In vitro Anthelmintic Activity of *Murraya Paniculata* Aqueous Crude Extract On *Mecistocerus Digitatus*

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Abstract

Gastrointestinal nematodes are accounted for major economic losses in ruminant worldwide; however, several drug resistance has been reported. In the search for natural anthelmintic, *Murraya paniculata*, a medicinal plant indigenous to Thailand, was selected due to the reported anthelmintic properties of its leaf. The present study aimed to evaluate *in vitro* antiparasitic activity of *M. paniculata* aqueous extract. The *in vitro* killing assay was studied by incubated the *Mecistocerus. digitatus* with levamisole at a concentration 0.25, 0.5, 1, and 2 mg/ml or the aqueous extract of *M. paniculata* at 1, 10, 20, and 40 mg/ml. During 0-24 hours. The worms in all groups were gradually decreased in motility, where the dead worms could be observed only at 24 hours. The lethal dose at 50% (LD50) of levamisole and *M. paniculata* aqueous extract-treated group was at 0.32 and 32.99 mg/ml, respectively. The worms that were exposed to the extract at 40 mg/ml at 12 and 24 hours showed the alteration on the cuticle with swollen, bleb, and erosion. The result of this study showed that there were active anthelminthic substances in *M. paniculata* leaves aqueous extract. Further study is in need to purify the active constituents from *M. paniculata* leaves and test the effectiveness against *M. digitatus* both *in vitro* and *in vivo*.

***Keywords*:** *Murraya paniculata, Mecistocerus. Digitatus, Medicinal plant*

1. Introduction

In tropical countries, gastrointestinal nematodes are of major veterinary importance due to their great impact on livestock health and production that are associated with economic losses (Bliss & Todd, 1974; Coop & Holms, 1996; Steel et al., 1982). *Mecistocerus digitatus* is an abomasal nematode prevalent in Thailand. The parasite is categorized in the same superfamily of *Haemonchus* species, the *Tricostrongyloidea* superfamily, and causes pathological consequences that can lead to fatalities in young animals. The use of chemical anthelminthic drugs is the first line of strategies to combat the parasite. However, drug resistance was reported after mass drug treatment was approached repeatedly for a long period (van Wyk et al., 1997; Waller, 2003; Jabbar et al., 2006; Saddiqi et al., 2006; Saeed et al., 2007). The cost of treatment and drug residues in animal products awakened the interest in alternative sources of anthelmintic drugs such as medicinal plants (Herd, 1996; Vieira et al., 1999; Melo et al., 2003). Thailand is located in a tropical zone and extremely rich in plant diversity. The country houses over 11,000 plant species, and at least 2187 species were used in traditional medicine (Pooma & Suddee, 2014). However, there has little information about scientific knowledge that supports ethnoveterinary medicinal plants. *Murraya paniculata* or “Kaew” in local Thai name, is a small tropical evergreen shrub, native to the tropical part of Asia and Australia. In traditional medicine, *M. paniculata* is commonly used for the treatment of abdominal pain, dysentery, stomach ache, and diarrhea and is used as a local anesthetic (Dosoky et al., 2016). Previous reports showed that bark and leaf extracts are anti-inflammatory, antidiarrheal (Rahman et al., 2010), antimalarial, antitrypanocidal, antifungal, antibacterial, antioxidant activities (Sundaram et al., 2011; Narkhede et al., 2012), antinociceptive (Sharker et al., 2009), and anti-

amoebic (Sawangjaroen et al., 2006). Recently, Chalobol Wongsawad & Pheravut Wongsawad (2005) demonstrated that the aqueous extracts of *M. paniculata* can kill the *Haplorchis taichui* (*in vitro*) trematodes by causing the alteration on the tegumental surface of the worm. Tresia et al. (2016) demonstrated that 0.7% *M. paniculata* leaves infeed meal could reduce the egg per gram (EPG) of *Strongylida* in goats. They confirm the anthelminthic properties of *M. paniculata* leaves by *in vitro* experiments and found that *M. paniculata* leaves aqueous extract expressed the efficiency in decreasing the larval development, infective larvae, and the adult *Trichostrongylidae* by 93.16%, 94.39%, and 90%, respectively.

In this study, the authors investigated the anthelminthic property of *M. paniculata* leaves aqueous extract against abomasal nematodes in the *Tricostrongyloidea* superfamily by using *M. digitatus* as a model.

2. Objectives

1) To determine the *in vitro* antiparasitic effect of *M. paniculata* aqueous extract on *M. digitatus*
2) To investigate the changes on the cuticle of *M. digitatus* after exposed to the *M. paniculata* aqueous extract

3. Materials and Methods

3.1 Parasite

M. digitatus were collected from cattle at the local slaughterhouses in Phetchaburi Province. The worms were kept in 0.9% phosphate-buffered saline (PBS; pH 7-7.2) and washed with PBS several times. Only healthy worms that showed good motility and normal appearance were used in this study. They were kept in culture medium 199 containing antibiotics (penicillin 50 IU/ml; streptomycin 50mg/ml) until the incubation experiment began. An identification of the worm was done according to morphological criteria (Soulsby, 1987). The worms were then fixed in alcohol-formal-acetic acid (AFA) and stained with Carmine, differentiated in acid-alcohol, dehydrated in ascending concentrations of ethanol, cleared in xylene, and whole-mounted in a Permount (Saowakon et al., 2013).

3.2 Plant extract

M. paniculata leaves were collected from the trees located at latitude 13.0745502 and longitude 99.9786385 from March to April 2009. The leaves were washed and then air-dried at room temperature. The air-dried leaves of *M. paniculata* (200 grams) were ground and extracted with 700 ml distilled water at room temperature. The water extract was centrifuged at 5,000 rounds per minute for 30 minutes and then collected supernatant. The water was removed from the supernatant under vacuum at 40-50°C to obtain 6.5 g of an amorphous dark green solid, which was stored in a glass bottle in the dehumidification cabinet for a future experiment.

3.3 Bioassay

The worms were randomly divided into nine groups (10 flukes per group). The worms in Group 1 were incubated in an M199 medium containing 0.1% (v/v) DMSO and antibiotics (penicillin 50 IU/ml, streptomycin 50 µg/ml, and gentamycin 30 IU/ml). The parasites in Groups 2- 5 were incubated in the same medium containing levamisole at a concentration of 0.25, 0.5, 1, and 2 mg/ml, respectively. Flukes in Groups 6-9 were incubated in the medium containing the aqueous extract of *M. paniculata* at 1, 10, 20, and 40 mg/ml, respectively. The worms in all groups were kept in an incubator aerated with 5% CO_2 at 37°C. After 3, 6, 12, and 24 hours of incubation. Motility, survival, and tegument alterations were assessed by examination under the Olympus SZ-ST stereomicroscope (Tokyo, Japan). The experiment was repeated in three replicates.

3.4 Assays for the drug's activities

3.4.1 Motility criteria

Motility scores were determined by using the following criteria: 3 = whole body of the worm still moving, 2 = some parts of the body moving, 1 = worm was immobile but not dead, which determined when the worm was unstained with the vital dye (1% (w/v) methylene blue in 0.85% NaCl solution), and 0 = the worm was immobile and stained with the vital dye. The efficacies of the treatment were calculated as the relative motility (RM) value using the formula listed below (Kiuchi et al., 1987). A small RM value indicated stronger drug activity, and when all flukes died, this value was 0.

$$\text{Motility index (MI)} = \sum Nn / N$$

$$\text{RM value} = \text{MI test} \times 100 / \text{MI control}$$

n = motility score, N = number of flukes with the score of n

3.4.2 Determination of LC50

The lethal concentration that was able to kill half of the population was determined by linear regression analysis.

3.4.3 Specimen preparation for scanning electron microscopic (SEM) observation

The worms incubated in each treatment were collected, fixed in 2.5% glutaraldehyde-phosphate buffer (0.1 mol/L, pH 7.4) at 4°C for 24 hours, and post-fixed in 1% osmium tetroxide for 1 hour. They were dehydrated through a graded series of ethanol and dried in a Hitachi HCP- 2 critical point dryer using liquid carbon dioxide as a transitional medium. After drying, they were mounted on aluminum stubs and coated with platinum and palladium in an ion-sputtering apparatus, Hitachi E-102, set at 10–15mA for 6 minutes. They were examined and photographed in a Hitachi scanning electron microscope S-2500 (Hitachi High-Technologies, Hitachi-Naka City, Japan), operating at 15 kV.

3.4.4 Statistical analysis

The statistical tests were performed with the R program (version 4.0.3). Comparisons of relative mobility between groups were performed with one-way analysis of variance (ANOVA) by applying Duncan's test for multiple comparisons with the level of significant difference set at p-value <0.05.

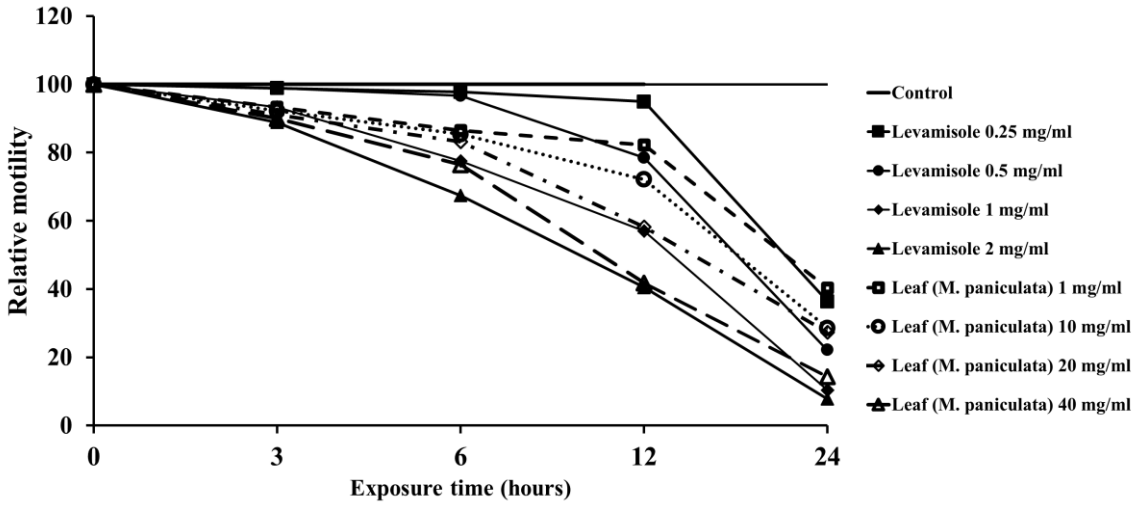

Figure 1 Relative motility (RM) values of the control and the experimental worms treated with levamisole and *M. paniculata* aqueous extract at various concentrations between 0-24 hours.

4. Results and Discussion

4.1 Results

4.1.1 Relative motility (RM) of the parasites treated with levamisole and extract of *M. paniculata*

All worms incubated in the control medium were alive and remained active by showing whole-body movement throughout the study period of 24 hours (RM = 100). The worms in the levamisole-treated group at all concentrations were decreased in motility since the 3rd hour (RM mean: 88.89-98.899). The groups treated at the concentrations of 0.25 - 0.5 mg/ml were slowly decreased in motility during 6-12 hours (RM mean: 68.89-83.33). The RM was sharply decreased at 24 hours (RM mean: 18.89-31.11) (Figure 1). In contrast, the levamisole-treated group at the concentrations of 1-2 mg/ml were decreased in motility gradually from 3 to 24 hours, with the lowest motility at 24 hours, and the RM mean values were 8.89 and 11.11, respectively. During 0-12 hours, all worms were still alive. Only at the 24th hour that some worms were dead (immobile and stained with the vital dye). Using linear regression analysis in determining the LD50, the results from the viability and motility assay showed that the LD50 of the levamisole-treated group was 0.49 ± 0.39 mg/ml. The worms in the aqueous extract of the *M. paniculata*-treated group at all concentrations were slowly decreased in motility from 3-6 hours (RM mean: 75.55-87.78). At 12 hours, only the group that treated with concentrations of 20 and 40 mg/ml were decreased in motility with the RM value of lower than 50. At 24 hours, the RM was sharply decreased in all groups. The lowest RM value was presented in the aqueous extract of the *M. paniculata*-treated group at 40 mg/ml with the mean RM value of 15.56, which was not significantly different from the RM value of the worm treated with 2 mg/ml of levamisole. All worms were still alive during 0-12 hours. Only at 24 hours that some worms were dead (immobile and stained with the vital dye). The LD50 of the *M. paniculata* aqueous extract-treated group was 25.82 ± 5.74 mg/ml.

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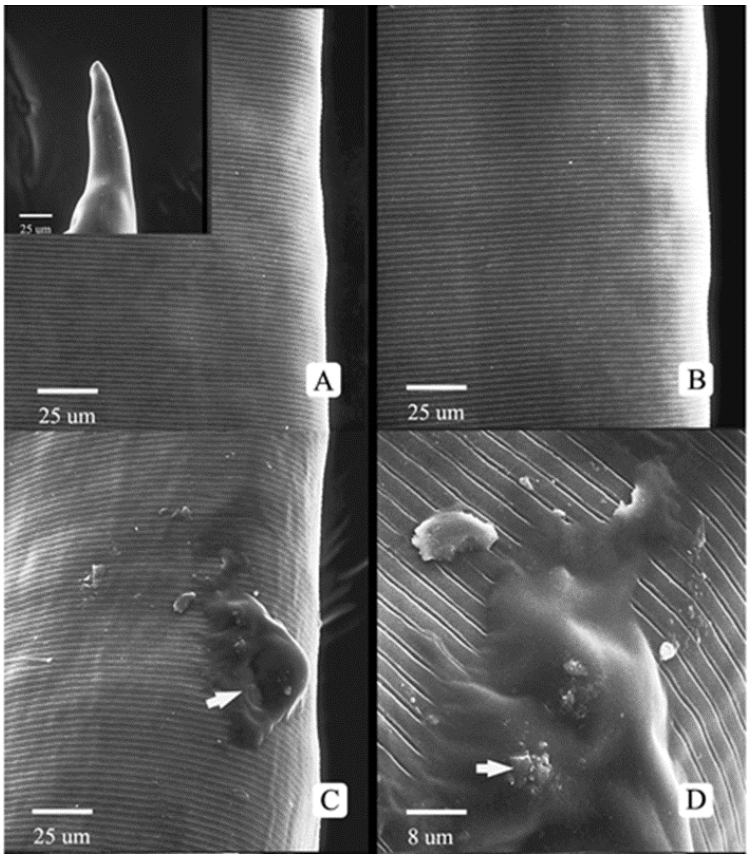

Figure 2 Scanning Electron Microscope (SEMs) of *M. digitatus* in medium M199 containing 0.1% DMSO or levamisole and *M. paniculata* aqueous extract A) *M. digitatus* in medium M199 containing 0.1% DMSO at 24 hours (Control), which the normal appearance of smooth cuticle surface with transversal folds was observed, B) *M. digitatus* in medium M199 containing 0.1% DMSO with 2 mg/ml levamisole at 24 hours showed the normal surface appearance of cuticle similar as in control group, C) A low magnification of *M. digitatus* in medium M199 0.1% DMSO containing 40 mg/ml *M. paniculata* aqueous extract at 24 hours showed the swollen of the cuticle (arrow), and D) A high magnification of *M. digitatus* in medium M199 0.1% DMSO containing 40 mg/ml *M. paniculata* aqueous extract at 24 hours showed blebs formation and erosion of the cuticle (arrow).

4.1.2 Scanning electron microscopic observations

In the control group, the surface topography of the cuticle of untreated *M. digitatus* showed a normal appearance of smooth cuticle surface with a normal aspect of transversal folds throughout 24 hours of incubation in Medium-199 containing 0.1% DMSO. The levamisole treated group showed a normal surface appearance similar as present in the control group from 0 to 24 hours. However, the cuticle topography of the worms treated with aqueous extract of *M. paniculata* at a concentration of 40 mg/ml showed the swollen cuticle at 12 hours, and a formation of blebs and erosion was observed at 24 hours (Figure 2).

4.2 Discussion

This study demonstrated that aqueous extract of *M. paniculata* affects abomasal roundworm *M. digitatus* by decreasing its motility, which some worms died within 24 hours. Even though the LD50 indicated that the potential in killing the parasite by the crude extract was lower than levamisole, this research revealed that the *M. paniculata* aqueous extract has an active biological substance against the

worm. The finding was supported by the evidence of alteration on the cuticle of the worms exposed to the extract at 40 mg/ml at 12 and 24 hours. The result of this research was correlated with the study by Tresia et al. (2016), who demonstrated that 0.7% *M. paniculata* leaves infeed meal decreased 43.67% of the egg per gram (EPG) of *Strongylida* in Ettawa crossbred lactating dairy goats. They also confirmed the anthelminthic properties of *M. paniculata* leaves by in-vitro experiments and found that 7% of *M. paniculata* leaves aqueous extract expressed the highest efficiency in decreasing the larval development, infective larvae, and adult *Trichostrongylidae* by 93.16%, 94.39%, and 90%, respectively. Chalobol Wongsawad and Pheravut Wongsawad (2005) demonstrated that the aqueous extracts of *M. paniculata* can kill the *Haplorchis taichui* trematodes *in vitro* by causing the alteration on the tegumental surface of the worm as blebbing, rupturing, and loss of spines. Khanijou (2009) demonstrated that the aqueous extract of *M. paniculata* can kill mosquito larva 100% within 24 hours, and changes in the morphology and histology of the larvae such as shape change, swelling, necrosis, and nuclear lysis were observed.

Besides, Mollah & Islam, (2008) found that *M. paniculata* leaf-derived materials or their constituents could use as insect-control agents against an important and destructive pest, *Callosobruchus maculatus* F., which correlated with the studies of Slama et al. (1974) and Ghani (1998) who found that *M. paniculata* leaf essential oil could affect the disruption of the growth of the insects. Sawangjaroen et al. (2006) also found that the *M. paniculata* extract showed anti-amoebic activity with IC_{50} 116.5 µg/ml. Tresia et al. (2016) demonstrated that the major constituent of the *M. paniculata* leaves aqueous extract was tannin and saponin, which accounted for *in vitro* ovicidal, larvicidal, and nematocidal activity against nematode in the *Trichostrongylidae* family.

Saponin is a triterpene glycoside in which water-soluble sugars are attached to either a lipophilic steroid or triterpenoid. This hydrophobic–hydrophilic asymmetry means that these compounds have the capacity to lower surface tension and are soap-like. The phytochemical saponins interact with the cell membranes causing changes within the cell membranes and subsequent changes in the cell wall (Radwan et al., 2012). Tannin is a polyphenolic compound found in various species of plant. It is astringent in nature and has the ability to form strong complexes with proteins and other macromolecules. Some tannin-rich plants can have direct antiparasitic effects against gastrointestinal nematodes of goats and sheep (Alonso-Díaz et al., 2008; Max, 2010). Some researchers reported a relatively good effect of feed with condensed tannins in decreasing worm burden in the abomasum (Ibanez et al., 2009). One hypothesis has been proposed to explain the effect of tannins against gastrointestinal nematodes: It is possible that condensed tannins bind with proline- and hydroxyproline-rich structure that covers the nematode body, esophagus, buccal cavity, cloaca, and vulva (Thompson & Geary, 1995). The capacity to bind to proteins of the worms could change their physical and chemical properties and impair vital processes like feeding, reproduction of the parasite, and disrupt the integrity of the cuticle. This ability could explain the cuticular changes observed by scanning electron microscopy after contact with condensed tannins (Hoste et al., 2006).

5. Conclusion

To our knowledge, this study is the first experiment that demonstrated the effect of the aqueous extract of *M. paniculata* leaves against *M. digitatus*. Even though the killing potential of the extract was lower than the commercial anthelminthic drug, levamisole, this study showed the scientific evidence which supports that there were active biological substances with anthelminthic property present in *M. paniculata* leaves aqueous extract. Thus, future study is in need to purify the active constituents from *M. paniculata* leaves and test the efficacy against *M. digitatus* both *in vitro* and *in vivo*.

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