibit the growth of microorganisms by affecting their genetic materials. In this respect, we suspect that C. citratus aqueous extracts caused more rapid disintegration of Giardia trophozoites than P. undulata, so it caused earlier removal of the parasite from the intestine, while P. undulata, needed a longer time of contact with the parasite to achieve the complete cure. However, the antioxidant activity and the medicinal effects of the herbal extract depend on the amount of total phenolic or flavonoids contents.

Our results whether in vitro or in vivo are in agreement with several studies used other medicinal plant extracts which significantly showed antigiardial affects, such as the chloroform extract of garlic, Zingiber officinale, and Myrtus Communis and Olibanum. Also, Commiphora molmol induced membrane erosions of Giardia trophozoite with increased cytoplasmatic vaculation.

The small intestine of Giardia-infected untreated mice showed many pathological lesions and these findings are in accordance with many previous studies. These results were also supported by the results of TEM examination of the intestinal mucosa. Giardiasis-induced changes could be as a sequence of direct attachment of the Giardia trophozoites with the intestinal epithelium causing microvillus alterations and epithelial dysfunction.

Regarding the pathological changes in treated mice groups, in our study, the improvement of intestinal mucosa was more obvious in C. citratus treated mice than those received P. undulata or MTZ and this might be due to early complete clearance of the parasite from intestinal mucosa and C. citratus could have a direct healing effect on the injured mucosal linings. Also, the mucosal ultrastructure of the treated mice with the tested plants and MTZ showed progressive improvements to be more or less near to the normal. This could be attributed to the antioxidant activity of phenolics and flavonoids contents of both C. citratus and P. undulata which can protect the host cells against oxidative damage caused by the produced free radicals, peroxides and various poisonous substances. Moreover, the methanol extract of P. undulata was found to have a cytoprotective effect and it has a pronounced healing effect in the treatment of gastric ulcer.
Similar improvements achieved by Cinnamon and ginger extracts were detected by histopathological and TEM examination of the intestinal mucosa of Giardia infected-treated groups. Additionally, improvement effects were obtained with many herbs used for giardiasis treatment in different studies which supported our results regarding the importance of medicinal plants.

CONCLUSION

The present study proved the effectiveness of C. citratus and P. undulata aqueous extracts against G. lamblia both in vitro and in vivo and we consider them as safe and economical promising natural therapeutic agents with wide biological activities. We recommend further investigations to identify and determine the phytochemical difference between both extracts and their anti giardial effectiveness by different extraction methods mainly for P. undulata.

Conflicts of interest: The authors declare that they have no financial or non financial conflicts of interest related to the work done in the manuscript.

- Each author listed in the manuscript had seen and approved the submission of this version of the manuscript and takes full responsibility for it.
- This article had not been published anywhere and is not currently under consideration by another journal or a publisher.

REFERENCES


