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Alkaloids content, cytotoxicity and anti-*Toxoplasma gondii* activity of *Psidium guajava* L. and *Tinospora crispa*

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Abstract

The alkaloids content, cytotoxicity activity and anti-parasitic activity of *Tinospora crispa* Miers and *Psidium guajava* L. against parasite *Toxoplasma gondii* were investigated. Both of the crude plant extracts contained high amount of crude alkaloids and were not toxic to Vero cells (EC_{50} >100 µg/mL). Following non-toxicity finding of the extracts, the *in vitro* anti-parasitic assay was carried out with clindamycin served as the positive control. *T. crispa* stem crude extract with EC_{50} value of 7.7 ± 1.6 µg/mL, showed potential anti-*T. gondii* activity as the results were comparable to clindamycin (EC_{50} = 6.2 ± 0.5 µg/mL). These findings suggest that stem extract of *T. crispa* Miers contained some potential active compounds against *Toxoplasma gondii*. Selective important alkaloids in the *T. crispa* stem fractions may be the key factor for their anti-parasitic activity. Further study is being carried out to investigate for potential bioactives present in the *T. crispa* extract through bioactivity guided fractionation.

Introduction

Toxoplasma gondii is an obligate intracellular apicomplexan parasite that has the ability to infect and propagate in virtually all nucleated host cells (Werk, 1985; Derouich-Guergour et al., 2002). Infection with *T. gondii* causes toxoplasmosis, a disease that occurs world-wide. In human, the outcome of *T. gondii* infection is influenced by a person's immune state, and immunocompetent individuals usually are asymptomatic (Luft and Remington, 1992). The parasites are able to establish a chronic infection with cysts residing in a number of tissues. Relapse of the quiescent infection or acquisition of a primary infection in the immunocompromised patient (e.g., people with AIDS) frequently results in encephalitis or disseminated disease, or both (Luft and Remington, 1992). Standard therapies for toxoplasmosis, involving combinations of pyrimethamine with sulfadiazine, clindamycin, azithromycin, or atavaquone (Djurkovic-Djakovic et al., 1999; Chirgwin et al., 2002) do not eliminate the intracellular parasite and are often associated with severe side effects (Montoya and Liesenfeld, 2004). Continuous drug therapy is essential for patients to ensure prevention of severe complications. Alternative drugs with lesser side effects are needed to combat the disease.

Tinospora crispa Miers (family Menispermaceae) is used to treat jaundice, rheumatism, intermittent fever (Siddiqi et al., 1974; Noor and Ashcroft, 1998), malaria (Bertani et al., 2005), filariasis (Zaridah et al., 2001) and also well known for anti-inflammatory, antispasmodic properties (HMR, 2002; Singh et al., 2003). *Psidium guajava* L. (from family Myrtaceae) has been employed

for treating various diseases such as relieving cough, pulmonary disorders, wounds, and ulcers. The fruit have tonic, cooling, laxative, and anthelmintic activities (Shen et al., 2008).

In the present study, crude methanolic extract from *T. crispa* stem and crude aqueous extract from *P. guajava* leaves were investigated in their respective crude alkaloids content, potential cytotoxicity activity and anti-*Toxoplasma gondii* effect *in vitro*.

Materials and Methods

Plant material

The whole study was conducted from 10th August 2011 to 25th April 2012. The stem of *T. crispa* Miers and leaves of *P. guajava* L. were purchased from Herbagus Sdn. Bhd. (Kepala Batas, Pulau Pinang, Malaysia). Voucher specimens (no. 11261 for *P. guajava* L. and no. 11262 for *T. crispa* Miers) were authenticated and deposited at Herbarium of School of Biological Sciences, Universiti Sains Malaysia. The plant materials were washed to remove the debris and air-dried in the shade. The dried plant materials were then blended into powder form and kept at 4°C until use.

Extraction

The dried powdered stem of *T. crispa* was extracted exhaustively with methanol at room temperature. The filtered methanolic extract was then evaporated to dryness to yield a sticky dark brown crude residue which was freeze-dried to become dark crude *T. crispa* extract. The crude extract was stored at 4°C until use. The dried powdered leaves of *P. guajava* were macerated with distilled water for 1 day. The filtered extract was then lyophilized to powder form. The dried powder was stored at 4°C until use. Stock solutions of 10 mg/mL of both crude extracts were prepared by dissolving in dimethyl sulfoxide (DMSO). The stock solutions were diluted before use.

Alkaloid determination

Five gram of the plant sample was weighed into a 250 mL beaker and 200 mL 20% acetic acid in ethanol was added and covered to stand for 4 hours. It was then filtered and the extract was concentrated using a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitation was collected by filtration and then weighed (Obadoni and Ochuko, 2001; Harborne, 1973).

Cell culture

Vero cell line was obtained from the Tissue Culture Laboratory of the Institute for Research in Molecular Medicine (INFORMM), Universiti Sains Malaysia. Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin (PS) was used as the growth medium (GM). The cells were grown and maintained in a humidified 5% CO_2 incubator at 37°C.

In vitro assay for cytotoxic activity (MTT assay)

Cytotoxicity of the extracts on Vero cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (Mosmann, 1983) with some modifications. The assay evaluates the percentage of viable cells remaining after the treatment. Briefly, after harvesting from the culture flask, Vero cells were seeded at a density of 12 x 10^4 cells/mL in each well of a 96-well plate containing 100 µL of GM. The plate was incubated at 37°C in 5% CO_2 incubator for 24 hours. The cells were treated with the plant extracts, which were serially diluted with GM to obtain various concentrations (200, 100, 50, 25, 12.5, 6.2, 3.1 µg/mL). An amount of 100 µL of each concentration was added into each well. Clindamycin, a drug used to treat toxoplasmosis, served as positive control. GM with 0.1% DMSO served as negative control. After 24 hours of treatment, the medium (200 µL) was aspirated out and the 100 µL of MTT- phosphate buffered saline (PBS) (5 mg/mL) solution in GM in ratio of 1:9 was added to each well. The plate was covered with aluminum foil and incubated for 4 hours in the incubator. The medium was then discarded and 100 µL of DMSO was added into each well to solubilise the dark-blue MTT formazan salt. The plates were shaken with microplate shaker (Heidolph Titramax 101, Germany) for 5 min before measuring the absorbance (abs) at 540 nm with a microplate reader (Thermo Scientific Multiskan® Spectrum, USA). The percentage of growth inhibition (GI) was calculated using the following formula:

$$GI = \frac{\text{Average abs of treatment well} - \text{Average abs of control wells}}{\text{Average abs of control wells}} \times 100\%$$

The median effective concentration (EC_{50}) value refers to the concentration of the plant extracts necessary to inhibit 50% of the control values. All tests and analyses were run in triplicates.

Parasite

Tachyzoites of *T. gondii* RH strain were maintained by intraperitoneal passages in Swiss albino mice and were collected in DMEM media supplemented with 5% PS, four days after infection. The peritoneal fluid obtained from infected mice, which contained the parasites, was collected and centrifuged at 200 x *g* (Eppendorf Centrifuge 5804R, Germany) for 5 min to separate mice cells. The supernatant was centrifuged at 1400 x *g* for 10 min. The pellet containing parasites was washed with

DMEM supplemented with 5% PS and suspended to a density of 6 x 10^5 parasites/mL. The parasites were used within 1 hour post-harvest, and 100% viability was observed using trypan blue dye-exclusion test.

In vitro anti-parasitic assay on Toxoplasma gondii

Vero cells were harvested during exponential growth and cultured in 96-well plates at 12 × 10^4 cells/mL (final volume of 100 µL). On day 2, 6 × 10^5 parasites/mL of parasites were added to each well (parasite: Cell ratio = 5:1, final volume 200 µL) (Belloni et al., 2003). Six hours after inoculation, the cultures were washed with DMEM-PS medium to remove any non-adherent parasites. A volume of 100 µL of fresh DMEM-PS medium was then added to each well. After 18 hours incubation, 100 µL of DMEM-PS medium with 2% FBS was added per well along with different concentrations of the plant extracts. Clindamycin was used as the positive control. After 24 hours of treatment, anti-*T. gondii* activity of the plant extracts was determined using MTT assay, as described above. All tests were assayed in triplicates. Selectivity index was calculated, this refers to the ratio of EC_{50} value determined on Vero cell line to the EC_{50} value determined on *T. gondii*.

Fractionation

Plant extract (crude methanolic *T. crispa* stem extract) that showed lowest EC_{50} values in anti-*T. gondii* assay was chosen for further fractionation by column chromatography. First, the crude *T. crispa* extract was tested with TLC Silica Gel 60 F254 (Merck) with different combinations of solvents to determine the best mobile phase as eluting solvent for the crude extract in the column chromatography. It was found that a mixture of hexane-acetone (70:30) was best in separating the different components. Following this, column chromatography was performed to fractionate the crude *T. crispa* extract with polarity gradient elution of the hexane-acetone. An amount of 200 g of silica gel 60 (0.06-0.20 mm) (Merck) was packed into a column with hexane. The silica gel was left to settle overnight. A sample amount of 10 g of the crude *T. crispa* extract was dissolved in hexane and loaded into the column. The elution of the crude extract in hexane with increasing degrees of polarity of acetone yielded eight subfractions (F1-F8). The eight fractions were dried under vacuum to dryness and stored at 4°C. A stock solution of 10 mg/mL of the fractions was prepared by dissolving the extract in DMSO. The stock solutions of all eight fractions were further investigated for potential cytotoxicity and anti-*T. gondii* activity.

Statistical analysis

The results were analysed using the Statistical Package for Social Sciences (SPSS) version 10.0 for windows. All the data are expressed as Mean ± SD (n = 3).

Results

Both *T. crispa* stem and *P. guajava* leaves contained high amount of crude alkaloids. *P. guajava* leaves contained higher alkaloids content (7.5 ± 0.2%) compared to *T. crispa* stem (5.2 ± 0.2%). Both *T. crispa* and *P. guajava* crude extracts, 8 fractions from *T. crispa* crude extract and clindamycin (positive control used in anti-parasitic assay) were tested for the potential cytotoxic activity *in vitro* using Vero cells. All of the extracts tested were not toxic to Vero cells with EC_{50} values more than 100 µg/mL.

Table I

***In vitro* anti-parasitic activity of plant extracts and clindamycin on *Toxoplasma gondii* and cytotoxicity on vero cell**

Plant species	Cytotoxicity assay EC_{50} (µg/mL)	*In vitro* anti-*toxoplasma gondii* assay EC_{50} (µg/mL)	SI
Clindamycin	613.8 ± 1.9	6.2 ± 0.5	98.3
P. guajava leaves extract	111.0 ± 2.2	121.2 ± 6.6	0.9
T. crispa stem extract	147.5 ± 2.3	7.7 ± 1.6	19.1
T. crispa F1	251.9 ± 2.9	34.2 ± 4.1	10.3
T. crispa F2	172.1 ± 2.2	23.0 ± 2.7	7.5
T. crispa F3	160.3 ± 2.5	11.3 ± 1.6	14.2
T. crispa F4	164.6 ± 1.8	6.7 ± 1.9	24.5
T. crispa F5	156.9 ± 2.1	6.1 ± 2.5	28.4
T. crispa F6	177.7 ± 2.6	7.3 ± 1.2	24.2
T. crispa F7	259.6 ± 2.7	21.9 ± 2.9	11.9
T. crispa F8	142.4 ± 2.5	26.0 ± 0.3	5.5

EC_{50} values are the mean ± standard deviations from three independent experiments; SI: selectivity index, defined as the ratio of the EC_{50} value determined on the Vero cell line on the EC_{50} value determined on *T. gondii*

T. crispa stem extract (EC_{50} = 7.7 ± 1.6 µg/mL) showed significant inhibitory effect on parasite growth compared to *P. guajava* leaves extract (EC_{50} = 121.2 ± 6.6 µg/mL; Table I). The result of *T. crispa* was comparable to positive control clindamycin (EC_{50} = 6.2 ± 0.5 µg/mL). Among the fractions that were tested for anti-parasitic assay, F4 (EC_{50} = 6.7 ± 1.9 µg/mL) and F5 (EC_{50} = 6.1 ± 2.5 µg/mL) showed the most significant inhibitory effect on parasite growth.

Discussions

A decoction of the stems, leaves and roots of *T. crispa* is used to treat inflammation, fever, cholera, diabetes and rheumatism in Thai and Malay traditional medicine (Dweck and Cavin, 2006). The extracts of roots, bark, and leaves of *P. guajava* are used to treat inflammation and fever. The guava leaves are used as an ingredient in the preparation of fever "teas" (Dweck, 2005). Both *T. crispa* and *P. guajava* are used to treat inflammation and fever in traditional medicine. Both plant species contained chemical components with potential anti-parasitic bioactivities. *P. guajava* stem-bark extract contained anthraquinones, flavonoids, seccoirridoids and terpenoids and has been found to be effective for the treatment and/or prophylaxis of malaria in KwaZulu-Natal province of South Africa (Dweck, 2005). *T. crispa* stem contained alkaloids, triterpenes, flavonoids and glucoside that may hold the key to combat parasites (Dweck and Cavin, 2006). Hence, both plants are worthy to be investigated for presence of potential anti-parasitic compounds.

Pure isolated plant alkaloids and their synthetic derivatives are used as a basic medicinal agent for its analgesic, antispasmodic and bactericidal effects (Stray, 1998; Okwu, 2004). Alkaloids exhibit marked physiological activity when administered to animals. Most of the plant parts used in the cure of diseases have been reported to contain traces of alkaloids. For instance, *Azadirachta indica* used in the cure of malaria contain alkaloids (Harborne, 1988). Quinine, an alkaloid isolated from *Cinchona* bark, is the oldest known effective anti-malarial agent. Being bases, alkaloids are normally extracted from plants into a weakly acid alcoholic solvent and are then precipitated with concentrated ammonia (Harborne, 1988). Both *T. crispa* and *P. guajava* contained significant amount of alkaloids. The presence of alkaloids suggests that both *T. crispa* stem and *P. guajava* leaves have medicinal properties.

In a previous study by Saadatnia et al. (2010), they reported 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 in order to determine the anti-parasitic activity of the plant extracts. However, it was very important to first establish whether the plant extracts affect the Vero cells performance. According to Zirihi et al. (2005), an extract is considered safe when EC_{50} is >20 µg/mL. As shown in Table I, *T. crispa* extract and *P. guajava* extract as well as the eight fractions obtained from *T. crispa* crude extract and clindamycin (as positive control) showed no cytotoxicity against Vero cells.

Following the non-toxic findings in the cytotoxicity assay, both crude *T. crispa* and *P. guajava* extracts were further tested for the potential anti-parasitic assay against *Toxoplasma gondii* parasite. Crude methanolic *T. crispa* stem exhibited potential *T. gondii* inhibitory activity while crude aqueous *P. guajava* extract was found to exhibit less significant activity. The most potent crude extract (*T. crispa* stem extract) was selected for fractionation by column chromatography with gradient elution of hexane:acetone. A total of eight fractions were pooled. All the fractions were subjected to cytotoxicity and *in vitro* anti-parasitic assays.

All eight fractions of *T. crispa* possessed no cytotoxicity activity against Vero cells. The fractions (F1-F8) were expected to be non-toxic since the crude extract was proved to be not toxic against Vero cells. All fractions were further investigated for the anti-parasitic assay through *in vitro* method. The results showed that *T. crispa* subfraction F4 and F5 were of particular interest, since their anti-*T. gondii* activities were comparable to the standard drug clindamycin: *T. crispa* subfraction F4 (EC_{50} = 6.7 ± 1.9 µg/mL, SI = 24.5) and F5 (EC_{50} = 6.1 ± 2.5 µg/mL, SI = 28.4). The SI values for crude *T. crispa* extract as well as subfractions F4 and F5 were found to be high. Extracts that demonstrated high selectivity (high SI value) are expected to offer the potential safer therapy. Based on the moderate amount of crude alkaloids content of the plant extracts, this suggested that important structures of alkaloid may have attributed to the anti-*T. gondii* activity of *T. crispa* extract.

Conclusion

Crude methanolic extract of *T. crispa* stem and its moderately polar subfractions F4 and F5 showed potential anti-parasitic activity against the parasite *T. gondii* and this could be due to the selective important alkaloids present in the *T. crispa* stem.

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