

ANTI-PLASMODIAL ACTIVITY OF ENGELETIN ISOLATED FROM *Artocarpus scortechinii*

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ABSTRACT

Ethnomedicinal use of plants from the *Artocarpus* genus has been extensively documented to include treatment of inflammation and malarial fever. Anti-plasmodial effects of *Artocarpus scortechinii* (synonym *A. elasticus*) reported against *P. falciparum* 3D7 culture in erythrocytes remain to be validated *in vivo*. The present study involved re-evaluation of anti-plasmodial activity of ethanolic extract of *A. scortechinii* *in vitro*, assessment of the extract for chemo-suppressive effects *in vivo*, and fractionation and identification of bioactive compounds. The ethanolic extract of *A. scortechinii* displayed moderate inhibitory activity towards growth of *P. falciparum* 3D7 (IC₅₀=14.4±8.1 µg/mL) with minimal toxicity effect in mammalian Chang liver cells (selectivity index, SI=13.9). Administration of 25, 50 and 100 mg/kg body weight extract into *P. berghei*-infected mice for four consecutive days resulted in dose-dependent chemo-suppression of 43.2, 74.8 and 78.4% respectively. Vacuum liquid chromatography fractions of the ethanolic extract exhibited moderate to good *in vitro* anti-plasmodial activities. Further fractionation using Sephadex Column Chromatography yielded engeletin identified by nuclear magnetic resonance spectroscopy and mass spectroscopy. Upon further testing the isolated engeletin was shown to display an IC₅₀ of 33.1±1.3 µM against the 3D7 parasite strain (SI=3.5). Data from the present study is the first report on anti-plasmodial activity of engeletin and its isolation from *Artocarpus scortechinii*.

Key words: Anti-plasmodial, *Artocarpus* sp., flavonoid, engeletin

INTRODUCTION

In recent years, anti-malarial research initiatives to discover and develop new anti-malarial therapeutics from ethnopharmacological plants resources have increased dramatically primarily due to the development of resistance to existing anti-malarial agents. The *Artocarpus* genus of plants has received extensive scientific attention due to the medicinally important secondary metabolite constituents with useful biological activities. Many of the widely-documented investigations have been restricted to primary screening studies using *in vitro* approaches. For example, Mustapha *et al.* (2010) showed anti-malarial activities in *A. elasticus* (also known as *A. scortechinii*) employing blood smear analysis of plasmodium-infected parasites and candle jar method of Trager & Jensen (1975). *In vivo* experiments to validate *in vitro* anti-plasmodial

activities of the *Artocarpus* plants remain scarce. The registry of studies on anti-plasmodial effects of *Artocarpus* preparations in murine models of infection is limited to investigations by Widyawaruyanti *et al.* (2007) on *A. champeden*. Identification of compounds responsible for anti-plasmodial activities in this genus of plants have led to many bioactive compounds among the secondary metabolite constituents. These include artoindonesianin Z-5, gemichalcone B, dihydromorin, artocarpesin, cycloartocarpesin, artonin E, 12-hidroxy artonin E, cycloartocarpin and artobiloxanthone which have all been further evaluated for anti-malarial activity against chloroquine-resistant *P. falciparum* K1 strain (Mustapha *et al.*, 2010). Phytochemical characterization of bioactive compounds is not only required as a basis for development of new anti-malarial compound(s) but is also an essential step in the production of standardized herbal preparations.

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MATERIALS AND METHODS

Parasites and Animals

P. falciparum 3D7 (chloroquine-sensitive) strain and *P. berghei* NK65 (chloroquine-sensitive) strain were obtained from Malaria Research and Reference Reagent Resource (MR4, USA) whereas Chang liver cells were obtained from American Type Culture Collection (ATCC, USA). Male ICR mice (6-8 weeks old, 25±4 g in weight) were obtained from the Animal House Facility at Universiti Kebangsaan Malaysia. Permission and approval for animal studies were obtained from the Universiti Kebangsaan Malaysia Animal Ethics Committee (UKMAEC-FST/2012/NOOR/21-NOV./465-DEC.-2012-DEC.-2014).

Extraction and Fractionation of Plant Materials

Air-dried stem bark of *A. scortechinii* collected from Jengka forest in the state of Pahang, Malaysia (voucher specimen no UKMB 40193) were ground into a powder prior to soaking in 95% ethanol for three days at room temperature. Filtered extract was then concentrated using a rotary evaporator (Fisher scientific, UK) and freeze dried (Labconco, UK). Further fractionation and separation was carried out using vacuum liquid chromatography (VLC) (solvent system; n-hexane: ethyl acetate, 3:7) and Sephadex column chromatography (SCC) (solvent system; 100% methanol). Structure of isolated compound was identified using spectroscopic techniques such as ultraviolet, infrared, NMR and mass spectrometry.

In vitro Anti-plasmodial Assay

Parasite lactate dehydrogenase (pLDH) assays (Makler & Hinrichs, 1993) were performed in flat-bottomed 96-well plates by Nkhoma *et al.* (2007) with slight modifications. Parasitized red blood cells and un-parasitized O+ red blood cells without treatment were used as positive and negative controls respectively. *P. falciparum* 3D7 culture was plated in the asynchronized phase at 1.5% hematocrit and 2% parasitemia (60% rings) in 100 µL of extract or fraction from *A. scortechinii* or anti-malarial drug. Extract or fraction from *A. scortechinii* was serially diluted (0.01 to 1000 µg/mL) whereas reference drug, chloroquine diphosphate (CQ) purchased from Sigma Aldrich (USA) was diluted serially (0.02 to 20 µM). Plates were placed in a sterile incubator (Thermo Scientific, USA) with a gas mixture of 4% O₂, 5% CO₂, and 97% N₂ at 37°C for 48 hours. Reagents required for detecting and measuring the parasite LDH enzyme were prepared as follows: Malstat reagent by dissolving 400 µL Triton X-100 in 80 mL deionized water, and adding 4.00 g L-lactate, 1.32 g Tris buffer and 0.022 g 3-acetylpyridine adenine dinucleotide

(APAD⁺) then adjusting pH to 9.0 and final volume topped up to 200 mL with deionized water; and NBT/PES solution by dissolving 0.160 g nitrotriazolium blue salt (NBT) together with 0.008 g phenazine ethosulfate (PES) in 100 mL deionized water and stored at 4°C in a foil-covered container. Upon completion of incubation, cultures were subjected to three 30-minute freeze-thaw cycles. This was followed by the addition of 100 µL Malstat reagent and 25 µL NBT/PES to each well of a fresh second set of flat-bottomed 96-well plate in triplicates. The culture in each well from the first set of plates was re-suspended and 15 µL cultures were added to the second set of 96-well plates to initiate the lactate dehydrogenase enzymatic reaction. Color development of the pLDH plate was then monitored colorimetrically at 650 nm (Fluorostar OPTIMA) after one hour of incubation in the dark. Data obtained was analyzed for IC₅₀ values (inhibition concentration at 50% parasite growth) by Graph Prism 5 software through non-linear regression.

In Vitro Cytotoxicity Assay

Cytotoxicity of extracts was determined using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) assay (Mossmann, 1983). On the day of test, Chang liver cells were harvested, counted and cell number adjusted to 2 × 10⁴ cells per mL. Then, 100 µL cell suspensions in complete Dulbecco's Modified Eagle Medium (DMEM) were seeded into each well of a 96-well plate. At the end of 24 hours of culture, medium was removed, and 100 µL of diluted extracts added in triplicates. The final concentration of the extracts in test wells ranged from 0.01 to 10 mg/mL. Cell suspension without test substance (100 µL) was used as positive control. The culture was incubated in the presence or absence of extract for 48 hours, 37°C, 5% CO₂ (Thermo Scientific, USA). MTT reagent was prepared in phosphate buffered saline (PBS) at 5 mg/mL. Then, 10 µL of MTT-PBS reagent was added to each well containing 100 µL complete DMEM. The plates were further incubated for 3 hours (37°C, 5% CO₂). The medium was then removed and replaced with 100 µL of dimethyl sulphoxide (DMSO) to dissolve the MTT formazan product. The mixture was thoroughly mixed for 15 minutes before measuring the absorbance at 540 nm (Fluorostar OPTIMA). The growth curve and the concentration at 50% growth inhibition (IC₅₀) were estimated through non-linear regression software Graph Prism 5.

In vivo Anti-malarial Activity Evaluation

Anti-malarial activity evaluation of extract was carried out based on the four-day suppressive test described by Peters (1975). Inoculum used consisted of 5 × 10⁶ *P. berghei*-parasitized erythrocytes/mL,

determined based on the percentage of parasitaemia and the erythrocyte count in stock mouse and blood dilution with Alsever's buffer. Extracts were diluted with 0.9% saline solution with 10% dimethyl sulfoxide (DMSO) to yield 25, 50 and 100 mg/kg body weight (bw) final concentration in 0.2 mL injection volume. CQ was freshly prepared at 10 mg/kg bw in 0.9% normal saline. A total of 42 mice were randomly divided into six groups of seven mice each. *P. berghei* NK65 infection was done to all groups of mice on day 0 (D0). After three hours of infection, treatment groups were intraperitoneally administered with the first dose of 0.2 mL extract. Administration of extract was repeated for three additional days (D1, D2 and D3). Two groups (n=7) served as negative and positive controls; the negative control group received a placebo (0.9% saline solution) while the positive controls received 10 mg/kg bw CQ. Dose of 10 mg/kg bw CQ was chosen because of the reported 100% parasite clearance (Peters 1975) associated with this dose in *in vivo* four-day suppressive tests. In addition, 10 mg/kg bw CQ did not cause death in *P. berghei*-infected mice (Peters 1975). Thin and thick blood smears from mice tail were prepared beginning D1 until D20 to measure parasitaemia levels and observe morphology of infected erythrocytes. Average chemo-suppressive activities were calculated according to Gathirwa *et al.* (2007):

$$\text{Chemo-suppression (\%)} = [(A - B)/A] \times 100$$

where A is the mean parasitaemia for negative control group on day 4 (D4) and B is the corresponding parasitaemia for treatment or positive control group.

In vivo Toxicity Evaluation

This investigation was aimed at evaluating the extract for toxicity effects at the dosages used in the *in vivo* experiments. Male, white, ICR strain, 6-8 weeks old mice were randomly grouped into seven mice in each group. Five groups were administered with 0.2 mL of each 25, 50, 100 and 250 mg/kg bw respectively. One positive control group was given 0.9% saline. Extract administration was given to mice (i.p.) for four consecutive days (D0-D3). Death of mice was monitored for two weeks and data analyzed using Kaplan-Meier analysis (Bewick *et al.*, 2004). Gross changes in behaviour, like gasping, low activity, vomiting and death were also observed and recorded.

Statistical Analysis

Data obtained from *in vitro* and *in vivo* activities were analyzed statistically using Student's *t*-test and Kaplan-Meier analysis. Treatments given were considered significant if $p < 0.05$.

RESULTS

Extraction and Fractionation

A. scortechinii plant materials (0.36 kg) which were air-dried at room temperature and extracted using ethanol yielded 19.8 g extract. Ethanolic extract of *A. scortechinii* was further fractionated using vacuum liquid chromatography (VLC) to yield five fractions; fraction A (303.3 mg), fraction B (22.3 mg), fraction C (129.1 mg), fraction D (100.1 mg) and fraction E (771.7 mg). Fraction C was further purified through Sephadex column chromatography (SCC) to afford pale yellow crystals.

Identification of Compounds

The pale yellow crystals obtained from *A. scortechinii* were subjected to spectroscopic analysis: ESI-MS (positive mode); m/z 457.1102 $[M+Na]^+$ calculated for $C_{21}H_{22}O_{10}$, 434; (UV) λ_{max} nm; 215, 291 and 320 nm and infrared (IR); 1644, 1432, 1736, 3445, 1227 and 1092 cm^{-1} suggested the isolated compound is a flavonoid (Mabry *et al.*, 1970). The 1H and ^{13}C NMR of data (Table 1) is in accordance to a flavanone glycoside and characterized as 5,7,4'-trihydroxyflavanone-3-O- α -L-rhamnopyranoside or engeletin previously isolated and identified by Xu *et al.* (2005) from roots of *Smilax brockii*. Interestingly, identification of engeletin from stem bark of *A. scortechinii* has not been reported.

Table 1. 1H and ^{13}C -NMR data for engeletin isolated from *A. scortechinii* (600MHz and 150 MHz respectively, CD_3COCD_3)

No.	Chemical shift, δ_H , ppm (double spin, constant coupling, Hz)	Chemical shift, δ_C , ppm
2	5.24 (d, 10.8)	82.4
3	4.71 (d, 10.8)	76.6
4	—	195.3
5	—	164.4
5-OH	11.92 (s)	—
6	5.95 (d, 1.8)	96.3
7	—	166.8
8	5.98 (d, 1.8)	95.1
9	—	162.9
10	—	100.7
1'	—	127.5
2'	7.43 (d, 8.4)	129.1
3'	6.90 (d, 8.4)	115.3
4'	—	158.2
5'	6.90 (d, 8.4)	115.3
6'	7.43 (d, 8.4)	129.1
1''	4.04 (d, 1.2)	101.6
2''	3.53 (m)	70.5
3''	3.64 (ddd, 9.6, 6.0, 3.6 Hz)	71.3
4''	3.30 (ddd, 13.6, 11.4, 3.6 Hz)	72.6
5''	4.21 (m)	69.0
6''	1.13 (d, 6.0 Hz)	17.1

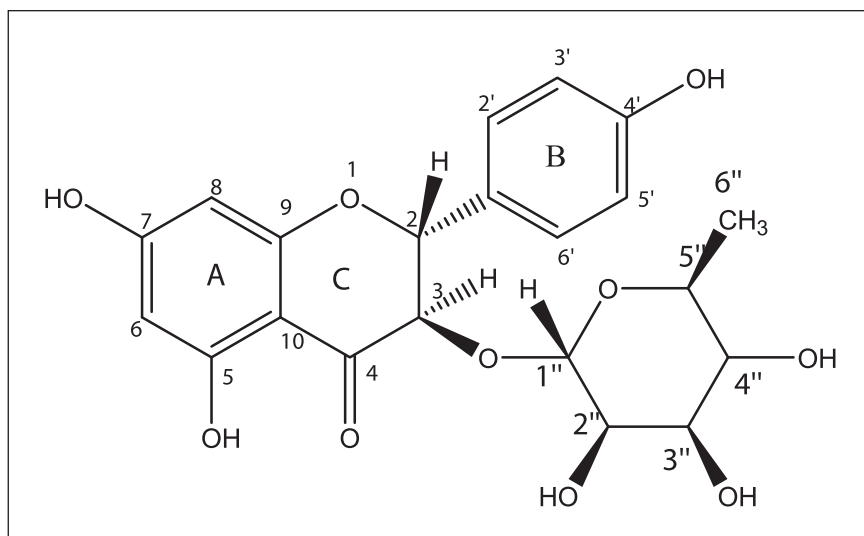


Fig. 1. Structure of Engeletin from Stem Bark of *A. scortechinii*

Table 2. *In Vitro* Anti-plasmodial and Cytotoxic Activities of Extract/Fractions and Isolated Compounds from *A. scortechinii*

Sample from <i>A. scortechinii</i>	Cytotoxicity Activity against Chang liver cell, $IC_{50} \pm SD$ ($\mu\text{g/mL}$)	Anti-plasmodial Activity against <i>P. falciparum</i> 3D7, $IC_{50} \pm SD$ ($\mu\text{g/mL}$)	Selectivity Index (SI) $= \frac{IC_{50} \text{ MTT}}{IC_{50} \text{ pLDH}}$
Ethanollic extract	>500	14.4 ± 8.1	34.7
Fraction A	122.4 ± 1.3	3.7 ± 1.3	33.2
Fraction B	30.5 ± 1.7	47.0 ± 1.4	0.7
Fraction C	155.2 ± 2.3	1.8 ± 0.1	85.7
Fraction D	186.6 ± 1.4	47.1 ± 1.3	4.0
Fraction E	290.8 ± 1.6	57.9 ± 0.1	5.0
Engeletin	$116.0 \pm 0.2 \mu\text{M}$	$33.1 \pm 1.3 \mu\text{M}$	3.5
Choroquine	ND	58.1 nM	ND

Susceptibility of 3D7 strain of *P. falciparum* to *A. scortechinii* extracts and fractions were evaluated. SI (index of selectivity towards parasite relative to mammalian cell) = $IC_{50} \text{ MTT}/IC_{50} \text{ pLDH}$. Experiments were carried out in triplicates and repeated at least twice. CQ was used as reference anti-malarial drug. (SD; standard deviation, ND; not determined)

In Vitro Anti-plasmodial and Cytotoxic Assays

Evaluations of pLDH in *P. falciparum* 3D7 cultures incubated with varying concentrations of ethanolic extract, fractions and pure compound from *A. scortechinii* yielded typical sigmoid dose response curves. Inhibition(s) of *P. falciparum* 3D7 growth was moderate for ethanolic extract of *A. scortechinii* ($IC_{50}=14.4 \pm 8.1 \mu\text{g/mL}$) (Table 2). Minimal toxicity effects were observed with extract toward Chang liver cells ($IC_{50} > 500 \mu\text{g/mL}$) thus the extract displayed high selectivity index (SI) towards the parasite of 34.7. Fractions A, B, C, D and E displayed moderate to active anti-plasmodial activities *in vitro* against 3D7 ($IC_{50}= 3.7 \pm 1.3, 47 \pm 1.4, 1.8 \pm 0.1, 47.1 \pm 1.3$ and $57.9 \pm 0.1 \mu\text{g/mL}$ respectively) and minimal effects on Chang liver cells with SI values ranging from 0.6 to 33.3 (Table

2). Engeletin isolated from fraction C exhibited moderate anti-plasmodial activity and low inhibition of Chang liver cell growth ($IC_{50}=33.1 \pm 1.3 \mu\text{M}$ and $116.0 \pm 0.2 \mu\text{M}$ respectively and $SI=3.5$). The reference drug CQ displayed an IC_{50} of 58.1 nM (0.03 $\mu\text{g/mL}$).

In vivo Anti-malarial Test

Administration of 25 to 100 mg/kg bw of ethanolic extract of *A. scortechinii* for four consecutive days did not cause any deaths in mice (Table 3). However, at a dosage of 250 mg/kg bw, one death occurred on day 7 implicating mild toxicity effect. Three selected dosages of 25, 50 and 100 mg/kg bw administered for four consecutive days into *P. berghei* NK65-infected ICR mice caused significant inhibition of parasite development. Good

chemo-suppressive activity exceeding 70% was achieved for both 50 and 100 mg/kg bw dosages tested (Table 3).

The observed dose-dependent chemo-suppressive activities for ethanolic extract of *A. scortechinii* is summarized together with experimental animal survival data in Table 3. *P. berghei*-infected mice administered with extracts survived beyond the survival period for non-treated mice. Noteworthy is the 100% survival in mice injected with 50 and 100 mg/kg bw extract 20 days

after start of experiment as observed in animals administered the reference drug, chloroquine diphosphate (CQ). Infected mice treated with CQ (10 mg/kg bw) was completely free from parasite on day 4 onwards (Table 3) (99.9% of chemo-suppression). However, at the lowest dosage of 25 mg/kg bw extract tested, low (43%) chemo-suppressive activity was obtained. This dose was not able to prolong mice survival compared to non-treated animals.

Table 3. *In vivo* parasitaemia level, chemo-suppressive activity and survival of ethanolic extract of *A. scortechinii* on day 4 (D4)

Extract/Drug	Dosage (mg/kg bw/day)	Average Parasitaemia at D4 \pm SD	Suppression of Parasitemia at D4 (%)	Median of Mice Survival (Day)
Ethanolic extract of <i>A. scortechinii</i>	25	1.9 \pm 0.9	43.2	14
	50	0.9 \pm 0.5*	74.8*	24
	100	0.7 \pm 0.7*	78.4*	25
Chloroquine 0.9% Saline Solution	10	0.1 \pm 0.1*	99.9*	>30
	0.2 mL	3.3 \pm 1.1*	–	18

Inoculation of *P. berghei* NK65 was carried out in all groups of animals three hours prior to administration. Mice (n=7 per group) were treated with extract at different dosages (25, 50 and 100 mg/kg bw) for four consecutive days (D0 to D3). CQ (10 mg/kg bw) and 0.2 mL saline solution were used as positive and negative controls respectively. (SD; standard deviation, *shows significant value, $p < 0.05$).

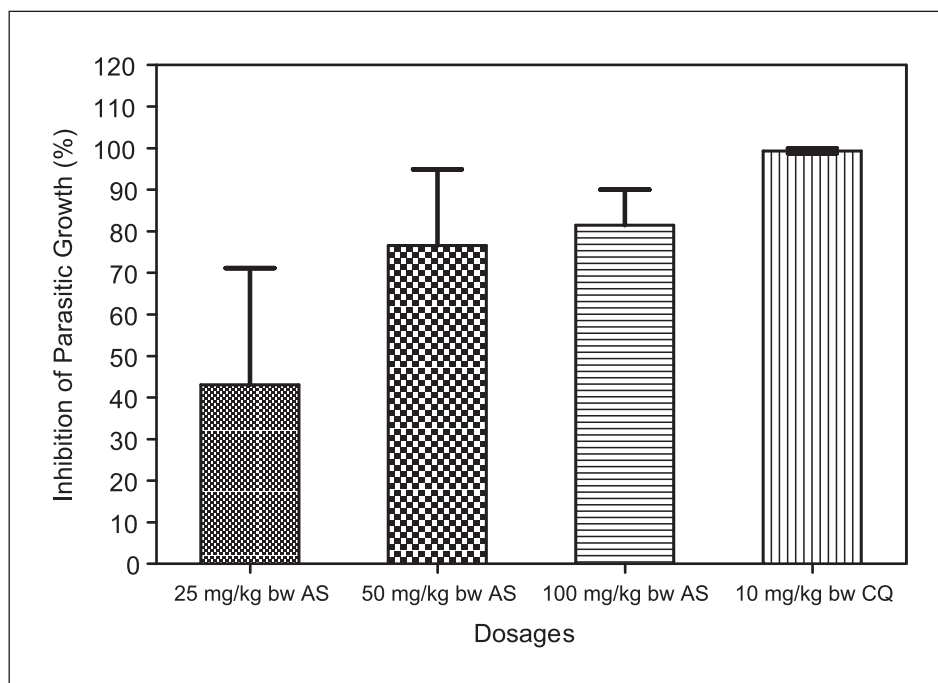


Fig. 2. Percentage of inhibition of *P. berghei* growth

Percentages of inhibition of parasite growth *in vivo* and standard deviations at day 4 post-infection in all groups of mice infected with *P. berghei* NK65 intraperitoneally and treated with indicated doses of ethanolic extract of *A. scortechinii* compared with non-treated controls. Day 0 corresponds to the day of *P. berghei* infection.

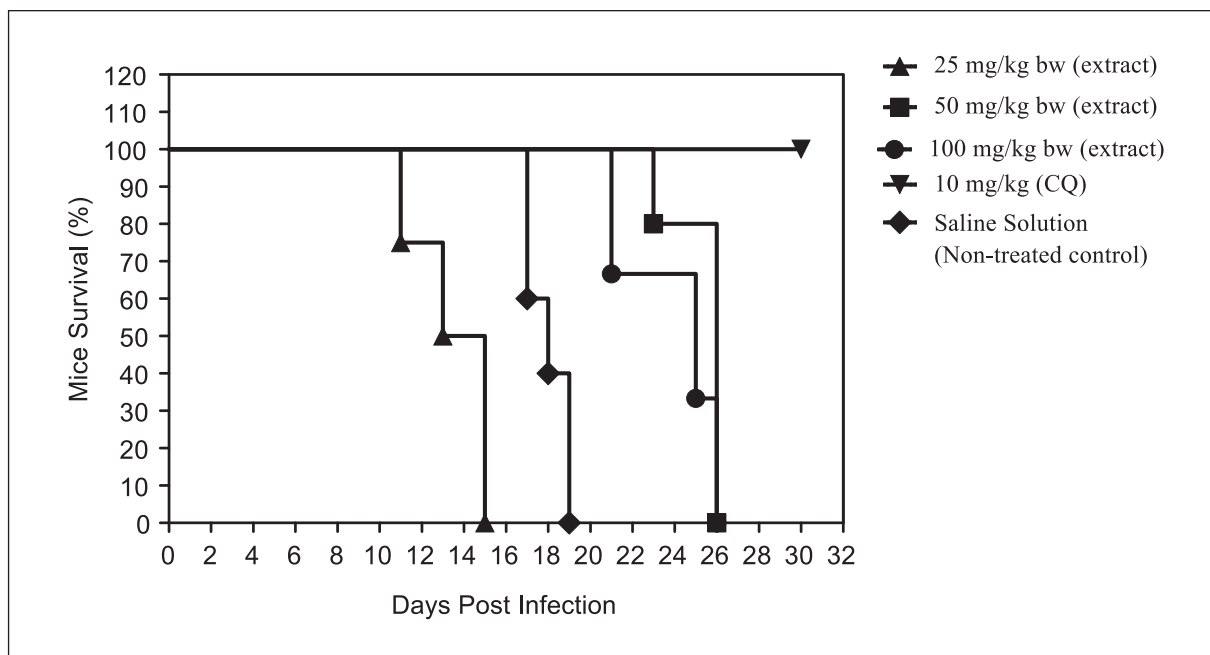


Fig. 3. Survival of mice after infection with *P. berghei* NK65 and administered with ethanolic extract of *A. scortechinii* intraperitoneally at different dosages (25, 50 and 100 mg/kg bw). Chloroquine diphosphate (CQ) (10 mg/kg bw) and 0.9% saline solution (0.2 mL) were used as positive control and negative control respectively.

DISCUSSION

Here we re-evaluated crude ethanolic extract of *A. scortechinii* (synonym *A. elasticus*) for anti-plasmodial activity *in vitro* and showed similar moderate inhibition against chloroquine-sensitive *P. falciparum* 3D7 as previously reported by Mustapha *et al.* (2010). Consequent *in vivo* assessment of the ethanolic extract provided for the first time direct evidence that *A. scortechinii* was capable of suppressing chloroquine-sensitive *P. berghei* NK65 parasitaemia development in a murine model of malarial infection. As an extension to the investigation using crude extract from *A. scortechinii*, our study revealed that all fractions prepared from ethanolic extract displayed moderate to good anti-plasmodial activities implicating the presence of potential anti-plasmodial lead compounds in the fractions (Rasoanaiva *et al.*, 2004). Of these fractions, fraction C was selected for purification and isolation of bioactive compounds based on strong inhibitory activity toward parasite and more efficient chromatographic separation. A previous report on anti-plasmodial activity of *A. elasticus* attributed parasite inhibition to several isolated compounds; artoindonesianin Z-5, gemichalcone B, dihydromorin, artocarpesin, cycloartocarpesin, artonin E, 12-hydroxyartonin E, cycloartocarpin and artobiloxanthone (Mustapha *et al.*, 2010). Our study here revealed the presence of engeletin identified via spectroscopic analysis in

fraction C derived from *A. scortechinii* ethanolic extract. Engeletin or 5,7,4'-trihydroxyflavanone-3-O- α -L-rhamnopyranoside is a flavonoid compound isolated from many plants of importance in traditional medicine (McIntyre *et al.* 1989; Huang *et al.*, 2011; Wungsintaweekul *et al.*, 2011; Chen *et al.*, 2011; Ruangnoo *et al.*, 2012;) with reported bioactivities including anti-inflammatory properties (Huang *et al.*, 2011; Ruangnoo *et al.*, 2012), antioxidant and anti-HIV-1 integrase activities (Itharath *et al.*, 2012), estrogenic and anti-estrogenic effects (Wungsintaweekul *et al.*, 2011), a role in the prevention of immunological hepatocyte damage and anti-hyperuricemic and nephroprotective effects (Chen *et al.*, 2011). However, anti-malarial activity of engeletin has not been documented before. The relatively low selectivity indices (SI<10) for engeletin and the ethanolic extract the compound was derived from, was contrary to that earlier reported for the extract by Tanamatayarat *et al.* (2000). It is however interesting to note that fraction C itself exhibited better selectivity than engeletin or the crude extract implicating presence of other potential anti-malarial compounds in the fraction. *A. scortechinii* and plants from the *Artocarpus* sp. are rich in flavonoids, terpenoids, stilbenoids and arylbenzofurans (Hakim *et al.*, 2006) contributing toward a myriad of pharmacological effects, such as anti-bacterial (Khan *et al.*, 2003), anti-platelet (Weng *et al.*, 2006), anti-fungal (Jayasinghe *et al.*, 2004) and cytotoxic (Ko *et al.*, 2005 & Shah *et*

al., 2006) actions. *Artocarpus* sp. compounds already identified with anti-plasmodial activity include artocapones A and B, artonin A, cycloheterophyllin, artoindonesianin E, artoindonesianin R, heterophyllin, heteroflavanone C and artoindonesianin A-2 (Jagtap & Bapat, 2010). Therefore in addition to engeletin isolated in this study from *A. scortechinii*, other bioactive lead compounds remain to be identified in active fractions.

CONCLUSION

In essence, our findings provide *in vivo* evidence for the anti-malarial potential of *Artocarpus* genus in general and *A. scortechinii* in particular. Further isolation will be carried out to explore more potential anti-malarial leads.

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