Ethanol extracts from Thai plants have anti-*Plasmodium* and anti-*Toxoplasma* activities *in vitro*

Arpron Leesombun [a, b], Sookruetai Boonmasawai [b], Yoshifumi Nishikawa [a*]

Author affiliations:

[a] *National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan*

[b] *Department of Preclinic and Applied Animal Science, Faculty of Veterinary Science, Mahidol University, 999 Phutthamonthon Sai 4 Road Salaya, Phutthamonthon Nakhonpathom 73170, Thailand*

*Corresponding author: Yoshifumi Nishikawa

National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan

Tel.: +81-155-49-5886

Fax: +81-155-49-5643

E-mail: nisikawa@obihiro.ac.jp

Abstract

The ethanol extracts from seven Thai plants, *Kaempferia parviflora*, *Stemona tuberosa* Lour., *Ananas comosus*, *Punica granatum*, *Musa sapientum* L., *Pseuderanthemum palatiferum* and *Annona muricata* L., which are traditionally used in Thailand to support human health, were evaluated for their anti-*Plasmodium* and anti-*Toxoplasma* activities, and for their cytotoxicities against human foreskin fibroblasts *in vitro*. The *K. parviflora*, *P. palatiferum* and *A. muricata* extracts were active against *P. falciparum* (3D7) with selectivity index (SI) values > 10, while their half maximal inhibitory concentrations (IC_{50}) were 28.7 µg/ml, 78.8 µg/ml and 46.1 µg/ml, respectively. Extracts from *K. parviflora* and *M. sapientum* (ripe fruit peel) inhibited *T. gondii* (RH) growth with IC_{50} values of 53.5 µg/ml and 90.4 µg/ml, respectively. The SI values of the extracts from *K. parviflora* and *M. sapientum* (ripe fruit peel) were 9.0 and 10.8, respectively. Our data show that some of the aforementioned ethanol extracts are potential sources of new drugs to treat *Plasmodium* and *Toxoplasma* infections.

Keywords: natural plants; traditional medicine; *Plasmodium falciparum*; *Toxoplasma gondii*

Introduction

Plasmodium spp. and *Toxoplasma gondii* are obligate intracellular parasites within the Apicomplexa phylum. These parasites have a hugely negative impact on human health, and on social and economic wellbeing via the inestimable morbidity and mortality they cause. Human malaria is caused by *Plasmodium* species and is regularly found in tropical and subtropical countries. According to the latest WHO estimates in December 2016, 212 million cases of malaria occurred in 2015 and 429,000 deaths were reported. Human infections with *Plasmodium falciparum* cause severe anemia, acidosis, acute respiratory distress syndrome, and acute renal and hepatic failure, which can all prove fatal (Phillips *et al*. 2017). In contrast, *Toxoplasma gondii* is an opportunistic pathogen that infects approximately one-third of the world's population. *T. gondii* infections cause miscarriage or adverse fetal effects, including neurological and ocular diseases (Montoya and Liesenfeld 2004). The appearance of drug-resistant parasites and the side effects of the current drugs used to treat malaria and toxoplasmosis cause serious concern; hence, developing new drug treatments for these diseases remains challenging.

Medical plants have been used for therapeutic purposes since ancient times. They often contain highly diverse biological compounds and are very promising sources of new drugs (Dias *et al*. 2012). Several drugs for treating infectious diseases such as malaria and toxoplasmosis are derived from plants. For example, quinine and artemisinin are both used to treat malaria (Batista *et al*. 2009). Because no licensed vaccines for preventing malaria and toxoplasmosis are available in human, chemotherapy is currently the only option for treating these diseases. However, for long-term treatment, drugs should be both effective and lacking in toxicity. Drug resistance is also a problem in malaria parasites with many drugs targeting them being, or starting to be, ineffective (Batista *et al*. 2009). For discovering novel compounds with new mechanisms of action and/or lower side effects, plants have great

potential (Giang *et al*. 2003; Yenjai *et al*. 2004; Lin *et al*. 2008; Waghmare and Kurhade 2014; Fadaeinasab *et al*. 2015; Shaygannia *et al*. 2016). Medicinal plants have been traditionally used to treat various illnesses, relieve their symptoms, or to promote wellness. The rhizomes of *Kaempferia parviflora* (Thai black ginger) have been traditionally used for health-promoting purposes, to treat gastrointestinal ulcers, and for impotence (Yenjai *et al*. 2004). *Stemona tuberosa* is traditionally used for respiratory disorders, parasitic infections, and inhibiting the growth of bacteria and fungi (Lin *et al*. 2008). *Ananas comosus* (pineapple) is used for wound healing (Pavan *et al*. 2012) and *Punica granatum* (pomegranate) has anti-inflammatory, antibacterial, and anticancer properties (Shaygannia *et al*. 2016). *Musa sapientum* (banana) is used to treat diarrhea, constipation, and allergies (Waghmare and Kurhade 2014). *Pseuderanthemum palatiferum* is primarily used for treating inflammation, cancer and digestive troubles (Giang *et al*. 2003), while *Annona muricata* (soursop) is used to treat cancer and parasitic infections (Fadaeinasab *et al*. 2015). In this study, we investigated the potential anti-*Plasmodium* and anti-*Toxoplasma* activities of ethanol extracts from the aforementioned Thai plants; namely, *K. parviflora*, *S. tuberosa*, *A. comosus*, *P. granatum*, *M. sapientum*, *P. palatiferum*, and *A. muricata*.

Materials and methods

Parasites

P. falciparum 3D7 was maintained according to the method of Trager and Jensen (Trager and Jensen 1976). The parasites were routinely transferred to 5% O-positive washed human erythrocytes in fresh complete RPMI-1640 medium (Sigma, St Louis, MO, USA) supplemented with 6 g HEPES (Sigma), 5 g albumax II (Gibco, Carlsbad, CA, USA), 2 g $NaHCO_3$, 25 mg hypoxanthine and 250 µl gentamicin (stock 50 mg/ml), which was previously made up to one liter with MilliQ water. The complete medium was sterilized

through a 0.20 μm membrane filter (IWAKI, Saitama, Japan). The parasite cultures were incubated at 37°C in a 5% CO_2, 5% O_2 atmosphere. *T. gondii* RH-GFP (a green fluorescent protein expressing-RH strain) (Nishikawa *et al*. 2003) was maintained in human foreskin fibroblast (HFF) cells or African green monkey kidney (Vero) cells according to the previous methods (Leesombun *et al*. 2016).

Plant materials and chemicals

The plants used in this study (Table 1) were obtained from different regions of Thailand. *K. parviflora* and *S. tuberosa* came from northeastern Thailand. *A. comosus* came from eastern Thailand, and *P. granatum*, *M. sapientum*, *P. palatiferum* and *A. muricata* came from the central region of Thailand. *K. parviflora* was harvested in the winter season (January). *A. comosus* and *P. granatum* were harvested in the summer season (April). *S. tuberosa*, *P. palatiferum*, *M. sapientum* and *A. muricata* were harvested in the raining season (July). All plant materials were identified by Dr. Sookruetai Boonmasawai, Faculty of Veterinary Science, Mahidol University, Thailand. After plant identification, the parts were cleaned, cut into small pieces, and dried in a hot air oven at 70°C. The plant materials were ground and extracted with ethanol and the solvent was evaporated. The final crude extracts were prepared at 100 mg/ml in dimethyl sulfoxide and kept at −30°C. Chloroquine (Sigma) was prepared at 100 mM in distilled water.

Cytotoxicity

Cytotoxicity was evaluated in HFF cells. Cell suspensions (1×10^5 cells/ml in DMEM supplemented with 10% FBS) were plated at 100 μl/well in 96-well plates and incubated at 37°C in a 5% CO_2 atmosphere. The plant extracts were added to the cells at final concentrations of 100 to 1,000 μg/ml. To evaluate cell viability, HFF cell proliferation

inhibition (%) was calculated as described previously by Leesombun et al (Leesombun *et al*. 2016). IC_{50} values were calculated from the percentage inhibition using GraphPad Prism 6 software (GraphPad Software Inc., La Jolla, CA, USA).

Anti-Plasmodium activity

P. falciparum 3D7 was synchronized to the ring stage with 5% sorbitol, as verified by light microscopy on Giemsa-stained thin blood smears, and cultures with > 90% rings were used. The parasites were prepared at 0.5% parasitemia and 2% hematocrit in complete media. The suspension was added to a 96 well-plate containing each extract at several concentrations (1 to 100 μg/ml). Chloroquine (Sigma) was used as the positive control and medium alone was used as the negative control (total volume, 100 μl per well) and then incubated at 37°C in a 5% CO_2, 5% O_2 atmosphere. Following the description of Johnson et al 2007 (Johnson *et al*. 2007), after one cycle of the parasite's intraerythrocytic life cycle (48 h), 100 μl of SYBR Green I in lysis buffer was added to each well, mixed gently, and then incubated in a dark room at 37°C for 1 h. Fluorescence intensity was measured with a Fluoroskan Ascent instrument (Thermo Scientific, Waltham, USA) with the excitation and emission wavelengths set at 485 nm and 518 nm, respectively.

Anti-Toxoplasma activity

HFF cells (cell suspensions 1×10^5 cells/ml in DMEM supplemented with 10% FBS) were plated at 100 μl/well in 96-well plates, then *T. gondii* RH-GFP (5×10^4 tachyzoites/ well) was added to the cells for 4 h, the extracellular parasites were washed away, and new medium plus 10% FBS was added to each well. The plant extracts prepared in DMEM were added to the infected cells (final concentrations, 1 to 100 μg/ml), with sulfadiazine (Sigma) or media used as the positive and negative controls, respectively, and then incubated for 72 h.

The fluorescence intensity of RH-GFP was measured using a microplate reader (SH-900, Corona Electric Co., Ltd, Ibaraki, Japan) as described previously by Leesombun et al (Leesombun *et al*. 2016).

Results

The Table 1 shows the activities of the ethanol extracts from the Thai plants on *Plasmodium* and *Toxoplasma* parasites along with their cytotoxicities. The anti-*Plasmodium* activities of *K. parviflora*, *P. granatum* obtained from immature fruit, and *A. muricata* have already been reported (Yenjai *et al*. 2004; Dell'Agli *et al*. 2009; Somsak *et al*. 2016), whereas their anti-*Toxoplasma* effects have not. The ethanol extracts obtained from the *P. granatum* fruit peel and tree bark had high anti-*Plasmodium* activities (IC_{50} = 7.8 μg/ml and 4.9 μg/ml, respectively). However, the selective toxicities were relatively low because the SI values for *Plasmodium* were 2 and 7.4, respectively. Extracts from *K. parviflora*, *A. muricata* and *P. palatiferum* showed moderate to weak inhibition of *Plasmodium* growth (IC_{50}= 28.7 μg/ml, 46.1 μg/ml and 78.8 μg/ml, respectively), while the SI values for *Plasmodium* were higher than 10. Interestingly, the plant extracts that inhibited *P. falciparum* growth also affected *T. gondii* growth. The ethanol extracts from *K. parviflora* and *P. granatum* (from fruit peel and tree bark) inhibited *T. gondii* growth (IC_{50}= 53.5 μg/ml, 82.8 μg/ml, and 53.8 μg/ml, respectively), with lower IC_{50} values than that of sulfadiazine (IC_{50} = 99.4 μg/ml), while *K. parviflora* and *M. sapientum* (ripe fruit peel) especially, showed higher SI values (9.0 and 10.8, respectively) compared with the other extracts. Activities against *Plasmodium* and *Toxoplasma* were not seen with the ethanol extracts from *S. tuberosa*, *A. comosus* and *M. sapientum* (raw fruit peel), because their IC_{50} values were > 100 μg/ml.

Discussion

Identifying new chemotherapies for the treatment of malaria and toxoplasmosis is a challenging exercise. In contrast, scientific evaluation of medicinal plants can offer an effective and low-cost route to obtain drugs for the treatment of these diseases. In this study, the anti-*Plasmodium* activities of plant extracts were evaluated using a SYBR green I fluorescence assay to measure the DNA content of the parasite. The effects of the plant extracts against the *P. falciparum* ring, trophozoite and schizont developmental stages in the blood were investigated at 48 h post treatment (Bagavan *et al*. 2011; Kaushik *et al*. 2014; Berthi *et al*. 2018). The crude extract of *K. parviflora* exhibited anti-*P. falciparum* activity, with an IC_{50} of 28.7 μg/ml. Previously, the two flavonoids, 5,7,4′-trimethoxyflavone and 5,7,3′,4′-tetramethoxyflavone found in *K. parviflora* were found to exhibit anti-*P. falciparum* activities (IC_{50} = 3.70 μg/ml and 4.06 μg/ml, respectively) and low cytotoxicity to cancer cells (Yenjai *et al*. 2004). Because the *K. parviflora* ethanol extract inhibited *T. gondii* growth in this study, with an IC_{50} lower than that of sulfadiazine, which was used as the control, other flavonoids might also affect *T. gondii* growth. Therefore, flavonoid compounds should be investigated further to determine their anti-*Toxoplasma* activities.

We also found that the methanol extract from *P. granatum* fruit showed anti-*Plasmodium* activity (Dell'Agli *et al*. 2009). A fraction enriched in tannins (*Pg*-FET) found in *P. granatum* has potent activity against *Plasmodium*, and it has been suggested that *Pg*-FET may also inhibit the pro- inflammatory mechanisms involved in the onset of cerebral malaria (Dell'Agli *et al*. 2010). However, we noticed that the ethanol extracts from *P. granatum* fruit peel and tree bark had a strong growth inhibition effect on *Plasmodium* parasites, but a weak effect on *Toxoplasma* parasites. This plant had low SI values, suggesting that it is highly cytotoxic and therefore not recommended for use.

Leaf extracts from *A. muricata* by dichloromethane, methanol and sterile water (deionized) inhibit chloroquine-resistant blood-stage *P. falciparum* (EC_{50} < 10 μg/ml) with

non-toxic effects on Madin-Darby bovine kidney cells (SI values = 66-756) (Mohd Abd Razak *et al*. 2014). The pentane leaf extract from *A. muricata* has previously been tested on chloroquine-sensitive and chloroquine-resistant *Plasmodium* strains (IC_{50} = 16 µg/ml and 8 µg/ml) (Ménan *et al*. 2006). Several chemical compounds isolated from *A. muricata* leaves belong to alkaloid, annonaceous acetogenin, megastigmane and flavonol triglycoside classes and their anti-cancer activities have been confirmed (Fadaeinasab *et al*. 2015). Here, although *A. muricata* had anti-*Plasmodium* activity, it was less active against *Toxoplasma* (SI values = 12.2 and 5.0 respectively). Therefore, these phytoconstituents may be effective against *Plasmodium* growth. The compounds that were isolated from *M. Sapientum* peel have several pharmacological properties including antioxidant and antimicrobial ones. In particular, hexadecanoic acid ethyl ester has nematicide and pesticide properties (Waghmare and Kurhade 2014). Therefore, this ester alone or in combination with other compounds might be the active component responsible for anti-*Toxoplasma* activity. In this study, *M. Sapientum* was less effective at inhibiting *P. falciparum* growth, which is consistent with the results from a previous study (Kaou *et al*. 2008).

Additionally, the *P. palatiferum* leaf components contain n-pentacosan-1-ol, β-sitosterol and stigmasterol, and their corresponding 3-O-β-glucosides, kaempferol 3-methyl ether 7-O-β-glucoside and apigenin 7-O- β-glucoside (Giang *et al*. 2003), suggesting that the leaves may have anti-*Plasmodium* activities.

Our experiments have revealed the *in vitro* anti-*Plasmodium* activities of *K. parviflora*, *P. palatiferum* and *A. muricata*, and the anti-*Toxoplasma* activities of *K. parviflora* and ripe fruit peel from *M. sapientum*. The phytochemicals in these plant extracts will have various biological activities. Therefore, these plant extracts warrant further investigation in the search for new compounds to treat malaria and toxoplasmosis. In our future studies, these phytochemicals will be characterized to identify their active components.

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Conflict of interests

The authors declare, we have no conflict of interest.

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Plants	Plant part	Traditional use	References	IC_{50} (μg/ml)			Selectivity index (SI)	
				HFF cells	3D7	RH-GFP	3D7	RH-GFP
Kaempferia parviflora	Rhizome	- health-promoting, treating gastric ulcers	Yenjai *et al*. 2004	482	28.7	53.5	16.8	9
Stemona tuberosa Lour.	Root	- treating coughs, parasitic infections	Lin *et al*. 2008	751.4	144.9	214.7	5.2	3.5
Ananas comosus	Fruit peel	- used for wound healing	Pavan *et al*. 2012	612.9	104.1	127.3	5.9	4.8
Punica granatum	Fruit peel	- used for immune modulation, diuretic	Shaygannia *et al*. 2016	16.1	7.8	82.8	2	0.2
	Tree bark			36.3	4.9	53.8	7.4	0.7
Musa sapientum L.	Ripe fruit peel	- treating diarrhea, constipation, allergy	Waghmare and Kurhade 2014	980.2	454.9	90.4	2.2	10.8
	Raw fruit peel			405	114.4	165.2	3.5	2.5
Pseuderanthemum palatiferum	Leaf	- treating inflammation	Giang *et al*. 2003	797	78.8	211.5	10.1	3.8
Annona muricata L.	Leaf	- treating cancer, parasitic infections	Fadaeinasab *et al*. 2015	562.5	46.1	113.3	12.2	5
Sulfadiazine				>1,000*	N.D.	99.4*	N.D.	N.D.
Chloroquine				N.D.	0.02	N.D.	N.D.	N.D.

* Data from Leesombun *et al*. 2016

N.D. not determined

Table 1. Activities of ethanol extracts from Thai plants on *Plasmodium* and *Toxoplasma* parasites and their cellular cytotoxicities.