In Vitro and *In Vivo* Antiplasmodial Activity and Cytotoxicity of Water Extracts of *Phyllanthus emblica*, *Terminalia chebula*, and *Terminalia bellerica*

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Objective: *To evaluate the in vitro and in vivo antiplasmodial activity and the cytotoxicity of Phyllanthus emblica Linn, Terminalia chebula Retz, and Terminalia bellerica (Gaertn) Roxb extracts.*
Material and Method: *Standard phytochemical screening tests were used to detect metabolites in the plant extract. The water extracts of medicinal plants were tested for their antiplasmodial activity in vitro by assessing their ability to inhibit the uptake of [^{3}H] hypoxanthine into the Plasmodium falciparum K1 multidrug-resistant strain. Cytotoxicity of all extracts was determined on Vero cell line. The in vivo antiplasmodial activity in Plasmodium berghei infected mice was evaluated by the standard 4-day suppressive test.*
Results*: Phytochemical screening of the water extracts of three plants revealed the presence of flavonoids, hydrolysable tannins, saponin and terpenes. All plant extracts showed antimalarial activity (IC_{50} values ranging from 14.33 $\pm$ 0.25-15.41 $\pm$ 0.61 μg/ml). The water extract of Terminalia bellerica (Gaertn) Roxb had the highest in vitro antiplasmodial activity followed by Phyllanthus emblica Linn. and Terminalia chebula Retz. The cytotoxic activity was exhibited by all plant extracts on Vero cells with IC_{50} values of 157.86 to 238.70 mg/ml. All of the plant extracts showed selectivity with the selectivity index (SI) ranged from 11 to 17. A standard 4-day suppressive test on P. berghei infected mice was used to evaluate the in vivo antiplasmodial activity of the extracts at 250 mg/kg/day. The results revealed that in vivo antiplasmodial activity with good suppression activity ranged from 53.40% to 69.46%.*
Conclusion: *All of the plant extracts exhibited interesting in vitro and in vivo antiplasmodial activity with good selectivity.*

Keywords: *Phyllanthus emblica, Terminalia chebula, Terminalia bellerica, Antiplasmodial activity, Cytotoxicity*

J Med Assoc Thai 2010; 93 (Suppl. 7) : S120-S126
Full text. e-Journal: *http://www.mat.or.th/journal*

Malaria, a tropical disease caused by protozoan parasites of the genus *Plasmodium* is one of the most important infectious diseases in the world[1]. World Health Organization (WHO) estimates that throughout the world more than 300 million clinical cases of malaria arise every year, and over 1 million people deaths. According to the increasing incidences of multi-drug resistance *Plasmodium*, there is a need to look for a new anti-malarial agents[2] that are inexpensive, routinely available to people particularly those in the developing countries[3]. Thus, the communities in endemic areas have started to look for malaria remedies in natural products[4].

Plants have always been considered to be a possible alternative and rich source of the new drugs. The antimalarial drugs, quinine and artemisinin were either obtained directly from plants or developed using chemical structures of plant-derived compounds as templates[5]. The extracts of a large number of plant species including many that are used in traditional

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medicines have been evaluated for *in vitro* antiplasmodial activities and some have also been tested *in vivo*[6]. In some cases the constituent(s) responsible for their activities have been isolated but relatively few have been studied further to assess their potential as lead compounds for the development of new antimalarial drugs.

A traditional Ayurvedic herbal combination dating back 5,000 years is a mixture of three herbs, two of which are *Terminalia* species: *Terminalia bellerica* (Gaertn) Roxb, *Terminalia chebula* Retz, and *Phyllanthus emblica*[7]. This formulation, rich in antioxidant, is a frequently used Ayurvedic medicine to treat many diseases such as anemia, jaundice, constipation, asthma, fever and chronic ulcers. It is an important medicine of the Rasayana group and is believed to promote health, immunity and longevity[8]. Mixed crude extracts from their medicinal plants mainly used as antipyretics and analgesics[9] or claimed to be effective against malaria in daily traditional healers practices. In previously, the dried powder of fruits of *Terminalia bellerica* (Gaertn) Roxb, *Terminalia chebula* Retz and *Phyllanthus emblica* Linn are ground and mixed with neem leaves, stem bark, young roots, and fruits for treatment of malaria-type fever[10].

Scientific evaluation of relevant medicinal plant parts that are used in traditional medicine offer some hope for developing new agents that may be appropriate for the treatment of malarial infection and associated pathological complications during a malaria attack. We therefore evaluate the *in vitro* and *in vivo* antiplasmodial activity against *Plasmodium falciparum* and *Plasmodium berghei*, respectively. The cytotoxicity of *P. emblica* Linn, *T. chebula* Retz and *T. bellerica* (Gaertn) Roxb extracts also are determined.

Material and Method

Plant materials

The dried fruits of *Terminalia chebula*, *Terminalia bellerica*, and *Phyllanthus emblica* (Table 1) were collected from Nakhon Ratchasima, Srakeaw, and Nan provinces, respectively. The voucher specimen PBM 00485 (*T. chebula*), PBM 02678 (*T. bellerica*) and PBM 01402 (*P. emblica*) were kept at Department of Pharmaceutical Botany, Faculty of Pharmacy, Mahidol University.

Preparation of crude extracts

The dried fruits of three medicinal plants were extracted using boiling water. Then, the aqueous solutions were separated from the plant residues by filtration. Subsequently, the aqueous solution was spray-dried. The extracts were dissolved in media and diluted to the desired concentrations for antiplasmodial and cytotoxicity assays.

Phytochemical analysis

Phytochemical screening of three water extracts was carried out employing standard procedures and tests[11] to reveal the presence of chemical constituents such as alkaloids, flavonoids, tannins, terpenes, saponins, anthraquinones, reducing sugars, cardiac glycosides among others. The content of total tannins was assayed bt protein precipitation method[12]. The HPLC analysis of the extracts was carried out to determine the present of polyphenols[13].

In vitro anti-plasmodial assay

Quantitative assessment of antimalarial activity *in vitro* was determined by means of the microculture radioisotope technique based upon the method previously described by Desjardins et al (1979)[14]. The assay uses the uptake of [^{3}H]hypoxanthine by parasites as an indicator of viability. Continuous *in vitro* cultures of asexual erythrocytic stages of *Plasmodium falciparum* were maintained following the methods of Trager and Jensen (1976)[15]. Plant extracts were tested on K1 strain (multidrug pyrimethamine/chloroquine-resistant strain). Initial concentration of the plant extracts was 30 mg/ml

Table 1. List of medicinal plant species and phytochemical screening of plant extracts

Species (family)	Common name	Part used	Chemical compound (% w/w)
Terminalia chebula Retz. (Combretaceae)	Myrobalan wood	Fruit	26.75% Tannins 2.43% Gallic acid
Terminalia bellerica (Gaertn) Roxb. (Combretaceae)	Beleric Myrobalan	Fruit	34.46% Tannins 7.98% Gallic acid
Phyllanthus emblica Linn. (Euphorbiaceae)	Emblic Myrobalan, Malacca Tree	Fruit	42.51% Tannins 20.48% Gallic acid

diluted with two-fold dilutions to make seven concentrations. After 48 h incubation of the parasites with the extracts at 37°C, [^{3}H] hypoxanthine (Amersham, UK) was added to each well and the incubation was continued for another 24 h at the same temperature. The extract concentrations at which the parasite growth ([^{3}H] hypoxanthine uptake) was inhibited by 50% (IC_{50}) was calculated by linear interpolation between the two drug concentrations above and below 50%. Dihydroartemisinin was used as a positive reference. The values given in Table 2 were means of two independent assays. Each assay was performed in triplicate.

Cytotoxicity assay

The sulforhodamine B (SRB) assay was performed to assess growth inhibition by a colorimetric assay. Cell number was estimated indirectly by staining total cellular protein with the dye SRB[16]. Briefly, 100 ml/well of Vero cell suspensions (5 x 10^5 cells/ml) were seeded in 96-well microtiter plates and incubated at 37°C to allow for cell attachment. After 24 h, the cells were treated with the extract by adding 100 ml/well of each concentration in triplicate to obtain final concentration of 125, 250, 500 and 1,000 mg/ml of the extracts. The plates were incubated for 1 h (day 0) and 72 h (day 3) at 37°C. At the end of each exposure time, the medium was removed. The cells were fixed with 20% (w/v) trichloroacetic acid (TCA, Fluka), stained for 30 min with 0.4% (w/v) SRB (Sigma) dissolved in 1% acetic acid (Sigma) and washed four times. Protein-bound dye was solubilized with 10 mM Tris base pH10 (Sigma). The optical density (OD) of each well was read on an ELISA plate reader (Amersham, Buckinghamshire, UK) at 492 nm. Percentage of cell survival was calculated by the following formula. Percentage cell survival = [(OD test sample at day 3 OD day 0)/(OD vehicle control at day 3-OD day 0)] x 100. Selectivity index (SI) was used as parameter of clinical significance of the test samples by comparing general toxins and selective inhibitory effect on *Plasmodium falciparum* calculated as below[17]:

$$SI = \frac{IC_{50}\ (\text{Vero cells})}{IC_{50}\ (\textit{Plasmodium falciparum})}$$

In vivo anti-plasmodial assay

The extracts were prepared by dissolving the extracts in sterile water. Male ICR mice (8 weeks old, weighing 30-40 g) were used as the subjects. Permission and approval for animal studies were obtained from the Animal Ethics Committee of Faculty of Medicine, Thammasat University, Pathumthani, Thailand (No. 0004/2003). The mice were bred in standard cages in airconditioned rooms at 24°C, 50-70% relative humidity, were fed with the standard diet and water ad libitum. *P. berghei* strain P-line (chloroquine-susceptible strain) maintained by serial passage of infected blood through intraperitonial injection (i.p.) was used in the study. The test protocol was based on the 4-day suppressive test[18]. This procedure is proposed by the WHO as the first-line primary screen for *in vivo* testing of potential antiplasmodial compounds. Briefly, *P. berghei* infected blood was obtained by heart puncture, mixed with 1% (w/v) heparin in phosphate buffered saline (PBS) (1:1) and the experimental animals infected by i.p. injection with 0.2 ml (2 x 10^7 parasitized erythrocytes). The experimental groups (five mice per group) were treated with 250 mg/kg/day of water extract in 0.2 ml by oral administration 2-4 h post-infection. After 24, 48 and 72 h post-infection, the experimental groups were treated again with the same dose. Two groups (five mice per group) served as negative and positive controls. The negative control group received a vehicle (normal

Table 2. Antiplasmodial activity and cytotoxicity of three plant extracts in this study

Treatment	Antiplasmodial activity (*P. falciparum* K1 strain)	Cytotoxic activity (Vero cells)	
	IC_{50} (µg/ml) (mean ± SD)	IC_{50} (µg/ml) (mean ± SD)	Selectivity index (SI)
Terminalia chebula	15.41 ± 0.61	257.47 ± 12.53	17
Terminalia bellerica	14.33 ± 0.25	238.70 ± 8.45	17
Phyllanthus emblica	14.37 ± 0.17	157.86 ± 14.90	11
Dihydroartemisinine	0.0035 ± 0.0007	-	-

saline) while the positive group was treated with 5 mg/kg/day of artesunate through oral administration. Thin blood film was sampled from the tail of the experimental mice and stained in 10% Giemsa solution. Percentage of parasitemia was counted based on infected erythrocytes calculated per 1,000 erythrocytes. The difference between the mean number of parasites in the control group (100%) and those of the experimental groups on day 4 was calculated and expressed as percent parasitaemia suppression (% suppression) according to the formula below[19]:

$$\% \text{ suspression} = \frac{\text{(parasitaemia in the control-parasitaemia with plant extract)}}{\text{parasitaemia in the negative control}} \times 100$$

Number of dead mice was recorded daily from all the study groups to determine the average survival time of the infected mice after treatment.

Statistical analysis

Results were shown as means ± SEM from the analysis group. The difference between each group was analyzed by ANOVA followed by a post-test (Tukey-Kramer multiple comparison test) and p-values less than 0.05 were accepted as statistically significant.

Results

Phytochemical screening of water extracts of *T. bellerica* indicated the presence of flavonoids, hydrolysable tannins, saponin and terpenes while the two water extracts of *T. chebula* and *P. emblica* revealed the presence of flavonoids, hydrolysable tannins and terpenes. The water extracts of *P. emblica T. bellerica* and *T. chebula* were found to have tannins and gallic acid as main constituents (Table 1).

The IC_{50} values obtained with extracts of various plants against *P. falciparum* K1 strain using the radioactive method were summarized in Table 2. According to WHO guidelines[20,21], antiplasmodial activity was classified as follows: highly active at IC_{50} < 5 µg/ml, promising at 5-15 µg/ml, moderate at 15-50 µg/ml and inactive at > 50 µg/ml.

All of the plants tested showed an antiplasmodial activity with IC_{50} < 50 µg/ml. The extract of *T. chebula* showed a moderate level activity (IC_{50} at 15.41 ± 0.61 mg/ml). In addition, extracts of *T. bellerica* and *P. emblica* showed a promising level of activity, IC_{50} at 14.33 ± 0.25 µg/ml and 14.37 ± 0.17 µg/ml, respectively. Three extracts with the moderate and promising levels of activity were assessed for their cytotoxic activity in order to determine the selectivity index. Cytotoxic assays were performed on Vero cells to examine the specificity of selected extracts to the parasite. The IC_{50} values for three extracts ranged from 157.86 ± 14.90 to 238.70 ± 8.45 µg/ml (Table 2). The impact of the toxicity was established by analyzing the selectivity index (SI) values. The SI corresponds to the ratio between cytotoxic IC_{50} values and parasitic IC_{50} values. Selectivity index of *T. chebula* and *T. bellerica* are 17 and selectivity of *P. emblica* is 11 as shown in Table 2. All of the plant extracts that showed good selectivity indicating the potential of specific and safer therapy.

As a complement to *in vitro* assays, three water extracts were tested in the *in vivo* model of the rodent malaria *P. berghei* (P-line) (chloroquine-susceptible strain). Anti-malarial activity of water extracts was summarized in Table 3. The *in vivo* antiplasmodial activity results were classified by Munoz et al (2000)[22]. The *in vivo* results were classified as follows: at the dose of 1,000 mg/kg/day, when the percent growth inhibition was higher than 50% the activity was considered moderate, in other cases it was considered inactive. This dose was taken into account to detect an active product presented in small amount in the extract, at the dose of 500 mg/kg/day, if the extract displayed a percent growth inhibition equal or greater than 50%, the antimalarial activity was also considered moderate, at the dose of 250 mg/kg/day, if the percent growth inhibition was equal or greater than 50%, the antimalarial activity was considered good, and at the dose of 100 mg/kg/day if the percent growth inhibition was equal to 50%, the antimalarial activity was

Table 3. Effect of water extracts of three plants on *P. berghei* in mice (4-day suppressive test)

Treatment	% Suppression (n = 5)	Survival time (day) (n = 5)
Terminalia chebula (250 mg/kg/day)	68.89	6.80 ± 0.84
Terminalia bellerica (250 mg/kg/day)	53.40	6.60 ± 1.52
Phyllanthus emblica (250 mg/kg/day)	69.46	6.80 ± 0.97
Artesunate (5 mg/kg/day)	100	> 15
Normal saline	-	5.2 ± 0.45

considered very good.

The extracts of *P. emblica* and *T. chebula* showed the good suppression activities of the extracts at 250 mg/kg/day among all groups of mice with mean parasitaemia values at 11.85 (69.46 % suppression) and 12.07 (68.89 % suppression), respectively, which were significantly different from those of the normal saline-treated group ($p < 0.05$) (Table 3 and Fig. 1). The oral administration of *T. bellerica* could suppress by 53.40% of mean parasitemia in the control group (Table 3). All three treated groups also had longer survival time ranging between 6.6 $\pm$ 1.52 days and 6.8 $\pm$ 0.98 days compared to the negative control group while mice treated with artesunate could survive longer than 15 days (Table 3).

Discussion

Terminalia is a well-studied genus especially for antiplasmodial activity because of its traditional use against malaria in other endemic regions[21]. *Terminalia macrophtera* displayed high *in vitro* activity on chloroquine resistant (W2) strains and *Terminalia glaucescens* on chloroquine resistant and sensitive strains[23]. Whereas, the extract of *Terminalia bentzoe*[21] has already reported to have promising effect on 3D7 and W2 strains and *Terminalia mollis* on 3D7 strain[24]. In addition, the fractional extract of *T. bellerica* rind let to the isolation of lignans and flavan showing a promising effect on chloroquine-susceptible strain 3D7 of *P. falciparum*[25].

The antiplasmodial activity is already known in other species of Euphorbiaceae; the species *Phyllanthus niruri* L[26,27] have already been reported to have a highly active effect on chloroquine-resistant (FCR-3) and chloroquine-sensitive (D-10) strains of *P. falciparum*. Aqueous extracts of whole plant *Phyllanthus urinaria* displayed highly *in vitro* activity on W2 strain[28]. In addition, the extract of *Phyllanthus niruri* was also the most *in vivo* active extract with 72-73% of inhibition at 200 mg/kg[19] and an ED_{50} of 9.1 mg/kg/day[26] and leaves extract of *Phyllanthus acuminatus* displayed moderate activity with 52% of inhibition at 1000 mg/kg[22].

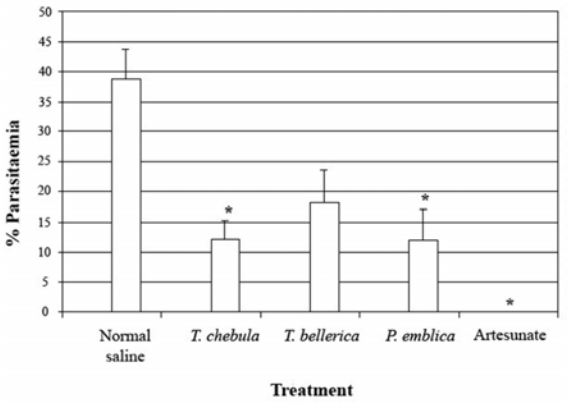

Fig. 1 *P. berghei* infected mice were treated with water extract, normal saline and artesunate at specified dosage by oral administration.
* Significantly different from the normal saline control group ($p < 0.05$) Results are mean count $\pm$ SEM ($n = 5$)

According to those previous findings, we are therefore interested in studying the antiplasmodial activity of these plant groups. Plant derived polyphenols, including tannins and gallic acid, were reported to be the main constituents in *P. emblica*, *T. chebula* and *T. bellerica*[29,30]. Although mechanism of action of these secondary metabolites has not been evaluated in the present study, some of these metabolites have been found to exert their antiplasmodial effect either by elevating red blood cell oxidation or by inhibiting protein synthesis[31]. Considering the chemical nature of constituents reported in the literature for this plant, the possibility that the antiplasmodial activity shown by *P. emblica*, *T. bellerica* and *T. chebula* extracts would be related by the presence of gallic acid[32,33], and tannins[34,35] contents with different structures and isolated from various plant species had been shown to possess at different levels antiplasmodial activity in both *in vitro* and *in vivo* tests.

Conclusion

It revealed that the three plants had the *in vitro* and *in vivo* antiplasmodial activity. All of plant extracts showed a good selectivity index. Further investigations on *P. emblica*, *T. bellerica* and *T. chebula* will be carried to identify the active antimalarial compounds. The isolation of the active compound and understanding the mechanism of inhibition of those plants would be of interest.

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ฤทธิ์การต้านเชื้อพลาสโมเดียมในหลอดทดลอง และในสัตว์ทดลอง และความเป็นพิษระดับเซลล์ของสารสกัดน้ำ *Phyllanthus emblica, Terminalia chebula* และ *Terminalia bellerica*

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วัตถุประสงค์: *เพื่อประเมินหาฤทธิ์ต้านเชื้อพลาสโมเดียมในหลอด และในสัตว์ทดลอง และความเป็นพิษระดับเซลล์ของสารสกัด Phyllanthus emblica, Terminalia chebula และ Terminalia bellerica*

วัสดุและวิธีการ: *การทดสอบคัดกรองพฤกษาเคมีแบบมาตรฐานนำมาใช้เพื่อตรวจหาสารเมตาโบไลท์ในสารสกัดพืช สารสกัดน้ำสมุนไพรถูกนำมาทดสอบหาฤทธิ์ต้านเชื้อพลาสโมเดียมในหลอดทดลองโดยการประเมินความสามารถของสารเหล่านี้ในการยับยั้งการรวมตัวเข้าของ [^{3}H] hypoxanthine ในสายพันธุ์ Plasmodium falciparum K1 ที่ดื้อยาหลายชนิด ความเป็นพิษระดับเซลล์ของสารสกัดถูกนำมากำหนดหาโดย Vero cell line ฤทธิ์ต้านเชื้อพลาสโมเดียมในหนูทดลองที่ติดเชื้อ Plasmodium berghei ถูกประเมินหาโดยการทดสอบการระงับเชื้อที่ 4 วัน แบบมาตรฐาน*

ผลการศึกษา: *การคัดกรองพฤกษาเคมีของสารสกัดน้ำจากพืชทั้ง 3 ชนิด พบสารฟลาโวนอยด์ ไฮโดรไลซ์แทนนิน ซาโปนินและเทอร์ปีน สารสกัดทั้งหมดแสดงให้เห็นถึงฤทธิ์ต้านเชื้อพลาสโมเดียม (ค่า IC_{50} อยู่ระหว่าง 14.33 $\pm$ 0.25-15.41 $\pm$ 0.61 mg/ml) สารสกัดน้ำของ Terminalia bellerica (Gaertn) Roxb มีฤทธิ์ในการต้านเชื้อพลาสโมเดียมในหลอด ทดลองสูงที่สุดตามมาด้วย Phyllanthus emblica Linn และ Terminalia chebula Retz ฤทธิ์ความเป็นพิษระดับเซลล์ ของสารสกัดทั้งหมดถูกนำมาทดสอบกับ Vero cells ได้ค่า IC_{50} ตั้งแต่ 157.86 ถึง 238.70 mg/ml สารสกัดทั้งหมดแสดง ให้เห็นถึงดัชนีการคัดเลือกในช่วงตั้งแต่ 11 ถึง 17 การทดสอบการระงับเชื้อที่ 4 วัน แบบมาตรฐานในหนูที่ติดเชื้อ ถูกนำมาใช้ในการหาฤทธิ์ต้านเชื้อพลาสโมเดียมของสารสกัดที่ระดับความเข้มข้น 250 มิลลิกรัมต่อน้ำหนักหนู 1 กิโลกรัมต่อวันแสดงให้เห็นถึงฤทธิ์ในการต้านเชื้อพลาสโมเดียมในสัตว์ทดลองที่มีฤทธิ์การระงับเชื้อที่ดีในช่วงระหว่าง 53.40% ถึง 69.46%*

สรุป: *สารสกัดทั้งหมดแสดงให้เห็นถึงฤทธิ์ต้านเชื้อพลาสโมเดียมในหลอด และในสัตว์ทดลองที่น่าสนใจ พร้อมกับมีการคัดเลือกที่ดี*