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Original Research Article

In vitro activity of Piper sarmentosum ethanol leaf extract against Toxoplasma gondii tachyzoites

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Abstract

Purpose: *To evaluate the activity of the ethanol leaf extract of Piper sarmentosum against toxoplasmosis.*

Methods: *An in vitro anti-Toxoplasma study was conducted using Vero cells as a host for T. gondii. Clindamycin used as the reference drug. Light microscopy technique was used to study the in situ antiparasitic activity of T. gondii. Non-toxic concentrations of the ethanol extract for Vero cells were determined by methyl thiazolyl tetrazolium (MTT) cell proliferation. The presence of Toxoplasma gondii was observed by Giemsa staining.*

Results: *The results showed that significant ($p < 0.05$) anti-toxoplasma activity of the ethanol extract, though lower than that of clindamycin (control drug), was achieved, with half-maximal inhibitory concentration (IC_{50}) of 12.4 and 7.2 µg/mL for the extract and reference drug, respectively. After 24 hours of exposure to the extract, the inoculated Vero cells showed lower parasitemia and no remarkable morphological changes.*

Conclusion: *The findings demonstrate that the ethanol extract of P. sarmentosum leaves are active against toxoplasmosis in vitro. However, further studies are required to determine the therapeutic significance of these findings in vivo.*

Keywords: *Toxoplasma gondii, Piper sarmentosum, Vero cell, Toxoplasmosis, Antiparasitic*

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INTRODUCTION

The sporozoan parasite, *Toxoplasma gondii*, is an intracellular coccidian tissue protozoa which is a common cause of toxoplasmosis in humans and animals, particularly immunocompromised hosts [1]. It is reported that approximately one third of the world's current population has been infected with this parasite [2]. The World Health Organization considers toxoplasmosis one of the major parasitic diseases infecting humans in the developing countries [3]. This status is reflected by the parasite's worldwide distribution and broad range of intermediate hosts [4] that have resulted in the infection of up to 1 billion people [5]. Most toxoplasmosis cases are reported in South America, Middle East and other low-income countries with high seropositivity against *T. gondii* infection in either normal or immune-compromised hosts [6].

Toxoplasmosis is treated with a combination of sulfadiazine and pyrimethamine which are both key enzyme inhibitors in the biosynthesis of

pyrimidines [6]. However, these drugs cause adverse reactions, including the suppression of bone marrow, teratogenic effects in the first trimester of pregnancy, and sulfadiazine hypersensitivity [7]. Other drugs such as clindamycin, atovaquone, daspone, trimethoprim, pentamidine, and azithromycin have also been used, but side-effects such as diarrhoea, nausea, abdominal pain, cholestatic hepatitis, hepatomegaly, and hepatitis have been reported [8]. Cultural knowledge pertaining to the application of medicinal plants in the treatment of parasitic infection represents a vital role in the discovery of safe and natural chemotherapeutic agents against toxoplasmosis [9].

Traditionally, the leaves of *P. sarmentosum* are used to treat malaria, coughs and colds, backache and joint pain, toothache, and worm infections [10]. Thus, the present study aims to investigate the anti-*Toxoplasma* activity of the ethanolic extract of *P. sarmentosum* leaves on *T. gondii* in the inoculated Vero cells.

EXPERIMENTAL

Plant materials

The leaves of *Piper sarmentosum* were collected from the vicinity of Universiti Putra Malaysia. Authentication of the species was based on published taxonomical nomenclatures [11]. The leaves of *Piper sarmentosum* were rinsed thoroughly with multiple changes of distilled water and oven-dried at 50 °C before being ground into powder. Extraction was carried out by macerating 100 g of powdered leaves with 500 mL of 95 % ethanol at room temperature for 24 h. The extract was then filtered through Whatman no. 1 filter paper. After filtration, the extract was evaporated under reduced pressure using rotary evaporation. The RPMI-1640 medium (Gibco BRL) was used as the solvent for the preparation of various dilutions of the ethanolic extract. Concentrated stock solution of each ethanolic extract was prepared by adding a known weight of each ethanolic extract to a known volume of RPMI-1640 medium, and then serially diluted (1:2) to obtain the working solutions at different final concentrations.

Vero cell line

Vero cells were maintained in a growth medium consisting of RPMI-1640 supplemented with 10 % FBS (Gibco BRL), (Gibco BRL), 100 units/mL penicillin (Gibco BRL) and 100 µg/mL streptomycin (Gibco BRL). Clindamycin (Sigma-Aldrich Corp) was used as the reference control drug. The cells were cultured and maintained at 37 °C in a humidified 5 % CO_2 incubator.

Toxoplasma gondii RH strain

The tachyzoites from the virulent RH strain of *T. gondii* were harvested from the peritoneal fluid layer of infected Balb/c mice in phosphate-buffered saline (PBS), pH 7.2. The peritoneal fluid obtained was then centrifuged at 1500 rpm for 10 min at room temperature to remove host cells and debris as mice host cells were found in the peritoneal fluid layer. The pellet was washed twice with RPMI-1640 supplemented with glutamine (2 raM), penicillin (100 units/mL), and streptomycin (100 µg/mL). The tachyzoites *T. gondii* were inoculated to the Vero cell and grown in a 25mL tissue culture flask at 37°C in a humidified 5 % CO_2 incubator.

MTT assay

Cytotoxicity of the ethanol extract of *P. sarmentosum* on Vero cells was determined using 3-(4, 5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide MTT assay which was published by Mosmann [12]; however, some modifications were made to this method. Vero cells were seeded at a density of 6 x10^4 cells/mL in each well of a 96-well plate containing 100µL of the growth medium. The plate was incubated at 37 ºC in a humidified 5 % CO_2 incubator for 24 hrs. The cells were treated with the ethanol extract of *P. sarmentosum*, which was serially diluted using the growth medium to obtain the various final concentrations of 200, 100, 50, 25, 12.5, 6.25 and 3.125 µg/mL. 100 µL of each concentration was added into each respective 96-well plate. In this study, clindamycin, a known drug for treating toxoplasmosis was used as the reference (positive) control drug, and RPMI was used as the control buffer. After 24 h of treatment, 200µL of the medium was aspirated out and 100 µL of MTT - PBS (5 mg/mL) solution in RPMI medium in the ratio of 1: 9 was added to each well. The plate was then covered with aluminium foil and incubated for 4hrs in a 37 ºC incubator. The medium was then discarded and 100µL of DMSO was added into each well to solubilise the dark-blue MTT formazan salt. The optical density was measured at 570 nm absorbance using a Dynex Microplate Reader; higher concentrations can cause toxicity in Vero cells. Growth inhibition (GI) was calculated as in Eq 1.

$$GI\ (\%) = \{(At - Ac)/Ac\}100 \quad \ldots\ldots\ldots\ldots\ldots\ldots \quad (1)$$

where At and Ac are the absorbance of treated cells and control, respectively.

Determination of anti-*Toxoplasma* activity and cytotoxicity

The inoculated Vero cells were harvested from the culture flask during the exponential growth of the cells at day 2, post-inoculation, with the cell density of 6×10^4 cells/mL cultured in 96-well plates. Following this, 3×10^5 mL of *Toxoplasma gondii* was added to each well with the parasite to cell ratio of 5:1 for Vero cell inoculation; this ratio was based on a previous study by Belloni [13]. Six hours after inoculation, the inoculated cells were washed twice with RPMI to remove any non-adherent parasites. After 18 hours of incubation, 200µL RPMI supplemented with 2 % FBS was added to each well following the methods developed by Sheng [14]. After 24 hours of treatment, the anti- *T. gondii* activity and cytotoxicity of the ethanol extracts of *P. sarmentosum* were evaluated using the MTT method. The optical density reading on the microplate reader at 570nm was represented by the mean of three independent experiments. The median inhibitory concentration (IC_{50}) value was calculated based on the concentration of ethanol extracts of *P. sarmentosum* and clindamycin that successfully inhibited 50 % of *T. gondii*. Anti-*Toxoplasma* activity was expressed as selectivity index (SI) based on the mean of the IC_{50} values for Vero cells, relative to the mean of the IC_{50} value of *T. gondii* as shown in in Eq 2 [15].

$$SI\ (\%) = (V\text{-}IC_{50}/T\text{-}IC_{50})100 \quad \ldots\ldots\ldots (2)$$

where $V\text{-}IC_{50}$ and $T\text{-}IC_{50}$ are the median inhibitory concentrations required to inhibit Vero cell and *T. gondii*, respectively.

Microscopic examination of tachyzoites

Vero cells were cultured on a glass cover slip inside a 35mm petri dish until confluent, before being inoculated with 1×10^4 tachyzoites of *T. gondii*. After incubation for 4 hours, the monolayers formed on the glass cover slip were washed with a Hanks balanced salt solution (HBSS) (Gibco BRL). The ethanol extract of *P. sarmentosum* and clindamycin was then added to each glass cover slip and incubated for 24 h at 37 ºC. After incubation, the glass cover slips were washed twice with HBSS and fixed with methanol prior to staining with Giemsa (Sigma-Aldrich Corp). All the prepared samples were observed using Nikon (model- Nikon Eclipse 50*i*) microscope and the images were captured using (NIS-Elements D, Japan). Qualitative analysis methods were used as an alternative to quantitative analysis.

Statistical analysis

The statistical analysis was performed using Student's t-test, and differences were considered significant at $p < 0.05$. The results were analysed using the Statistical Package for Social Sciences (SPSS) version 22 for Windows and are presented as mean ± standard deviation (SD).

RESULTS

Anti-*Toxoplasma* activity

The results of *in vitro* anti-*Toxoplasma* activity against the *T. gondii* RH strain and selectivity index are summarized in Table 1 and Figure 1. Anti-*Toxoplasma* activity was found in all the samples tested as well as high mortality. The highest anti-*Toxoplasma* activity was found in Clindamycin with IC_{50} = 84.0 ± 1.15 µg/mL and Selectivity index = 11.67. Anti-*Toxoplasma* activity of the ethanol extracts of *P. sarmentosum* was comparable to clindamycin with IC_{50} = 125.0 ± 1.53 µg/mL and selectivity index = 10.08.

Morphology of anti-*Toxoplasma* in inoculated Vero cells

Figures 2 and 3 illustrate the morphological effects of RPMI, clindamycin and *P. sarmentosum* extract on Vero cell and *T. gondii* inoculated Vero cells. After 24 hours, post-inoculation, higher parasitemia were observed in the inoculated Vero cells (Figure 3). Tachyzoites of *T. gondii* could be observed inside the Vero cells; the tachyzoites were also adhered to the glass coverslip in the group treated with RPMI. However, when 50 µg/mL clindamycin and *P. sarmentosum* extract was added, the parasitemia drops and quantities of tachyzoites of *T. gondii* decreased sharply.

Table 1: Cytotoxicity, *in vitro* activity and selectivity index of ethanol extract of *P. sarmentosum* (with clindamycin as control) against *T. gondii* tachyzoites

Tested material	**Cytotoxicity (IC_{50}, µg/mL)**	***In vitro* anti-*Toxoplasma* assay (IC_{50}, µg/mL)**	**Selectivity index (SI)**
Clindamycin	84±1.15	7.2± 1.53	11.67
P. sarmentosum	125± 1.53	12.4± 1.00	10.08

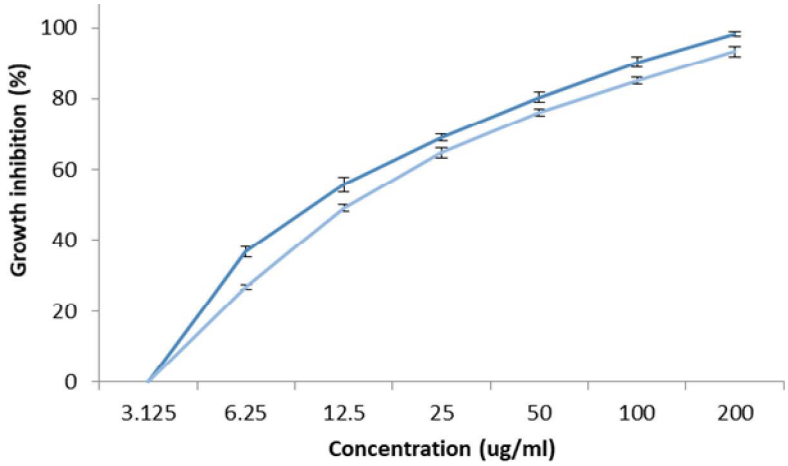

Figure 1: Anti-*Toxoplasma* activity of ethanol extracts of *P. sarmentosum* and clindamycin against *T. gondii* (−−−− Clindamycin, ------Piper)

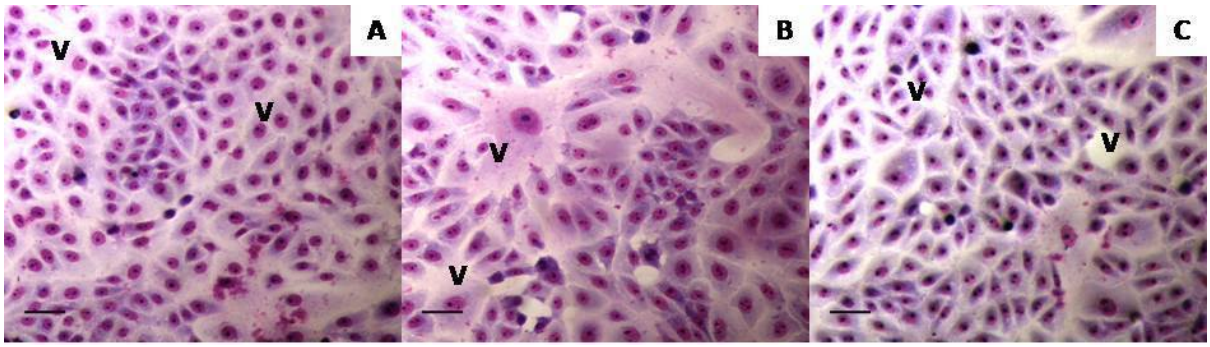

Figure 2: Morphology of Vero cells after cytotoxicity testing with A) Clindamycin, B) *P. sarmentosum* leaves and C) RPMI medium. After 24 hours of exposure to 50 µg/mL of *P. sarmentosum* leaves extract and clindamycin. Vero cells showed no remarkable morphological changes. Stained using Giemsa stain. (200 X magnification) Scale 20 µm

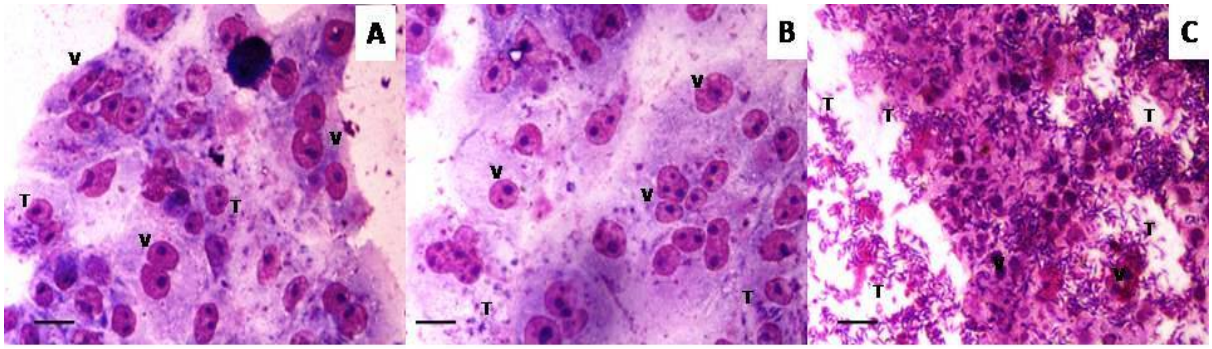

Figure 3: Morphology of Vero cells infected with *T. gondii* after adding a A) clindamycin, B) *P. sarmentosum* and C) RPMI medium. The tachyzoites of *T. gondii* (T) can be seen inside the inoculated Vero cells (V), as well as adhered to the glass cover slip. After 24 hours, post-inoculation and exposure to the 50µg/mL of *P. sarmentosum* extract and clindamycin, the inoculated Vero cells showed no remarkable morphological changes. Stained using Giemsa stain. A and B (400 X magnification), C (400 X magnification) Scale 10 µm

Following the first 24 hours, the tachyzoites of *T. gondii* were infrequently observed in the inoculated Vero cells. No counting was involved in this process, the method was exclusively based on observation. There was no significant change in morphology of Vero cells after exposure to *P. sarmentosum* extract (Figure 2 and Figure 3).

DISCUSSION

Parasitic diseases continue to have huge impact on human health, particularly in tropical regions [16]. New anti-parasitic drugs are urgently required in the treatment and control of parasitic infections such as toxoplasmosis [17]. The identification of an appropriate host to support

the growth of the parasite presents a major challenge in anti-parasitic drug discovery. Scientific evidence documenting the anti-parasitic properties of medicinal plant extract, derives mainly from *in vitro* studies. The main advantage of *in vitro* studies in this context is the ability to detect directly anti-parasitic activity of these extracts or compounds. It is necessary to ensure that the plant extracts are not toxic to the host and will not affect host performance [18]. Plant extracts can be involved in anti-parasitic activity assessment only if the parasitized host benefits from prolonged exposure to the extract. This net benefit can only be achieved if the antiparasitic properties of these extracts do not cause the adverse side effect on the host cell's morphology and performance [19].

Studies by Villena [20], demonstrated the efficacy of sulfadiazine and pyrimethamine combination in the treatment of toxoplasmosis. This combination leads to remarkable synergistic activity against the replicating form of *T. gondii* cysts in tissue through the sequential inhibition of parasite dihydropteroate synthase (DHPS) and dihydrofolate reductase (DHFR). These two major enzymes are responsible for the synthesis of the folate compounds that are essential for the survival and replication of parasites. However, several failures have been reported in the treatment of toxoplasmic encephalitis, chorioretinitis and congenital toxoplasmosis. These failures, relating to the host, may be due to several factors such as drug intolerance, malabsorption, poor compliance to the development of drug-resistant parasites, or lower levels of susceptibility to the parasite strain [20]. The ethanolic extract of *P. sarmentosum* was tested for potential cytotoxic activity *in vitro* against Vero cells. It was concluded that the extract was not toxic to Vero cells with IC_{50} values of more than 100 µg/ml (Figure 1).

It is imperative to first establish whether the plant extracts affect Vero cell performance using the cytotoxicity study. Following the findings (Table 1), the ethanol extracts of *P. sarmentosum* were further tested for anti-*Toxoplasma* and the potential (Figure 2). Anti-*toxoplasma* activity was found in the sample tested and high mortality of tachyzoites was observed. The extract also exhibited potential T. gondii inhibitory activity with the IC_{50} = 12.4 ± 2.08 µg/mL on the parasite growth (Figure 4). The results of the ethanol extracts of *P. sarmentosum* leaves were comparable to those of clindamycin (IC_{50} = 7.2 ± 1.52 µg/mL) (Figure 3 and Figure 4). A previous study by Saadatnia [21] concluded that the propagation of *T. gondii* parasites in Vero cells produced high yield and viability of tachyzoites, with minimal host cell contamination. Hence, Vero cells were used as the host cells for *T. gondii* in the present study to determine the anti-parasitic activity of the plant extracts.

Following the addition of plant extracts to monolayers of Vero cells, the cells remained metabolically active and viable after being infected with *Toxoplasma gondii*. This study confirmed that *Toxoplasma gondii* could invade Vero cells and proliferate quickly; it also demonstrated that plant extracts effectively inhibited the growth of *Toxoplasma gondii*, and were less toxic to Vero cells than clindamycin. This finding signifies that *P. sarmentosum* could potentially be an alternative to clindamycin for the treatment of toxoplasmosis; this warrants further study.

Normally the rapidly proliferating *Toxoplasma gondii* tachyzoites propagate by host cell lysis, regression, reattachment, and invasion of new host cells [22]. The significant changes observed in the morphology and confluence of Vero cells in an exclusively RPMI medium treated group (negative control) is likely due to the above fact. However, plant extract did not significantly affect the normal growth and morphology of the infected host cells, despite the potent anti *T. gondii* activities. The cells seemed to remain metabolically active and viable. In fact, cell proliferation and confluence was greater when compared to exposure to clindamycin. This observation verified that plant extracts had greater anti *T. gondii* activity and also had no selective toxicity against the infected host cells. Finally, this study confirms *P. sarmentosum* has potential as an alternative to clindamycin for the treatment of toxoplasmosis.

CONCLUSION

The ethanol leaf extract of *P. sarmentosum* shows potential anti-parasitic activity against *T. gondii.* Further study in the identification of the active compounds from this plant in *Toxoplasma gondii* infected animal models need to be carried out.

DECLARATIONS

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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