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Original article

Antiplasmodial effects of *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack extracts and their combination with chloroquine and quinine on *Plasmodium falciparum* in culture

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Abstract: Fruits of *Brucea javanica* (L.) Merr. ("Ratchadad" in Thai) and roots of *Eurycoma longifolia* Jack ("Plalaipeag" in Thai) are used as traditional medicines for the treatment of malarial fever. Ethanol, methanol, ethyl acetate, ethyl alcohol and aqueous extracts were tested against the multidrug-resistant *Plasmodium falciparum* strain K1. Ethanol and methanol-ethanol extracts, together with methanol residue, from fruits of *B. javanica* (L.) Merr. showed the highest antiplasmodial activities with IC_{50} values of 0.5 ± 0.3, 0.3 ± 0.1 and 0.3 ± 0.05 μg/mL, respectively, comparable to the IC_{50} values of chloroquine (0.17 ± 0.02 μg/mL) and quinine (0.3 ± 0.1 μg/mL). Similarly, ethanol and methanol-ethanol extracts of roots of *E. longifolia* Jack showed higher activities than those of the other solvent extracts, but their activities were about 10-fold lower than those of extracts from *B. javanica* (L.) Merr. fruit. In drug combination tests, *B. javanica* (L.) Merr. and *E. longifolia* Jack extracts did not appear to antagonize antiplasmodial activity of chloroquine and quinine. Not only well-known quassinoid glycosides but also coumarins and flavonoids identified by thin-layer chromatography in ethanol and methanol-ethanol extracts and in methanol residue of *B. javanica* (L.) Merr fruit and *E. longifolia* roots may be responsible for the antimalarial activity. Taken together, our extraction conditions provided extracts containing novel active compounds that did not antagonize the inhibitory effects of the two widely used antimalarials. This finding could lend support to the future discovery of active antimalaria compounds of *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack as drugs for the treatment of malaria that could be employed as drug combinations in order to delay the onset of parasite drug resistance.

Keywords: *Plasmodium falciparum, Brucea javanica* (L.) Merr., *Eurycoma longifolia* Jack, traditional medicine, antimalarial activity, drug combination

INTRODUCTION

Malaria continues to be a serious public health problem in tropical countries. According to WHO, more than 500 million people worldwide contract malaria annually, resulting in more than one million deaths [1-3]. Of the four human *Plasmodium* species (*P. falciparum, P. vivax, P. ovale,* and *P. malariae*), *P. falciparum* is the most virulent and is the primary cause of death. It has developed resistance to almost all antimalarial drugs in current use, including quinine and its analogue, chloroquine [4]. Thus there is an urgent need to develop new effective antimalarials against drug-resistant parasites. One strategy is through discovery of natural antimalarial products, especially those that can be used in combination with chloroquine and quinine to treat malaria parasites more effectively, because chloroquine and quinine are still widely used as first-line drugs in malaria therapy [5-7].

The plants *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack, which belong to the family Simaroubaceae, are widely distributed throughout the Asia Pacific region, including China, Indonesia, Malaysia and Thailand [8, 9].

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Several parts of the plants (fruit, roots, seeds, stems and bark) provide promising sources of natural medicinal products that are used in those countries in traditional therapy for a variety of diseases, including babesiosis, malaria and cancer [8-11].

The *in vitro* and *in vivo* antimalarial activities of *B. javanica* (L.) Merr. and *E. longifolia* Jack against *P. falciparum* have been investigated [8, 12-14]. In the present study, extracts of the fruits of *B. javanica* and roots of *E. longifolia* were obtained by procedures that differed from those in previous reports and the extracts were evaluated for their inhibitory activity against multidrug-resistant *P. falciparum* strain K1 in culture, both alone and in combination with chloroquine or quinine.

MATERIALS AND METHODS

Plant materials and extraction procedure

Fruits of *B. javanica* (L.) Merr. (3 kg) and roots of *E. longifolia* Jack (2.5 kg) were collected from Pang Sida National Park, Sa Kaew Province, Thailand. Voucher herbarium specimens were identified by Thanongsak Jonganurak, a plant taxonomist, and deposited at the Forest Herbarium (BKF), Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand, with the numbers BKF.162339 for *B. javanica* and BKF.162340 for *E. longifolia.*

After air drying, the plant materials were ground and processed as shown in Fig. 1. *B. javanica* fruits with high oil content were defatted with hexane (100 g in 350 mL) by Soxhlet extraction for 4 h and dried at 40 ℃. Then, both plant materials were soaked in methanol (1 kg in 3.5 L) 3 times for 3 days each time. Solutions were evaporated at 40 ℃ to produce methanol extracts, while residues were soaked in 50% ethanol (1 kg in 3 L), concentrated in a rotary evaporator at 40 ℃ to produce methanol-ethanol extracts (53.48 g for *B. javanica*: BME, and 14.93 g for *E. longifolia*: EME), and kept at 4 ℃ until use. The residues were further soaked in 50% ethanol (1 kg in 3 L) 2 times for 2 days each time and evaporated to produce 50% ethanol extracts (125.77 g for *B. javanica*: BE, and 58.93 g for *E. longifolia*: EE). Methanol extracts were mixed with pure water (25 g in 500 mL) and dichloromethane (1,500 mL: 3 volume of pure water) to give two phases. The aqueous phase was stored at 4 ℃ after evaporation as aqueous-methanol residue (59.01 g for *B. javanica*: BAM). The dichloromethane phase was evaporated and then applied to silica gel 60 (63-200 mm, Merck 7734) open column (1 g/ 30 g silica gel), which was sequentially eluted with 200 mL each of ethyl acetate, ethyl alcohol and distilled water. Each obtained solution was evaporated to dryness at 40 ℃ to obtain ethyl acetate (29.41 g for *B. javanica*: BEtAc, and

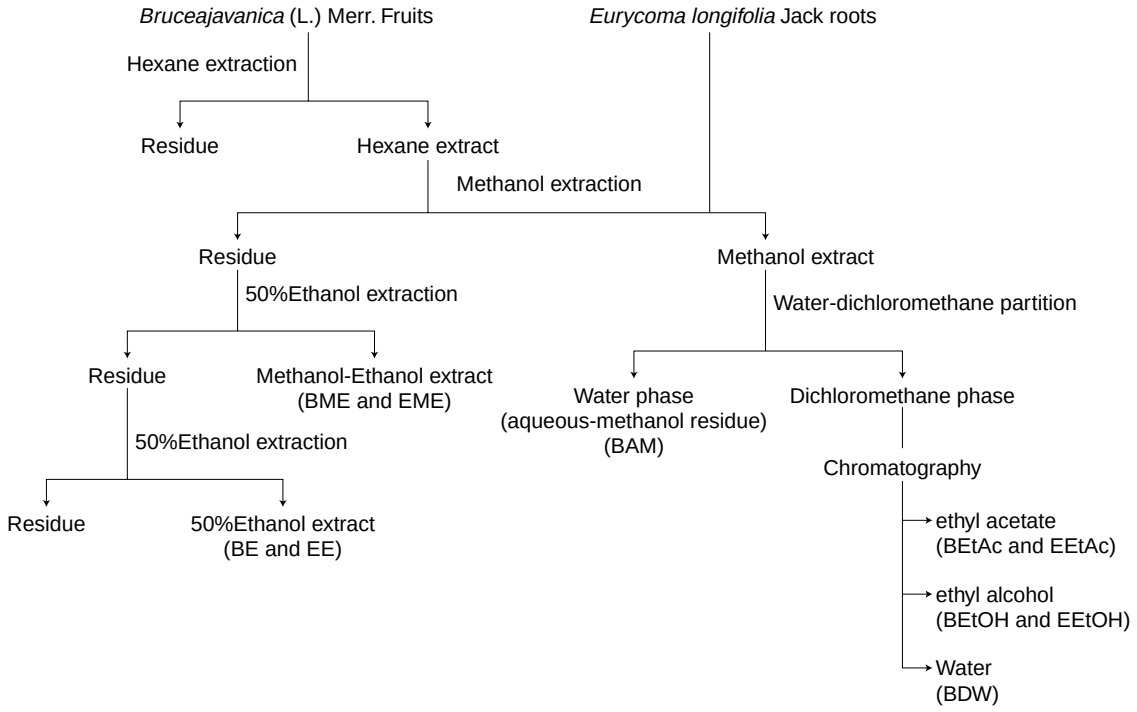

Fig. 1. Flow chart of extraction procedure of *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack by increasing polarity of solvents.

6.55 g for *E. longifolia*: EEtAc), ethyl alcohol (7.54 g for *B. javanica*: BEtOH, and 2.77 g for *E. longifolia*: EtOH) and water extracts (8.19 g for *B. javanica*: BDW, and 0.73 g for *E. longifolia*: EDW), which were kept at 4 ℃ until use.

Parasite culture and *in vitro* assessment of antimalarial activity

P. falciparum strain K1, which is resistant to chloroquine and pyrimethamine [15], was maintained in continuous culture as described by Trager and Jensen [16] under a gas mixture of 5% O_2, 5% CO_2, and 90% N_2 at 37 ℃ in human O-positive erythrocytes suspended in RPMI 1640 medium (Gibco, Life Technologies) supplemented with 25 mM $NaHCO_3$, 25 mM HEPES (Gibco, Life Technologies), 40-50 μg/L gentamicin sulfate and 10% human serum.

The activities of plant extracts, chloroquine and quinine against *P. falciparum* K1 were assessed using a [^{3}H]-hypoxanthine incorporation assay as described previously [17]. Each test material (10 mg/mL plant extracts, 1 mg/mL chloroquine and 1 mg/mL quinine) was prepared as a stock solution in 100% dimethylsufoxide (DMSO), sterile distilled water and 70% ethanol, respectively. Prior to use, the stock solution was freshly serially-diluted in medium without serum to yield appropriate final concentrations ranging from 3.2 ng/mL to 10 μg/mL in each well. Aliquots (25 μL) of each dilution were dispended into triplicate wells of a 96-well plate (Nunc). After addition of erythrocyte suspension (200 μL), at 1.5% hematocrit with 1% parasitemia, parasites were allowed to grow at 37 ℃ for 24 h. The final concentrations of DMSO and ethanol (0.01%) in each well had no effect on parasite growth. A 25 -μL aliquot of 10 μCi/mL of [^{3}H]-hypoxanthine (specific activity of 17.4 Ci/mmol, Perkin Elmer) in medium was added to each well and cultures were grown for another 18-24 h. Contents of the wells were harvested onto glass-fiber filters using a semiautomated 96-well harvester (Tomac, Orange, Conn.), and cells were lysed with distilled water. Filters were dried and soaked with liquid scintillation fluid (0.35% (w/v) 2,4-diphenyloxazole and 0.005% (w/v) 1,4-bis [2-(5-phenyloxazoyl)]benzene in toluene), and the amount of [^{3}H]-hypoxanthine incorporated into parasites was measured in a liquid scintillation counter (Beckman LS 1801). IC_{50} values were estimated from drug dose-response curves (plot of percent radioactivity versus log of test material).

The effect of a plant extract on chloroquine or quinine action was evaluated using a checkerboard technique [18]. The experiment was performed as described above with the following modification: Drugs were prepared at double the concentrations used in a single drug test and plant extracts were prepared at 18 times the concentrations of their respective IC_{10} and IC_{25} values. The 12.5-μL aliquots of chloroquine or quinine were added to triplicate wells of a 96-well plate together with an equal volume of plant extract and 200 μL of erythrocyte suspension. The plate was processed as described above.

Thin-layer chromatography (TLC) analysis

The components of each extract from fruits of *B. javanica* and roots of *E. longifolia* were characterized by TLC (silica gel 60 F254, Merck) [19]. Each extract (0.1-0.2 g) was dissolved in 5 mL of 80% ethanol (for detection of alkaloids, antioxidants and glycosides), methanol (for detection of flavonoids) or dichloromethane (for detection of coumarins) and incubated at 40 ℃ for 15 min in order to obtain complete dissolution of the extracts. One mL of 28% NH_4OH together with 1 mL of $CHCl_3$ or 2 mL of $CHCl_3$ was added for detection of alkaloids and glycosides, respectively, and then the solutions were thoroughly shaken and the lower (organic) phases were transferred to new tubes. After evaporation to 0.5 mL, 3-5 μL of the mixture was spotted onto a TLC plate, which was developed with solutions of toluene:ethyl acetate:diethylamine (40:20:20, v/v/v), ethyl acetate:methanol:water (80:20:20, v/v/v), ethyl acetate:methanol:water (81:11:8, v/v/v) and ethyl acetate:formic acid:acetic acid:water (100:11:11:27, v/v/v/v) to detect alkaloids, antioxidants, coumarins and glycosides (developed by the same solvent mixture), and flavonoid, respectively. The plates were dried and sprayed with Dragendorff's reagent, DPPH reagent, KOH reagent, Kedde's reagent and natural product-polyethlene glycol reagent for detection of alkaloids, antioxidants, coumarins, glycosides and flavonoids, respectively. The sprayed TLC plates for detection of alkaloid, antioxidant and glycoside were developed to orange, white and pinkish purple colors, respectively, in visible light. Presence of coumarins and flavonoids was observed under UV light (365 nm) as yellow, blue, green, orange or red color.

RESULTS AND DISCUSSION

An *in vitro* test by a [^{3}H]-hypoxanthine uptake assay confirmed that the multidrug-resistant *P. falciparum* strain K1 is strongly resistant to quinine and chloroquine with IC_{50} value of 0.318 ± 0.117 and 0.165 ± 0.023 μg/mL respectively, but sensitive to dihydroartemisinin with an IC_{50} value of 1.133 ± 0.080 ng/mL comparable with values previously reported [20, 21]. Then, extracts of fruits of *B. javanica* (L.) Merr. and roots of *E. longifolia* Jack were evaluated for their antiplasmodial activities *in vitro* against the K1 strain. As shown in Table 1, antiplasmodial activities of *B. javanica* (L.) Merr fruit alcoholic extracts were 10

Table 1. *In vitro* antiplasmodial activities of extracts isolated from *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack against *P. falciparum* strain K1

Plant species	Part used	Extract	Mean IC_{50} ± SD (μg/mL)
Brucea javanica (L.) Merr.	Fruit	Ethanol	0.5 ± 0.3
		Methanol-ethanol	0.3 ± 0.1
		Aqueous-methanol residue	0.3 ± 0.05
		Ethyl acetate	1.4 ± 0.4
		Ethyl alcohol	1.3 ± 0.9
		Distilled water	>10
Eurycoma longifolia Jack	Root	Ethanol	2.6 ± 0.8
		Methanol-ethanol	2.2 ± 0.9
		Ethyl acetate	>10
		Ethyl alcohol	>10
		Distilled water	>10

Table 2. Effects of *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack extracts on antiplasmodial activity of chloroquine and quinine against *P. falciparum* K1 growth *in vitro*

Compound	Mean IC_{50} (μg/mL) ± SD	
	Chloroquine	Quinine
Chloroquine alone	0.17 ± 0.02	-
Quinine alone	-	0.3 ± 0.1
Chloroquine or quinine plus		
BAM extract at IC_{10} (0.05 μg/ml)	0.16 ± 0.02	0.2 ± 0.1
BAM extract at IC_{25} (0.13 μg/ml)	0.16 ± 0.03	0.3 ± 0.1
EME extract at IC_{10} (0.45 μg/ml)	0.15 ± 0.03	0.2 ± 0.1
EME extract at IC_{25} (1.12 μg/ml)	0.12 ± 0.02	0.19 ± 0.05

BAM: *Brucea javanica* (L.) Merr. aqueous-methanol residue extract, EME: *Eurycoma longifolia* Jack methanol-ethanol extract.

-fold higher than those of roots of *E. longifolia* Jack, but water extracts of both plants were inactive (IC_{50} > 10 μg/mL). The IC_{50} values of 50% ethanol, methanol + 50% ethanol and aqueous-methanol residue extracts from fruits of *B. javanica* (L.) Merr (0.5 ± 0.3, 0.3 ± 0.1 and 0.3 ± 0.05 μg/mL, respectively) were comparable to those of the standard antimalarial drugs, chloroquine (0.17 ± 0.02 μg/mL) and quinine (0.3 ± 0.1 μg/mL) (see Table 2). Several parts of *B. javanica* and *E. longifolia* collected in different countries have been shown to be active against *P. falciparum* with different IC_{50} values, ranging from 0.34 to 94.8 μg /mL, depending on the type of solvent extraction [8, 12-14].

To evaluate whether the plant extracts can be employed without an antagonistic effect with chloroquine and quinine, which are widely used for *falciparum* malaria treatment [5-7], fixed concentrations at IC_{10} and IC_{25} values of the most active plant extracts were added in the presence of various concentrations of chloroquine or quinine in tests on *P. falciparum* K1 growth. Neither IC_{10} and IC_{25} concentrations of *B. javanica* (L.) Merr aqueous-methanol residue extract nor *E. longifolia* Jack methanol-ethanol extract exerted any significant effect on chloroquine and quinine antimalarial activities (Table 2), suggesting that these extracts did not antagonize the actions of the two drugs. *E. longifolia* methanol root extract has been reported to act synergistically with another antimalarial drug, artemisinin, in suppression of *P. yoelii* infection in mice [22].

All extracts from *B. javanica* and *E. longifolia* were screened for the presence of bioactive compounds, including alkaloids, antioxidants, glycosides, coumarins and flavonoids, by TLC. Ethanol and methanol-ethanol extracts (BE and BME) with high antimalarial activity showed very similar TLC patterns (Fig. 2) and chemical constituents (Table 3). Several derivatives of antioxidants, glycosides, coumarins and flavonoids, but not alkaloids, were detected in these two extracts. Antioxidants, glycosides, coumarins and flavonoids have been reported to possess antimalarial activity [23-25]. The aqueous-methanol residue (BAM) with the highest antiplasmodial activity showed a TLC pattern similar to the TLC patterns of ethanol and methanol extracts

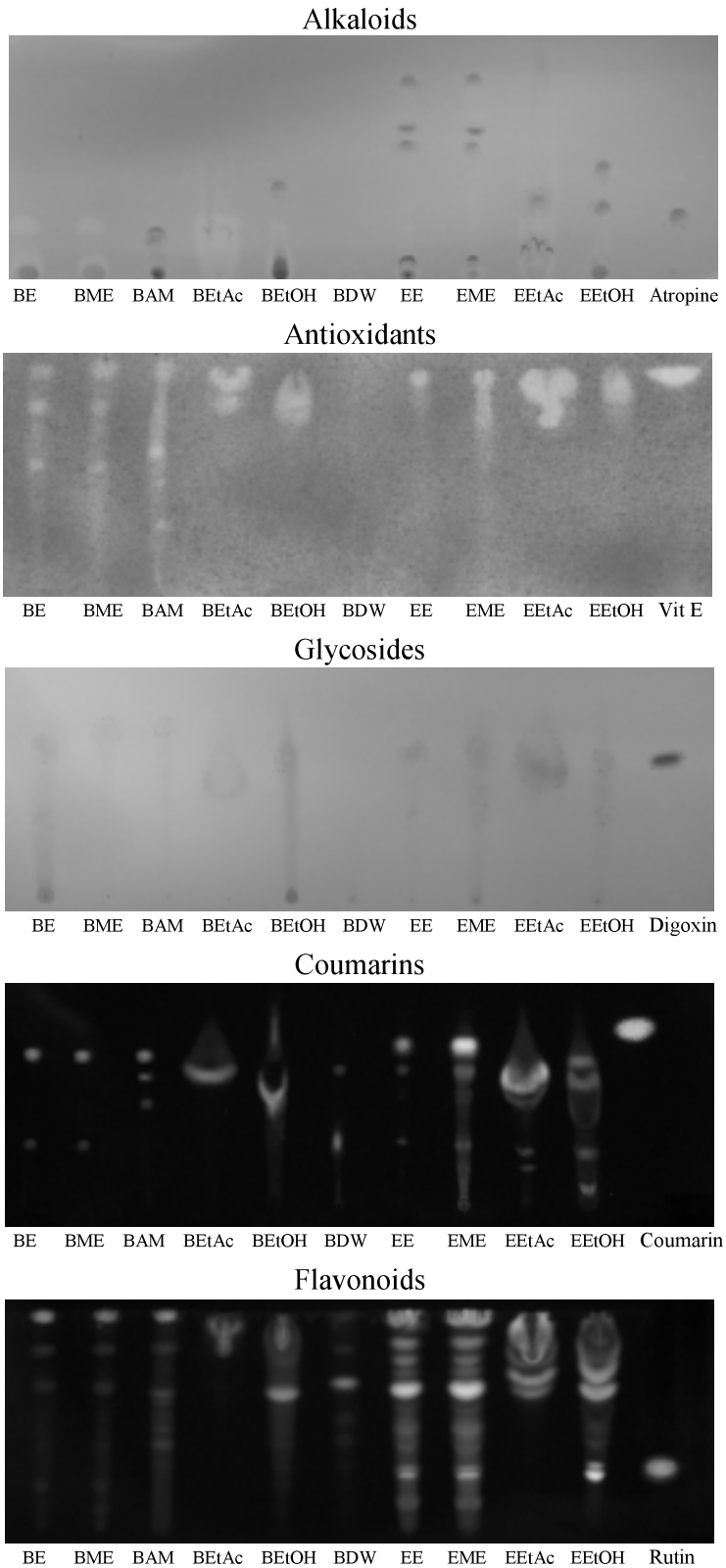

Fig. 2. Thin-layer chromatography of *Brucea javanica* (L.) Merr. and *Eurycoma longifolia* Jack extracts. Each extract of 50% ethanol (BE and EE), methanol-ethanol (BME and EME), aqueous-methanol residue (BAM), ethyl acetate (BEtAc and EEtAc), ethyl alcohol (BEtOH and EEtOH), and distilled water (BDW) from *Brucea javanica* (L.) Merr. (Lane 1-6) and *Eurycoma longifolia* Jack (Lane 7-10) was dissolved in organic solvent and processed as described in MATERIALS AND METHODS. After developing and spraying with appropriate reagents, plates were visualized by the naked eye for detection of alkaloids, antioxidants and glycosides and under UV light (365 nm) for detection of coumarins and flavonoids.

Table 3. Relationship between chemical constituents and antimalarial activity of plant extracts

Plant species	Extract	IC_{50} (µg/mL)	Constituents									
			Alkaloids	Rf	Antioxidants	Rf	Glycosides	Rf	Coumarins	Rf	Flavonoids	Rf
Brucea javanica (L.) Merr.	BE	0.5 ± 0.3	-	-	+	0.53 0.76 0.90	+	0.32 0.63	+	0.28 0.67	+	0.20 0.37 0.44 0.52 0.65 0.82 0.98
	BME	0.3 ± 0.1	-	-	+	0.53 0.76 0.90	+	0.70	+	0.28 0.66	+	0.10 0.22 0.37 0.44 0.53 0.66 0.84 0.98
	BAM	0.3 ± 0.05	+	0.12	+	0.60 0.93	+	0.72	+	0.44 0.56 0.67	+	0.40 0.46 0.53 0.57 0.66 0.84 0.98
	BEtAc	1.4 ± 0.4	-	-	+	0.79 0.89	+	0.51	+	0.56 0.64	+	0.58 0.64 0.88 0.94
	BEtOH	1.3 ± 0.9	+	0.33	+	0.82	+	0.59	+	0.44 0.59 0.71	+	0.22 0.37 0.61 0.7 0.89 0.94
	BDW	>10	-	-	-	-	-	-	+	0.29 0.60	+	0.29 0.40 0.50 0.64 0.80 0.96
Eurycoma longifolia Jack	EE	2.6 ± 0.8	+	0.47	+	0.89	+	0.37 0.64	+	0.13 0.18 0.27 0.47 0.58 0.70	+	0.12 0.25 0.28 0.35 0.45 0.54 0.62 0.70 0.76 0.84 0.97
	EME	2.2 ± 0.9	+	0.44 0.59	+	0.89	+	0.37 0.63	+	0.25 0.45 0.58 0.68	+	0.13 0.25 0.28 0.37 0.45 0.54 0.62 0.69 0.76 0.84 0.97
	EEtAc	>10	+	0.28	+	0.73 0.87	+	0.60	+	0.16 0.24 0.45 0.56 0.68	+	0.60 0.65 0.68 0.73 0.86 0.92
	EEtOH	>10	+	0.21 0.36	+	0.87	+	0.37 0.62	+	0.06 0.16 0.21 0.28 0.36 0.44 0.54 0.62 0.72	+	0.24 0.28 0.36 0.45 0.52 0.61 0.69 0.77 0.89
Control			Atropine	0.20	Vitamin E	0.95	Digoxin	0.60	Coumarin	0.79	Rutin	0.42

+: Present, -: Absent, Rf: Retention factor, which is distance of solute from origin relative to distance of solvent from origin

(BE and BME), but this extract contained derivatives not only of antioxidants, glycosides, coumarins and flavonoids but also of alkaloids. The other extracts with lower antiplasmodial activity *in vitro* had different TLC patterns. The water extract (BDW), inactive against *P. falciparum* K1, contained only two derivatives of coumarins and six derivatives of flavonoids, and no alkaloids, antioxidants or glycosides (Table 3). The two extracts (EE and EME) with highest antiplasmodial activity among the extracts from *E. longifolia* Jack showed similar TLC patterns. The other two extracts (EEtAc and EEtOH), which were inactive extracts, from the same plant had very different TLC patterns from those of EE and EME. These findings indicated the loss of some active antimalarial compounds in EEtAc and EEtOH. Antimalarial activity of *B. javanica* and *E. longifolia* has been previously shown to be attributable to quassinoid glycosides [26-30]. However, the correlation of loss of some coumarin and flavonoid derivatives with reduction of antimalarial activity (Table 1 and Fig. 2: compare TLC patterns of the highest active extracts BE, BME and BAM with those of active extracts BEtAc and BEtOH and inactive extracts BDW; compare active extracts EE and EME with inactive extracts EEtAc and EEtOH) suggested that these derivatives may also play an important role in the antimalarial activity of *B. javanica* and *E. longifolia.* Further studies are needed to isolate and characterize these inhibitory molecules and to investigate the mechanism of their action.

In summary, alcoholic extracts of fruits of *B. javanica* (L.) Merr. displayed antiplasmodial activity comparable to that of chloroquine and quinine, whereas those from roots of *E. longifolia* Jack extracts were about 10 times less potent. The antiplasmodial effects might be due to derivatives of glycosides, coumarins and flavonoids present in these plant extracts. The most potent antiplasmodial plant extracts had no significant effect on the action of currently used antimalarial drugs, chloroquine and quinine.

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