IN VITRO ANTHELMINTHIC ACTIVITY OF *PIPER RETROFRACTUM* AGAINST *STRONGYLOIDES STERCORALIS* THIRD STAGE INFECTIVE LARVAE

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Abstract. Emergence of anthelminthic resistance due to widespread and indiscriminate applications of conventional drugs has stimulated the need for alternatives in treatment and prevention of strongyloidiasis. This study evaluated anthelminthic activity of tropical plants against *Strongyloides stercoralis*, the most common soil-transmitted threadworm. *In vitro* anthelminthic potential of plant-based products was screened against *S. stercoralis* third stage infective larvae. Of 11 plants tested, three ethanolic extracts showed *in vitro* helminthicidal efficacy, causing 97-100% larval mortality. A dose-response anthelminthic bioassay demonstrated *Piper retrofractum* as the most effective extract, followed by *Abelmoschus esculentus* and *Carica papaya*, with LC_{50} value of 0.037, 0.092 and 0.101 mg/ml, respectively. Anthelminthic bioassays of *P. retrofractum* hexane extract, diethyl ether extract, dichloromethane extract, and essential oil demonstrated LC_{50} value of 0.060, 0.078, 0.086, and 1.080 mg/ml, respectively, much higher than that of the reference drug ivermectin (LC_{50} value = 0.021 µg/ml). *P. retrofractum* extracts will be further tested for their *in vivo* anti-strongyloides activities to evaluate their potentials for development as novel anthelminthic agents in treatment and prevention of strongyloidiasis.

Keywords: *Piper retrofractum, Strongyloides stercoralis*, natural anthelminthic, plant extract

INTRODUCTION

Strongyloides stercoralis is the most common soil-transmitted threadworm distributed worldwide. This nematode is capable of producing infection in humans and mammals. Strongyloidiasis, an intestinal parasitic disease caused by *S. stercoralis*, affects an estimated 30-100 million in 70 countries, particularly in tropical and subtropical regions (Genta, 1989; Jorgensen *et al*, 1996; Asdamongkol *et al*, 2006; Bethony *et al*, 2006; Nolan *et al*, 2011).

Infection of *S. stercoralis* is acquired through direct contact, mainly of bare feet with contaminated soil, which results in direct skin penetration of the infective free-living filariform larvae. However, the unique parthenogenesis property of female worm reproduction within the hu-

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man host permits cycles of autoinfection. *S. stercoralis* infective larvae are, therefore, not only produced externally in soil, but also internally by autoinfection, especially in immunodeficient or immunocompromised individuals, which lead to persistent infections for decades without further exposure to new infection (Liu and Weller, 1993; Grove, 1996; Speare and Durrheim, 2004). The phenomenon of autoinfective larvae results in hyperinfection and dissemination, which is the main reason why strongyloidiasis is a serious disease.

The prevalence of *S. stercoralis* is likely to be underestimated due to difficulty in detecting the parasite in uncomplicated cases and chronic infections because of low and fluctuating parasite burden. Furthermore, clinical manifestations in both acute and chronic infections are mostly asymptomatic or mildly symptomatic (Ramanathan and Nutman, 2008; El-Sherbini and Osman, 2013). Intermittent signs and symptoms, with non-specific or generalized complaints, including gastrointestinal (*eg*, abdominal discomfort, diarrhea, nausea, anorexia, and constipation), respiratory (*eg*, cough, wheezing and chronic bronchitis), and cutaneous (*eg*, pruritus and urticaria) manifestations, are found frequently in strongyloidiasis patients (Grove, 1989; Woodring *et al*, 1996; Johnston *et al*, 2005; Holt *et al*, 2010). Nevertheless, *S. stercoralis* can be severe in cases of altered immune status, leading to increased parasite burden, producing hyperinfective and/or disseminated strongyloidiasis. Potentially life-threatening conditions of pneumonia, meningitis, peritonitis, and septicemia are reported mostly in patients with deficient, compromised or suppressed immune status, resulting in a fatality rate of 87-100% (Olsen *et al*, 2009; Miller *et al*, 2014). Thus, all patients with strongyloidiasis, whether symptomatic or asymptomatic, should be diagnosed and treated promptly to prevent development of hyperinfective and disseminated disease.

At present, several synthetic anthelminthic medicines are available for management and treatment of strongyloidiasis. The current drug of choice is oral ivermectin; a broad-spectrum drug that is a semi-synthetic macrocyclic lactone derivative of avermectin B (Ramanathan and Nutman, 2008; Forbes *et al*, 2014). Alternative pharmacologic agents include benzimidazole compounds, such as albendazole, mebendazole and thiabendazole (Ramanathan and Nutman, 2008; Forbes *et al*, 2014). However, medication with these drugs is fraught with unreliable effectiveness and a number of disadvantages, particularly in cases that require repeated treatment with multiple dosages (Most, 1984; Grove, 1989; Forbes *et al*, 2014). Although ivermectin is highly effective against strongyloidiasis, its use is relatively limited due to its cost and undesirable side effects, including diarrhea, gastrointestinal upset, headache, fever, rash, and itching (Markell *et al*, 1999; Boonmars *et al*, 2005; LiverTox, 2015). Likewise, treatment with the benzimidazole drugs not only requires multiple doses but also is frequently associated with considerable frequencies of adverse effects, particularly of gastro-intestinal and neuropsychiatric systems (Most, 1984; Grove, 1989; Forbes *et al*, 2014). These unpleasant events reduce compliance of the patients. Furthermore, widespread and indiscriminate administration of current anthelminthic drugs is posing a major problem of parasite resistance, leading to decreasing medical effectiveness (Gill *et al*, 1991; Shikiya *et al*, 1992; Carvalho *et al*, 2012). These critical drawbacks have stimulated a search for alternative ways

to replace synthetic anthelminthics in treating and preventing strongyloidiasis.

Phytochemicals with anthelminthic property are recognized as potential alternatives to synthetic chemicals in helminth control programs. The importance of herbal medicine has tended to increase significantly because of better consumer acceptance due to its impressive efficacy with fewer undesirable effects (WHO, 2004; Benzie and Wachtel-Galor, 2011; Yuan *et al*, 2016). Several helminthicidal screening studies have been performed in a wide range of botanical species traditionally used for the treatment of helminth infections, with the goal of identifying and developing new anthelminthics from plant products. The *in vitro* anthelminthic bioassays of selected Jamaican plants, including *Mimosa pudica*, *Cuscuta americana*, *Stachytarpheta jamaicensis*, *Salvia serotina*, and *Artocarpus altilis* revealed their potential to immobilize filariform larvae of *S. stercoralis* (Robinson *et al*, 1990). The *in vitro* filaricidal and larvicidal activities of extracts derived from the medicinal plant, *Cardiospermum halicacabum*, were recorded against *Brugia pahangi* (Khunkitti *et al*, 2000) and *S. stercoralis* (Boonmars *et al*, 2005). Sanderson *et al* (2002) reported that ethyl acetate extract of *Zingiber officinale* (ginger) not only killed almost all worms of *Schistosoma mansoni* within 24 hours, but also reduced the cumulative egg output of the surviving ones. *Mentha piperita*, *Piper tuberculatum*, and *Lippia sidoides* showed *in vitro* anthelminthic effect on *Haemonchus contortus*; a gastrointestinal nematode usually found in small ruminants (Carvalho *et al*, 2012).

Hence, this study evaluated the anthelminthic potential produced from plant-based products to identify bioactive components that may be useful for future management of *S. stercoralis*.

MATERIALS AND METHODS

Drug and chemicals

Ivermectin (reference drug) was from Sigma-Aldrich (St Louis, MO) and chemicals and reagents of analytical grade were from local suppliers, Chiang Mai Province, Thailand.

Fecal specimens

Fresh feces samples used for culture of *S. stercoralis* were obtained from patients admitted to Maharaj Nakorn Chiang Mai Hospital, Chiang Mai and submitted for routine parasitological examination at the Parasitology Laboratory, Department of Parasitology, Faculty of Medicine, Chiang Mai University (CMU), Thailand.

Fecal culture and collection of infective third-stage (L_3) *S. stercoralis* larvae

Infective *S. stercoralis* L_3 larvae were harvested from fecal specimens cultured using a modified Harada-Mori technique (Harada and Mori, 1955). In brief, filter paper (15 × 18 cm) smeared with fresh fecal material was inserted into a plastic bag (17 × 23 cm) containing 2-3 ml of distilled water. The culture bag was sealed and kept in the dark at 28-30°C for 7-10 days, during which time the larvae developed and fell into the water at the bottom of the bag. Presence of larvae was checked periodically and confirmed microscopically. Diagnostic criteria for *S. stercoralis* L_3 larva were size (500-700 μm × 14-24 μm), a cylindrical esophagus (1/2 body length) and notched tail (WHO, 1991; Garcia, 2009). *S. stercoralis* L_3 larvae, collected at the bottom of the bag, were washed three times with 10 ml of 0.1 M phosphate-buffered saline solution pH 7.4 (PBS), centrifuged at 3,000*g* for 5 minutes, re-suspended in PBS and counted microscopically.

Plant species

Eleven indigenous plants belonging

to 10 families (Table 1) were obtained by either collecting from their natural habitats or from herbal suppliers in Chiang Mai Province. Scientific identification of plant specimens were performed by Mr James Franklin Maxwell, a botanist at the CMU Herbarium, Department of Biology, Faculty of Science, CMU and by Ms Wannaree Charoensup, a scientist at the Department of Pharmaceutical Science, Faculty of Pharmacy, CMU. Voucher specimens were deposited at the Department of Parasitology, Faculty of Medicine, CMU. The principal criterion of plant species chosen in this study was their report as having anthelminthic property in the scientific literature (Diehl *et al*, 2004; Githiori *et al*, 2006; Klimpel *et al*, 2011; Carvalho *et al*, 2012). Furthermore, in searching for safe materials, selection also focused primarily on plants used as food, in spices and in traditional medicine (Thiengburanathum, 1996; Wutythamawech, 1997).

Preparation of plant extracts

Plant samples were washed with water and air dried in the shade of an open area with ventilation for 5-10 days at ambient temperature of $30 \pm 5°C$. After grinding, 500 g of plant powder were extracted by maceration for at least two days with two liters of 95% ethanol at room temperature and vacuum filtered (11 µm pore) to obtain ethanolic extract. This process was repeated three times with an interval of 7 days between each extraction. The combined filtrates of each plant were concentrated under reduced pressure at 60°C, and then lyophilized at -55°C for complete removal of residual ethanol, weighed (for yield determination) and kept at -20°C until used.

The most effective plant established from a dose-response anthelminthic bioassay (see below) was selected for further extractions by either isolating for essential oil using steam distillation (Champakaew *et al*, 2015) or macerating with three individual chemical solvents of increasing polarity, namely, hexane, diethyl ether, and dichloromethane, as described above. Each solvent extract was filtered, concentrated in vacuo at 20°C, 35°C and 40°C for diethyl ether, dichloromethane and hexane extract, respectively, freeze-dried at -55°C and stored at -20°C. The essential oil was stored at 4°C.

Preliminary screening for anthelminthic activity of ethanolic plant extracts

Ethanolic extract of each of the 11 plants at 2 mg/ml Tween 80 in PBS (T80-PBS) was screened *in vitro* for anthelminthic activity against *S. stercoralis* L_3 larvae (Boonmars *et al*, 2005). Approximately 100 L_3 larvae were incubated in one ml of plant solution at 37°C under a humidified atmosphere containing 5% CO_2; control group was maintained in T80-PBS only. All experiments were performed in four replicates under aseptic conditions. After a 24-hour incubation, larval mortality was determined under an inverted microscope, with larvae were considered dead when after stimulation with light for 5 minutes there was no motility. Each mortality test was conducted by two independent observers, and results are expressed as percent mortality, corrected for control mortality using Abbott's formula (Abbott, 1925). Plant extracts producing 95-100% larval mortality were selected for the anthelminthic dose-response bioassay.

Anthelminthic dose-response bioassay

Dose-response assessment was performed according to the screening protocol as described above but using a serial diluted concentrations of plant material. Each assay was conducted in two independent experiments in four replicates.

Table 1

Ethnobotanical data, color and appearance, percent yield, and anthelminthic activity against *Strongyloides stercoralis* third stage larvae of ethanolic extracts derived from eleven plant species in Thailand.

Family/Species	Common name	Voucher specimen	Part used	Color and appearance	% Yield	% Mortality
Alliaceae						
Allium sativum L.	Garlic	PARA-AL-002-Rh/1	Rhizome	Pale yellow powder	3.98	80
Caricaceae						
Carica papaya L.	Papaya	PARA-CA-005-Se/1	Seed	Brown viscous	5.53	97
Cucurbitaceae						
Cucurbita moschata Decne.	Pumpkin	PARA-CU-008-Se/1	Seed	Green viscous	4.15	76
Momordica charantia L.	Bitter gourd	PARA-MO-001-Fr/1	Fruit	Green viscous	6.09	85
Lamiaceae						
Ocimum basilicum L.	Sweet basil	PARA-OC-001-Le/1	Leaves	Dark green viscous	7.42	90
Malvaceae						
Abelmoschus esculentus L. Moench	Lady's finger	PARA-AB-001-Fr/1	Fruit	Green powder	6.63	100
Meliaceae						
Azadirachta indica	Neem	PARA-AZ-002-Fr/1	Fruit	Brown viscous	22.29	90
Piperaceae						
Piper retrofractum Vahl	Long pepper	PARA-PI-006-Fr/1	Fruit	Red brown viscous	10.05	100
Rubiaceae						
Morinda citrifolia L.	Indian mulberry	PARA-MO-003-Fr/1	Fruit	Brown powder	3.12	30
Rutaceae						
Zanthoxylum limonella Alston	Indian ivy-rue	PARA-ZA-002-Se/1	Seed	Green viscous	11.96	60
Zingiberaceae						
Zingiber officinale Roscoe	Ginger	PARA-ZI-008-Rh/1	Rhizome	Brown viscous	4.97	88

Plant material that produced the greatest efficacy was then subjected to steam distillation and extraction with the three solvents of different polarity for subsequent dose-response bioassay against *S. stercoralis* L_3 larvae.

Statistical analysis

A bioassay test was discarded if control mortality was >20% and repeated. If 5-20% control mortality was observed, percent mortality (% M) was calculated using the following formula (Abbott, 1925):

$$\% \, M = \frac{(\% \text{ test mortality} - \% \text{ control mortality})}{(100 - \% \text{ control mortality})} \times 100$$

Regression analysis of data employed SPSS version 19.0 (IBM, Armonk, NY). Lethal values of 50% (LC_{50}), 95% (LC_{95}) and 99% (LC_{99}) with corresponding 95% confidence intervals (95% CI) were calculated using chi-square method for each bioassay to assess significance and measure differences among test samples.

RESULTS

Ethanolic extracts (EEs) of 11 plant species had different physical characteristics (color and appearance) and yields, latter ranging from 3.12% dry weight (w/w) for *Morinda citrifolia* fruit to 22.29% for *Azadirachta indica* fruit (Table 1). Preliminary screening for *in vitro* anthelminthic activity revealed that all EEs were capable of killing *S. stercoralis* L_3 at 2 mg/ml with 30-100% mortality after 24 hours of incubation (Table 1). Plants with EE mortality >95% (*Abelmoschus esculentus*, *Carica papaya* and *Piper retrofractum*) (appearance of plant part used shown in Fig 1) were then assessed for their efficacy in a dose-response bioassay, which showed *P. retrofractum* EE had the lowest LC_{50} value (Table 2).

a) *Piper retrofractum* fruit

b) *Abelmoschus esculentus* fruit

c) *Carica papaya* seeds

Fig 1-Dried parts of a) *Piper retrofractum*, b) *Abelmoschus esculentus* and c) *Carica papaya* used in the study.

Table 2

Larvicidal activity of effective ethanolic local plant extracts against third stage larvae of *Strongyloides stercoralis*.

Plant extract (mg/ml)	% Mortality (mean ± SE)	Helminthicidal activity (95% CI) (mg/ml)			χ^2	df	SE	Regression coefficient
		LC_{50}	LC_{95}	LC_{99}				
Piper retrofractum		0.037	0.100	0.126	19.535	2	1.468	26.181
0.015	22.50 ± 2.64	(0.004-0.062)	(0.070-0.287)	(0.086-0.392)				
0.031	40.00 ± 4.80							
0.062	70.50 ± 1.00							
0.125	86.50 ± 4.79							
Abelmoschus esculentus		0.092	0.449	0.597	6.471	2	0.288	4.611
0.062	24.75 ± 1.70	(-0.017-0.153)	(0.345-0.723)	(0.499-1.005)				
0.125	41.00 ± 2.40							
0.250	58.25 ± 1.49							
0.500	79.25 ± 1.93							
Carica papaya		0.101	0.372	0.484	5.577	2	0.415	6.070
0.031	29.00 ± 3.16	(0.053-0.143)	(0.280-0.630)	(0.357-0.849)				
0.062	44.00 ± 8.26							
0.125	58.00 ± 6.68							
0.250	80.50 ± 4.79							

The dried fruit of *P. retrofractum* was then extracted with three different solvents of various polarity and by steam distillation for extraction of essential oil. Extract of hexane (PHE) (lowest polarity), diethyl ether (PDE) (intermediate polarity) and dichloromethane (PCE) (highest polarity) (all orange semi-solids with pungent odor) had a yield of 6.82%, 1.88% and 9.67% (w/w), respectively. PEO (light-yellow with a pungent odor) had a yield of 0.16% (v/w). Dose-response bioassay against *S. stercoralis* L_3 larvae showed LC_{50} value of PHE, PDE, PCE, and PEO of 0.060, 0.078, 0.086, and 1.080 mg/ml, respectively compared to 0.021 μg/ml of ivermectin, the current helminthicidal drug (Table 3).

DISCUSSION

As an attractive alternative to conventional drugs, plant-derived products are now being investigated at a large scale for their helminthicidal potential, with the aim of establishing and developing a novel anthelmintic agent (Robinson *et al*, 1990; Boonmars *et al*, 2005; Anantaphruti *et al*, 2009; Carvalho *et al*, 2012; El-Sherbini and Osman, 2013; Eriso *et al*, 2016). In the present study, from screening of ethanol extracts from 11 local plants with established nematocidal activity, that of *P. retrofractum* showed the best larvicidal activity against *S. stercoralis* L_3 stage. Subsequent tests demonstrated hexane extract of *P. retrofractum* fruit with LC_{50} value three folds higher than that of ivermectin, the standard drug for treating strongyloidiasis.

This finding is not surprising because these plant-derived products are crude extracts with complex combinations of both active and inactive constituents. Despite being less effective than conventional drugs, products of plant origin still have promising potential, as their composition complexity can be exploited for identifying anthelminthic compounds with a wide variety of novel mechanisms, essential in future management of drug resistant helminths (Okumu *et al*, 2007; Tak and Isman, 2015). However, more research on improving extractability, efficacy, toxicology, and user acceptability is needed for practical application of plant-derived drugs in human helminth therapy.

The yields of extracts of *P. retrofractum* dried fruit using three solvents with different polarity were higher than that of the essential oil using steam distillation. These findings correspond to those of earlier studies reporting extracts from different parts of *P. retrofractum* using various solvents, such as water, *n*-hexane, ethyl acetate, methanol, and acetone, produce varying yields, ranging from 0.6-4.68% (Chansang *et al*, 2005; Bodiwala *et al*, 2007; Kubo *et al*, 2013). Low yields of *P. retrofractum* essential oil, from 0.02-0.3%, using water and/or steam distillation have been reported (Jamal *et al*, 2013; Hieu *et al*, 2014; Subsuebwong *et al*, 2016). In order to obtain higher yields of essential oils, chemical solvents were used, with hexane being better than steam distillation for isolating rose essential oil (Younis *et al*, 2007). Nevertheless, solvent extraction is not suitable for obtaining plant oils that are to be applied on the body or for therapeutic purpose because residual solvents often remain in the end product, resulting in adulterated essential oils (HEO, 2017). Thus, steam distillation is currently the most favored method for producing therapeutic grade essential oils.

Few research studies on the anthelminthic potential of herbal products against *S. stercoralis* have been conducted worldwide with mixed results. In Thailand, *in vitro* antiparasitic activity of *Car-*

Table 3

Helminthicidal activity of *Piper retrofractum* hexane, diethyl ether, dichloromethane extracts and essential oil in comparison with ivermectin against third stage larvae of *Strongyloides stercoralis*.

Test material	% Mortality (mean ± SE)	Helminthicidal activity (95% CI) (mg or μg/ml)			χ^2	df	SE	Regression coefficient
		LC_{50}	LC_{95}	LC_{99}				
PHE (mg/ml)		0.060 (0.025-0.105)	0.152 (0.106-0.402)	0.190 (0.130-0.535)	21.677	2	0.901	17.829
0.015	15 ± 5							
0.031	34 ± 2							
0.062	58 ± 12							
0.125	85 ± 1							
PDE (mg/ml)		0.078 (-0.86-0.159)	0.249 (0.164-1.197)	0.319 (0.206-1.689)	27.469	2	0.519	9.655
0.031	25 ± 3							
0.062	48 ± 1							
0.125	74 ± 5							
0.250	92 ± 2							
PCE (mg/ml)		0.086 (0.001-0.150)	0.257 (0.178-0.693)	0.327 (0.222-0.947)	20.715	2	0.506	9.658
0.031	24 ± 5							
0.062	43 ± 4							
0.125	72 ± 2							
0.250	92 ± 3							
PEO (mg/ml)		1.080 (0.152-2.827)	2.808 (1.837-16.771)	3.524 (2.247-22.837)	29.462	2	0.051	0.952
0.265	14 ± 3							
0.531	34 ± 4							
1.062	57 ± 3							
2.125	80 ± 3							
Ivermectin (μg/ml)		0.021 (0.011-0.032)	0.057 (0.041-0.104)	0.072 (0.052-0.136)	42.357	3	2.078	45.647
0.004	15 ± 2							
0.008	26 ± 3							
0.016	54 ± 8							
0.032	70 ± 5							
0.064	96 ± 1							

PHE, hexane extract; PDE, diethyl ether extract; PCE, dichloromethane extract; PEO, essential oil.

diospermum halicacabum extracts against *S. stercoralis* L_3 larvae was evaluated in comparison with the reference drugs, ivermectin and piperazine by Boonmars *et al* (2005). Although *S. stercoralis* larvae are still viable after treatment with ivermectin, piperazine, and *C. halicacabum* extracts, their motility is reduced, particularly those treated with *C. halicacabum* extracts. The majority of *Strongyloides* larvae are immobilized within 48 and 72 hours after being exposed to alcohol and aqueous extracts of *C. halicacabum*, respectively, whereas ivermectin and piperazine takes 72-144 hours and >7 days, respectively to achieve the same rate of non-motility. Anantaphruti *et al* (2009) investigated the lethal effects of three Thai medicinal plants, namely, *P. retrofractum* (Javanese long pepper), *P. nigrum* (black pepper) and *Areca catechu* (Areca nut), against parasitic helminths *in vitro* and *in vivo*. These plants have the ability to kill eggs of intestinal helminths *in vitro*, infective larvae of hookworms and *S. stercoralis in vitro*, and adult-stage hookworms *in vivo*. *P. retrofractum* fruit solution *in vitro* has anthelminthic effect against *S. stercoralis* larvae (LC_{50} = 15.0 mg/ml) and against eggs of other parasitic nematodes, such as *Ancylostoma* sp, *Ascaris lumbricoides*, *Necator americanus*, and *Trichuris trichiura*.

Six Jamaican plant extracts and three commercial anthelminthic drugs, including albendazole, levamisole and thiabendazole, were investigated *in vitro* for nematode inactivation against the filariform larvae of *S. stercoralis* (Robinson *et al*, 1990). Among the drugs tested, levamisole inactivates 100% of the larvae in <1 hour and is therefore considered the most effective drug, followed by albendazole and thiabendazole, with time for inactivation of 50% of larvae (It_{50}) of < 1.0, 34.9, and 73.9 hours, respectively. All plant extracts,

including methanolic extracts of *Mimosa pudica*, *Cuscuta americana*, *Artocarpus altilis*, *Salvia serotina*, and *Stachytarpheta jamaicensis*, and methanol-water fraction of *A. altilis* are quite effective in immobilizing *S. stercoralis* larvae, with It_{50} values of <1.0, 2, 9.5, 20.1, 81.5 and 48.9 hours, respectively. The relative activity of *M. pudica* methanolic extract is comparable to that of levamisole, the most potent antifilariform drug.

In an *in vivo* investigation conducted in Thailand, a single 1-g oral dose of *P. retrofractum* water extract provides a 20% cure rate and an 80-90% egg-reduction rate among hookworm-infected dogs (Anantaphruti *et al*, 2009). Aqueous extract of immature mango, *Mangifera indica* L., is found to elicit 100% inhibition of larval development of *S. stercoralis* at 100 mg/ml (El-Sherbini and Osman, 2013). Eriso *et al* (2016) recently reported methanolic extract obtained from aerial parts of *Ajuga integrifolia* has an LC_{50} value of 50 mg/ml against rhabditiform larvae of *S. stercoralis*.

P. retrofractum belongs to the Piperaceae (pepper) family that contains approximately 2,000 species distributed widely worldwide (Schultes and Raffauf, 1990; Numba, 1993). Plants in this family, particularly the *Piper* species, have high commercial, economical and medicinal importance. In Thailand, *P. retrofractum* is grown widely throughout the country (The Forest Herbarium, 2001). This plant is listed as a source of botanical drugs in the Thai Herbal Pharmacopoeia (THP, 2000). The fruit of *P. retrofractum* is used in traditional medicine for its anti-flatulent, expectorant, antitussive, antifungal, uterus-contractile, sedative/hypnotic, appetizer, and counter-irritant properties (Tewtrakul *et al*, 2000) as well as for treatment for bronchial asthma, bronchitis, muscle pain and other maladies (Farnsworth

and Bunyapraphatsara, 1992). Extensive phytochemical studies of *P. retrofractum* have led to isolation of a number of bioactive compounds exhibiting a variety of pharmaceutical and biological activities (Chansang *et al*, 2005; Kim *et al*, 2011; Kubo *et al*, 2013; Muharini *et al*, 2015). However, few research studies have focused on anthelminthic potential, particularly the anti-strongyloides activity of *P. retrofractum*. The *in vitro* helminthicidal effects of *P. retrofractum* on *S. stercoralis* larvae, as demonstrated herein, reflect the potential anthelminthic property, particularly that of PEE and PHE, which warrants further investigation to isolate and identify new herbal drugs for the treatment, prevention and control of strongyloidiasis. However, future investigation of this work will be aimed at determining whether *P. retrofractum* extracts also are efficacious *in vivo* against *S. stercoralis* to develop an effective treatment for controlling strongyloidiasis.

In conclusion, this study contributes not only base-line information for phytochemical screening of helminthicidal property against the third stage larvae of *S. stercoralis*, but also the potential of *P. retrofractum* extracts in supporting the production and utilization as botanical-based anthelminthic agents. As this plant is inexpensive and regionally available in Thailand, commercial exploitation would reduce dependence on imported expensive chemical anti-helminth drugs and stimulate development of local herbs to enhance public health systems.

ACKNOWLEDGEMENTS

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