อุทธิย์ปัจจัยการเจริญเติบโตของเชื้อ *Crithidia fasciculata*  
ในทดลองทดลองของสารสกัดพืชสมุนไพรไทย

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บทคัดย่อ
ผลิตภัณฑ์จากธรรมชาติซึ่งมีสารสกัดที่มีฤทธิ์ทางชีวภาพ🕯️อยู่เป็นจำนวนมากมานำมาใช้ ในการพัฒนานี้เป็นหัวหน้าศิษย์จิตในนั้น เนื่องจากการศึกษาฤทธิ์ในการดั้งเชื้อ *Crithidia fasciculata* ของสารสกัดจากพืชชีวเสริมไม่ปรากฏในที่ใดที่หนึ่ง ซึ่งเซ็นซิชัดดังกล่าวถูกยืนยันในกลุ่ม Trypanosoma ได้ถูกกำหนดไว้เป็นเชื้อจะอยู่ในกลุ่มสกัดจากพืชชีวเสริมทางชีวภาพ ทางด้านหน้า และด้าน ผู้สรุปสาร จงขั้นนี้ได้ทำการประเมินฤทธิ์ทางชีวภาพในการเจริญเติบโตของเชื้อ *C. fasciculata*  
ในทดลองทดลองจากสารสกัดพืชในเรื่อง นอกจากนี้ 25 ตัวอย่างที่เรียงตามค่าพืชชีวที่สูงกว่า 8 วงที่ปริมาณกับทั่วไปทุกๆ โดยมีวัตถุประสงค์เพื่อดั้งทำการเป็นฐานฐานของฤทธิ์ทางชีวภาพของเชื้อนั้นล้วน การพัฒนาเป็นยา และเพื่อขั้นตอนถัดไปการพัฒนาการออกฤทธิ์ของพืชชีวเสริมแต่ละชนิด ฤทธิ์ของสารสกัด ที่ปรากฏถูกกำหนดประเมินแล้วว่า *Growth Inhibition test* และจำแนกฤทธิ์ของสารสกัดพืชออก เป็นฤทธิ์ขั้นต้นทางเศรษฐกิจภูมิศาสตร์ที่สูง ปานกลางและในฤทธิ์ ผลการศึกษาเบื้องต้นพบว่า สารสกัด ที่มาจากโดยแปลกเป็นเชื้อ *Micromelum* sp. ที่พบในและเป็นต้นเดี่ยวของพืชชีวตอมแบบ (Connaraceae) ที่พบในเชื้อ *Aglaipectabilis* และเป็นต้นเดี่ยวของ *Chisocheton* sp., *Dipterocarpus* sp. และ *Zanthoxylum* sp. ที่ผลดีที่สุดที่พบในการเรียงลำดับการเจริญเติบโตของ *C. fasciculata* ได้ร้อยละ 50 (IC₅₀) ในสารสกัดมีค่าต่ำกว่า 5000 หนักเกิร์ม/มิลิลิตร มีประสิทธิภาพในการ ออกฤทธิ์สูงสุด สารสกัดพบจากพืชที่มีมาจากพืชในงานวิจัยนี้พบในต้นชุดฤทธิ์ยั่งยืนการเจริญเติบโตของเชื้อในระยะยาว โดยมีค่า IC₅₀ อยู่ระหว่าง 5000-10000 หนักเกิร์ม/มิลิลิตร โดย สารสกัดพบที่มีค่าทางพืชออกฤทธิ์ระดับสูงสุดที่พบในงานวิจัยนี้สามารถนำไปสกัดแยกสารออก เป็นส่วน ๆ เพื่อศึกษาถึงองค์ประกอบของสารออกฤทธิ์ในงานวิจัยต่อไป

คำบรรณา : *Crithidia fasciculata*, *Trypanosomatidae*, ซีริน, หมู่ไทย, ความเข้มข้นของสารสกัดที่สามารถ ยับยั้งการเจริญเติบโตของเชื้อได้ 50%

ผู้รับผิดชอบบทความ: ดร.อุมาพร ทานแรก ภาควิชาเทคนิคการแพทย์ คณะศิษย์ศาสตร์ มหาวิทยาลัย ธรรมศาสตร์ ปทุมธานี 1221  
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The effect of crude extracts from Thai medicinal plants on in vitro growth of *Crithidia fasciculata*

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Abstract

Many natural products with bioactive properties can be used to develop new antiparasitic drugs. To date, studies about anticrithidial activity of Thai medicinal plants have not been investigated extensively. *Crithidia fasciculata* belonging to the family Trypanosomatidae has been used as model organisms for studying biochemical, cellular and genetic processes. This paper determined the biological activity against the growth of *C. fasciculata*, concentrating on twenty five methanolic crude extracts from eight plant families compared with a reference drug. The objectives of this study were to examine fundamental biological data prior to development of new drug and to understand the bioactive potential of higher plant materials. Activities of the methanolic crude extracts were assessed by Growth inhibition test and classified into active, moderately active and inactive. The empirical findings showed that the crude extracts from root and stem bark of *Micromelum* sp., leaf and stem bark of Connaraceous plant, leaf of *Aglaia spectabilis*, and stem barks of *Chisocheton* sp., *Dipterocarpus* sp. and *Zanthoxylum* sp. with 50% inhibitory concentration (IC₅₀) less than 5000 ng/ml were strongly active against the *Crithidia* growth. Most of the extracts with IC₅₀ between 5000–10000 ng/ml were moderately active against the *Crithidia* growth. The active extracts found in this study will need to be further examined using phytochemical investigations in future studies to identify their major components.

Key words : *Crithidia fasciculata*, Trypanosomatidae, Thai medicinal plants, 50% Inhibitory concentration (IC₅₀)

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Introduction

Human parasitic infections are one of the serious health problems worldwide, particularly in developing tropical and subtropical countries. Trypanosomatid and apicomplexan parasites are among major causes of morbidity and mortality in humans. The severity of side-effects and resistance to available drugs currently used to treat these parasitic diseases provide the impetus for development of new therapeutic approaches. For many centuries, medicinal plants have been used for the treatment of parasitic diseases. (1)

Crithidia fasciculata is a species of parasitic protists, often found in insects. It transmits from host to host as cysts in infective feces. Typically, it develops in the digestive tracts of insects and interacts with the intestinal epithelium using its flagellum. C. fascicul is often used as a model organism to understand the biology of human infective trypanosomatid (i.e., Trypanosoma brucei and Leishmania spp.). It is also used as feeder cells in the monoxenic culture of Entamoeba histolytica. (2) Unlike many other protists, C. fasciculata contains just one large mitochondria, and requires a very high quality nutrient broth (containing added heme and folic acid) to grow. (3)

According to recent estimates, up to 60 million people are exposed to the risk of contracting African trypanosomiasis (known as sleeping sickness). The human African trypanosomiasis is caused by the parasitic flagellates either Trypanosoma brucei gambiense or T. brucei rhodesiense. The trypanosomes can cross the blood–brain barrier and invade the central nervous system leading to behavioral changes, coma, and ultimately, if without treatment, death. (4) It is estimated that there are approximately 500,000 people infected with Trypanosoma. (5) Visceral leishmaniasis, caused by species of the Leishmania donovani group, is transmitted to humans by sand flies. It is reported that a total of 200 million people were at risk, and 500,000 new cases were diagnosed yearly in 62 countries. (6, 7)

In many Asian countries including Thailand, various animals were infected with trypanosome, particularly with Trypanosoma evansi and T. lewisi in which both are enzootic. (8–10) For instance, T. lewisi-like infection was found in infant’s blood in Lampang province. (11)

It has been reported that many Thai medicinal plants possess in vitro and in vivo antiprotozoal activity. In this study, we sought to determine the IC_{50} value of methanolic crude extracts from Thai plants against the growth of C. fasciculata promastigote. Selected plants with bioactive property against protozoan parasites other than Trypanosomatidae were collected to assess their in vitro anticrithidia activity.
Material and Methods

**Crithidia fasciculata** promastigote culture:

The strain of *Crithidia fasciculata* (Figure 1) was kindly provided by Professor Michael Duchêne, Medical University of Vienna, Austria. Stock cultures were maintained at 27 °C in the standard maintenance medium, TPS which contains 1.0% trypticase, 0.5% glucose, 2.0% liver extracts and 0.5% NaCl. The optimum temperature for the cultivation is between 25 and 28 °C. Dense populations of parasites were subcultured twice weekly, usually by 1:100 dilutions with fresh medium.\(^{(12)}\)

**Extraction:**

Parts of plants (50 gram each) were dried at room temperature for seven days, with no direct sunlight, then blended, and extracted with methanol at room temperature for seven days, followed by evaporation of solvent under reduced pressure at 40 °C. Then, the plant powders were dissolved with methanol or ethanol. The extracts were stored at -20 °C until use.

**Preparation of test formulations:**

The drug miltefosine (1-O-hexadecylphosphocholine) was purchased from Cayman Chemicals (Ann Arbor, MI, USA). It was dissolved in 5% ethanol to serve as inhibitory control.\(^{(13)}\) Drug solutions were prepared with 10 mM stocks in the appropriate medium immediately before the assay. The stock solutions of the crude extracts contained 1 and 5 mg dry extract/ml. Test solutions of miltefosine and plant extracts were diluted in test medium to the required concentrations. The crude extracts and miltefosine were sterilized using 0.22 μM filter prior to antircithidial analysis.

**In vitro** antircithidia activity by MTT assay:

MTT \([3-(4,5\text{-imethylthiazol-2-yl})\]

\[-2,5\text{-diphenyltetrazolium bromide}]\) cell proliferation assay is a colorimetric assay system,
The effect of crude extracts from Thai medicinal plants on in vitro growth of Crithidia fasciculata, which measures the mitochondrial enzyme reduction of tetrazolium dye (MTT) into an insoluble formazan product by the viable cells. The anticrithidia activity was performed by the MTT assay as described earlier. Briefly, TPS was used to maintain the C. fasciculata stock culture at 27 °C. For the first part of experiments, methanol or ethanol was used in the preparation of extracts and their solubilization was investigated whether they have affected on crithidial replication. In the second part of the experiments, a sensitivity testing was performed using a peptone-yeast extract medium, PYE (pH 7.2, containing 25 mM HEPES). The PYE medium contains 1% heat-inactivated fetal calf serum and 1% antibiotics (streptomycin-penicillin) which was originally developed for the short-term culture of leishmania and evaluated as a test medium. Sensitivity tests were conducted with miltefosine and the extracts, using tissue culture plates (FALCON 3070 BD). Promastigotes were counted in a Neubauer haemocytometer and seeded with at $1 \times 10^6$ well with a final volume of 200 μL. The extracts were tested over a concentration range of 50-10000 ng/ml at the factor 2. Compound controls were also tested in the same concentration to confirm that there was no optical density of compound-related effects. Meanwhile, untreated cell control was run in parallel. All test series were performed in triplicate. Miltefosine was used as a positive drug, covering a range from 23.4 to 6000 nM at the factor 2. The wells were incubated for 48 h under microaerophilic conditions. After 48 h incubation, MTT solution (5 mg/ml) was added to each well, and the plates were further incubated for 3 h at 37 °C. Absorbance was read on an ELISA microplate reader (Anthos 2020, Austria) at a wavelength of 570 nm. The amount of survivor (metabolically active) cells was determined based on the optical absorbance of the treated and untreated cells and blank wells expressed as

$$\% \text{ cell viability} = \frac{(\text{OD}_{\text{TC}} - \text{OD}_c)}{(\text{OD}_{\text{UT}} - \text{OD}_b)} \times 100$$

Where $\text{OD}_{\text{TC}}$ is an average of the absorbance of the treated cells, $\text{OD}_c$ is an average the absorbance of the compound control, $\text{OD}_{\text{UT}}$ is an average the absorbance of the untreated (control) cells, $\text{OD}_b$ is an average the absorbance of the blank. The cell viability was determined with the MTT assay, and was confirmed by comparing the amount of the parasite control via light microscopy.

**Determination of the inhibitory concentration:**

The determination of the inhibitory concentration was expressed as $\text{IC}_{50}$ which was calculated using GraphPad Prism 5.0 software, (Dose - response curve).
Results

The *in vitro* anticrithidia activity of 25 methanolic crude extracts from 8 species of Thai plants was evaluated by the MTT viability assay. The activities were classified into active, moderately active and inactive, according to their IC\textsubscript{50}. There was no sign of a significant impediment to the growth by methanol or ethanol measured at final concentration below 1.0% in medium (Figure 2). Inhibition of the crithidial growth by miltefosine is shown in Figure 3.\(^{(14)}\) Eighteen extracts out of 25 selected extracts indicated potential for anticrithidial activity (Table 1). Most active extracts were found in root and stem bark of *Micromelum* sp., leaf and stem bark of *Connaraceae* plant, stem barks of *Chisocheton* sp., *Dipterocarpus* sp. and *Zanthoxylum* sp., and leaf of *A. spectabilis*, with the IC\textsubscript{50} less than 5000 ng/ml. Conversely, the extracts from stem barks of *A. oligophylla*, *A. odorata* Lour. and *Azadirachta indica* A. Juss., leaf of *Micromelum* sp., rhizome of *Zingiberaceae*, leaf and stem bark of *Swietenia macrophylla* King with IC\textsubscript{50} not in the concentration range tested exhibited no anticrithidial activity. However, most of the methanolic crude extracts were moderately active, with IC\textsubscript{50} between 5000–10000 ng/ml against *Crithidae* growth (Table 1).

Interestingly, Table 1 also reported that rutaceous plants, mainly the *Micromelum* genus (Figure 4) and *Zanthoxylum* genus (Figure 5) as well as meliacceous plants, mainly the *Chisocheton* genus (Figure 6) and *A. spectabilis* (Figure 7) showed the strong activity compared with other plants. These findings suggested that Rutaceae and Meliaceae families were the most promising plants that can be further developed as new antiprotozoal agents.

Discussion

To date, the activity of methanolic crude extracts from Thai medicinal plants against the growth of *C. fasciculata* have not been studied extensively. This study aimed to fill this gap using the crithidial model. Unlike the human–pathogenic trypanosomatids, this approach has advantages because its operation is practical and does not require safety precautions in the laboratory.\(^{(12)}\)

Numerous studies of phytochemistry reported that antiprotozoal activity relies on the existence of alkaloids, terpenes, flavonoids, or saponins.\(^{(15,16)}\) Our study found that *in vitro* activity of the methanolic crude extracts from the species of the family Rutaceae, Meliaceae and Asteraceae on promastigote form of *C. fasciculata* exhibited significant activity. The family Rutaceae and Asteraceae, however, contain different chemical components. It is reported that limonoids and flavonoids are among the active chemical components of Rutaceae species.\(^{(17)}\) In addition, Asteraceae exhibited the antiprotozoal effects against *trypanosoma* sp., *leishmania* sp.\(^{(18-19)}\) and falciparum malaria.\(^{(20)}\) Therefore, the family Rutaceae and Asteraceae in this study need further phytochemical studies to identify bioactive groups.
**Table 1** Activity of crude extracts of plants after incubation with culture of *C.fasciculata* for 48 h expressed as 50% Inhibitory Concentration (IC$_{50}$).

<table>
<thead>
<tr>
<th>Plants</th>
<th>Family</th>
<th>Source</th>
<th>IC$_{50}$ (ng/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Micromelum</em> sp. No.1</td>
<td>Rutaceae</td>
<td>Roots</td>
<td>847.7</td>
</tr>
<tr>
<td><em>Micromelum</em> sp. No.2</td>
<td>Rutaceae</td>
<td>Stem bark</td>
<td>1149.8</td>
</tr>
<tr>
<td><em>Micromelum</em> sp. No.3</td>
<td>Rutaceae</td>
<td>Leaves</td>
<td>inactive</td>
</tr>
<tr>
<td><em>Zanthoxylum</em> sp.</td>
<td>Rutaceae</td>
<td>Leaves</td>
<td>2950.5</td>
</tr>
<tr>
<td><em>Zanthoxylum</em> sp.</td>
<td>Rutaceae</td>
<td>Stem bark</td>
<td>2482.0</td>
</tr>
<tr>
<td>Unknown 1</td>
<td>Sapindaceae</td>
<td>Stem bark</td>
<td>5920.5</td>
</tr>
<tr>
<td><em>Andrographis paniculata</em></td>
<td>Acanthaceae</td>
<td>Leaves</td>
<td>8780.3</td>
</tr>
<tr>
<td>Wall. ex Nees</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Chisocheton</em> sp.</td>
<td>Meliaceae</td>
<td>Stem bark</td>
<td>831.3</td>
</tr>
<tr>
<td><em>Acmella</em> sp.</td>
<td>Asteraceae</td>
<td>Leaves</td>
<td>7874.8</td>
</tr>
<tr>
<td><em>Acmella</em> sp.</td>
<td>Asteraceae</td>
<td>Fruits</td>
<td>3012.0</td>
</tr>
<tr>
<td><em>Acmella</em> sp.</td>
<td>Asteraceae</td>
<td>Roots</td>
<td>5039.0</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>Connaraceae</td>
<td>Leaves</td>
<td>2134.0</td>
</tr>
<tr>
<td>Unknown 2</td>
<td>Connaraceae</td>
<td>Stem bark</td>
<td>1478.3</td>
</tr>
<tr>
<td><em>Tectona grandis</em></td>
<td>Lamiaceae</td>
<td>Leaves</td>
<td>5100.0</td>
</tr>
<tr>
<td><em>Aglaia spectabilis</em></td>
<td>Meliaceae</td>
<td>Leaves</td>
<td>1817.2</td>
</tr>
<tr>
<td><em>Aglaia oligophylla</em></td>
<td>Meliaceae</td>
<td>Leaves</td>
<td>5996.1</td>
</tr>
<tr>
<td><em>Aglaia oligophylla</em></td>
<td>Meliaceae</td>
<td>Stem bark</td>
<td>inactive</td>
</tr>
<tr>
<td><em>Aglaia odorata</em> Lour.</td>
<td>Meliaceae</td>
<td>Roots</td>
<td>6715.0</td>
</tr>
<tr>
<td><em>Aglaia odorata</em> Lour.</td>
<td>Meliaceae</td>
<td>Meliaceae</td>
<td>inactive</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td>Meliaceae</td>
<td>Whole parts</td>
<td>6592.5</td>
</tr>
<tr>
<td><em>Azadirachta indica</em> A. Juss.</td>
<td>Meliaceae</td>
<td>Stem bark</td>
<td>inactive</td>
</tr>
<tr>
<td>Unknown 3</td>
<td>Zingiberaceae</td>
<td>Rhizome</td>
<td>inactive</td>
</tr>
<tr>
<td><em>Dipterocarpus</em> sp.</td>
<td>Dipterocarpaceae</td>
<td>Stem bark</td>
<td>1365.8</td>
</tr>
<tr>
<td><em>Swietenia macrophylla</em> King</td>
<td>Meliaceae</td>
<td>Leaves</td>
<td>inactive</td>
</tr>
<tr>
<td><em>Swietenia macrophylla</em> King</td>
<td>Meliaceae</td>
<td>Stem bark</td>
<td>inactive</td>
</tr>
<tr>
<td>Miltefosine</td>
<td>Positive control drug</td>
<td>-</td>
<td>172.4</td>
</tr>
</tbody>
</table>
Fig. 2 Influence of methanol and ethanol at concentrations of 0.2, 0.7, 2, 6 and 20% on the growth of *Crithidia fasciculata* in PYE medium compared with the parasite control (free from any extracts referred to the full growth).

Fig. 3 The growth inhibition (GI) of *C.fasciculata* by miltefosine ($IC_{50} = 172.4$ ng/ml or $422.96$ nM). Log-probit regression with data points.\(^{13}\)
The effect of crude extracts from Thai medicinal plants on in vitro growth of *Crithidia fasciculata*

**Fig. 4** Dose-response curve of *Micromelum* sp. roots extract against *C. fasciculata* growth ($R^2$: R squared correlation)

**Fig. 5** Dose-response curve of *Chisocheton* sp. stem bark extract against *C. fasciculata* growth ($R^2$: R squared correlation)
Fig. 6 Dose-response curve of *Zanthoxylum* sp. stem bark extract against *C.fasciculata* growth ($R^2$: R squared correlation)

Fig. 7 Dose-response curve of *Aglaia spectabilis* leaf extract against *C.fasciculata* growth ($R^2$: R squared correlation)
The effect of crude extracts from Thai medicinal plants on in vitro growth of Crithidia fasciculata

This study also found that the stem bark extract of Chisocheton sp. (Meliaceae) showed a good inhibitory activity while our earlier studies found that the stem bark extract of Aglaia elaeagnoida and the leaf extract of A. edulis exhibited a good antiprotozoal effect against Entamoeba histolytica and C. fasciculata, respectively.\(^{(21,12)}\)

Compared with other parts of the A. odorata and A. indica A. Juss, the leaf extracted from these plants were the most effective extract in inhibiting the growth of C. fasciculata\(^{(20)}\) whereas this study found that the extracts from stem bark and roots of both plants could not inhibit the growth of C. fasciculata.

Our findings showed that different parts of plants had different anticrithidia activity. In addition, the amount of their bioactive constituents varied greatly with the different and same plant species and it should be evaluated accordingly. For instance, plant leaves will contain high bioactive novel compounds because they are site of synthesis for different class of compounds.

A positive control drug; miltefosine, was tested in parallel to verify the precision of the anticrithidia activity test. The test showed the IC\(_{50}\) miltefosine had approximately 10 times lower than that of the crude extracts. The IC\(_{50}\) in the crude extracts was high because they were in forms of a mixture of the compounds. When the crude extracts are further isolated and purified as a single compound, their activity will provide clearer picture about phamocological effects.

The active extracts from root and stem bark of Micromelum sp., leaf and stem bark of Connaraceous plant, leaf of Aglaia spectabilis, and stem bark of Dipterocarpus sp. exhibited strong inhibitory efficacy. These results imply that these extracts contain compounds with high specific activity. More importantly, our finding (but not reported here) on cytotoxicity testing of these active extracts showed no sign of toxicity to the mammalian cell culture in vitro.

The extracts with no inhibitory activity against the Crithidia growth may be due to the facts that their plants have no existence of active components or the extraction process was not successful to extract a large amount of bioactive components.

Most importantly, three unidentified plants (unknown 1, 2, and 3) are belonged to the families of Zingiberaceae, cf. Connaraceae, cf. Sapindaceae, respectively. Therefore, taxonomy in plant selection should be applied to confirm plant species and identify the existence of their main constituents.

Further, it is valuable that the activity of the active extracts found in this study will be applied to investigate the growth inhibition of human-pathogenic trypanosomatids. This process is important for discovering new drug development in treatment of human-pathogenic trypanosome.
Conclusion

Currently available drugs used to treat the protozoan diseases; leishmaniasis, Chagas disease and African trypanosomiasis show highly toxic adverse effects and only a few of them available for the treatment. Moreover, most patients in endemic countries mainly from tropical countries have limited access to these drugs. More importantly, the currently available drugs are less effective because they often show a decline to a response of sensitive parasites. These problems lead to the development of new drugs as a treatment solution for these neglected diseases.

In this study, we reported in vitro anticrithidial activity of 25 methanolic crude extracts from 8 selected species of Thai plants. These findings are important because they can provide basic information to the use of the crude extracts from Thai plants in the therapy for diseases caused by human-pathogenic trypanosomatid. Both C. fasciculata and human-pathogenic trypanosomatid share inter alia mechanisms of polyamine biosynthesis and methionin salvage.\(^{(22)}\)

The empirical results indicated that the observed extracts with IC\(_{50}\) < 5000 ng/ml were strongly active against the Crithidia growth while those with IC\(_{50}\) between 5000-10000 ng/ml were moderately active. On the other hand, those with IC\(_{50}\) not in the concentration range were inactive against the Crithidia growth.

Based on our findings in this study, the active extracts from the plant samples with IC\(_{50}\) < 5000 ng/ml will need further phytochemical investigations; i.e. chromatographic fractionation, purification and structural identification of active compounds, respectively to confirm whether these plant samples are potential therapeutic agents.

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