Evaluation of antimalarial and toxicological activities of methanol and water leaves extracts of 

*Piper sarmentosum*

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**Abstract**

The discovery of new antimalarial drugs from medicinal plants is urgently needed due to the development of multidrug-resistant *Plasmodium falciparum*. Therefore, *Piper sarmentosum* (kaduk), a commonly used as a herbal medicine to treat malarial symptoms was screened for antimalarial as well as toxicological activities of their methanol and water leaves extracts. The inhibitory concentration (IC<sub>50</sub>) of *P. sarmentosum* methanol and water extracts against a chloroquine-sensitive strain (3D7) of *P. falciparum* was determined by using a malarial SYBR Green I-based fluorescence (MSF) assay. The lethal concentration (LC<sub>50</sub>) of the plant extracts was evaluated by using a brine shrimp lethality test (BSLT). The presence of heavy metal contents in the crude extract was also detected by using an atomic absorption spectrometry (AAS). Both methanol and water extracts showed an inactive antimalarial activity with an IC<sub>50</sub> value of 138.8 ± 0.122 µg/mL and 229.7 ± 0.125 µg/mL, respectively. The water extract was considered non-toxic (LC<sub>50</sub> = 2741.7 ± 3.16 ppm), while the methanol extract was toxic (LC<sub>50</sub> = 894.94 ± 0.018 ppm). The heavy metals such as plumbum (Pb), zinc (Zn), cadmium (Cd) and chromium (Cr) were identified in the crude extract but they were below the safety limits recommended except for arsenic (As). Further investigations are required to determine the toxicological profiles of *P. sarmentosum* extracts on mammalian models.

**Keywords:** antimalarial activity; *Piper sarmentosum*; *Plasmodium falciparum*; toxicological activity.


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Introduction

Malaria is mainly caused by *P. falciparum* that is transmitted to humans by female *Anopheles* mosquitoes. Despite significant declines of malaria cases globally, the disease continues to cause morbidity and mortality in tropical and subtropical countries [1]. The widely use of chloroquine to treat infected patients has led to the appearance of chloroquine-resistant strains of *P. falciparum* [2]. The occurrence of *P. falciparum* resistance has extended to other drugs such as sulfadoxine-pyrimethamine and artemether-lumefantrine [2]. This situation has heralded an urgent need for alternative drugs and therapies.

Malaysia is well-known with its tropical forest and vegetation that contain a huge source of herbal medicines. Many of herbal medicines are traditionally used by Malaysians for the treatment of various illnesses such as malaria [3]. Ethnobotanical surveys on plants in Malaysia that have been claimed to possess an antimalarial activity are readily available in the literature [5,6,7]. A survey of the Malay ethno-medic botany in Machang, Kelantan, Malaysia, has also identified several species of plants such as *Piper sarmentosum* or locally known as ‘kaduk’ used by the villagers to treat malaria fevers [4].

*P. sarmentosum* is a betel leaf-like plant that grows well on a damp soil in secondary-growth forests in tropical and subtropical regions of the world. It has an aromatic odor and a pungent taste [5]. Extracts of this plant have shown to have an antitumor [9], an insecticidal [10], an anti-allergic, an anti-inflammatory [11] and antitumoral [12] and an antitoxoplasmosis activity [13]. The evidence of the antimalarial activity of *P. sarmentosum* against *P. falciparum in vitro* was also demonstrated decades ago by using a conventional microscopic evaluation of Giemsa-stained thin blood films [14]. However, a very limited literature on its toxic effects has been reported.

Although there are highly sensitive antimalarial activity assays available today, the time and effort necessary to test a single microtiter plate remains substantial. The malaria SYBR Green I-based fluorescence (MSF) assay has been widely used for antimalarial drug susceptibility testing in *P. falciparum* cultures [15]. The method is accurate, reliable, requires less sophisticated equipment and involves a single processing step than that of the radioactive substrate incorporation assay or the colorimetric enzyme-linked immunosorbent assay (ELISA) [16,17].

It is therefore of interest to reexamine the antimalarial activity of *P. sarmentosum* leaves extracts against *P. falciparum in vitro* by using a MSF assay. These extracts were also studied in regard to their toxicity by using a brine shrimp lethality test (BSLT) and an atomic absorption spectroscopy (AAS) analysis to detect heavy metals. These toxicity tests are considered a useful tool for preliminary assessment of toxicity of the leaves extracts.

Materials and Methods

A. Collection and extraction of plant leaves

Leaves of *P. sarmentosum* were collected from Pasir Puteh, Kelantan, Malaysia and authenticated based on its physical appearances and a typical pungent taste [8]. Leaves samples (460 g) were washed, cut into small pieces and allowed to dry in an oven at 55°C for 10 hours [16]. Dried leaves were grounded into powder and powdered samples (100 g) were macerated in 95% methanol and water, respectively at a ratio of 1:10 (w/v) and constantly shaken at room temperature for overnight. Liquid extracts were separated from solid residues by using Whatman filter papers and concentrated by using a rotary evaporator (Heidolph Rotary Evaporator, Laborota 4000 series, Schwabach, Germany) at 45°C for 30 minutes. Concentrated extracts were allowed to dry in a fume hood for several days until the liquid was completely dried. Dried extracts were weighted three times to obtain a constant weight and kept in sterile airtight containers at 4°C until being used.

B. *In vitro* culture and maintenance of *P. falciparum*

A chloroquine-sensitive strain (3D7) of *P. falciparum* obtained from Institute for Research in Molecular Medicine (INFORMM) was maintained in culture flasks containing RPMI 1640 (GIBCO BRL, Invitrogen) supplemented with GlutaMAX I, 25 mM HEPES, 0.25% Albumax, 0.21 mM hypoxanthine (Sigma), 45% glucose (Sigma) and 50 mg/mL gentamicin (Duopharma) [9]. Human blood and serum were acquired from informed consent healthy donors recruited at School of Health Sciences, Universiti Sains Malaysia (USM). The nature and risks of the study approved by the Human Research Ethics Committee USM (USM/EPEM/15060227) were notified before the blood recruitment. Culture flasks in a candle jar with a lighted-up candle were incubated at 37°C. Synchronisation of ring stage parasites was performed by suspending a parasite pellet in a 10 cell pellet volume of 5% D-sorbitol (Sigma) for 10 minutes at room temperature [15]. After centrifugation (300 × g, 5 minutes), a pellet containing synchronised ring stage parasites was resuspended in RPMI medium and incubated at 37°C for at least 2 hours.

C. *In vitro* antimalarial test

For the antimalarial activity assay, a 96-well plate containing triplicates of two-fold serial dilutions of methanol and water extracts (0-2500 μg/mL), respectively was prepared before being added into respective wells in a plate containing synchronised cultures of ring stage parasites (2% hematocrit, 1% parasitemia) [10]. Solutions of the plant extracts and artemisinin, a highly potent antimalarial drug (used as a reference drug) were prepared in RPMI medium so that the final solvent concentration was less than 0.1%. Currently, artemisinin has been used as a first-line treatment of *P. falciparum* instead of chloroquine, which has developed resistance to most *P. falciparum* strains.

Parasite plates in a candle jar were incubated at 37°C for 48 hours. At the end of the incubation, each sample (90 μL) was incubated with 20 μL of 20× SBYR Green I solution for 30 minutes and the fluorescence signal was measured by using excitation and emission wavelengths of 485 and 530 nm, respectively by a microplate reader (Bio-Rad, USA). The 50% inhibition concentration (IC₅₀) of the tested samples was obtained by plotting a
log sample concentration-response curve by using a GraphPad Prism Software (Version 6.0.1).

D. In vitro toxicological test
i. Brine shrimp lethality test (BSLT)
For the toxicological activity assay, brine shrimp (Artemia salina) eggs (0.25 g) were hatched in well-aerated flasks containing 250 mL of 3.8% artificial seawater at room temperature for 48 hours [20]. Ten brine shrimp larvae (nauplii) were drawn by using a glass capillary and placed in petri dishes containing 5 mL of artificial seawater. Solutions of the methanol and water leaves extracts were prepared in artificial seawater so that the final solvent concentration did not exceed 0.05%. The extracts of various concentrations (0-1000 µg/mL) were added into larvae-containing petri dishes in triplicates, which were incubated at room temperature under the light for 24 hours. At the end of the incubation, samples were examined by using a magnifying glass and the number of dead and live nauplii was recorded. The 50% lethality concentration (LC$_{50}$) of the leaves extracts was obtained by plotting an extract concentration-response curve by using GraphPad Prism Software (Version 6.0.1).

ii. Heavy metal test
For the heavy metal detection, the leaves material (powder) and blank were digested using a dry ashing method [21]. The plant material (0.5 g) was placed in a porcelain crucible and dried to ash using a muffle furnace at 550°C for overnight. The resultant ash and deionised water (used as blank) were dissolved in nitric acid (5 mL) and hydrogen peroxide (5 mL). It was placed on a hot plate to gently heat the mixture. A distilled water (5 mL) was added and heated again until a colourless solution was obtained. The digested mixture was diluted to 50 mL of distilled water in a volumetric flask and filtered using filter paper. A stock solution of plumbum (Pb) (1000 ppm, 1 mL), cadmium (Cd) (1000 ppm, 0.1 mL), zinc (Zn) (1000 ppm, 0.05 mL), chromium (Cr) (1000 ppm, 0.25 mL) and arsenic (As) (1000 ppm, 5 mL) was pipetted into a 50 mL volumetric flask, respectively. Deionised water was added to the mark. Five serial dilutions were performed by pipetting 25 mL of the initial standard solution into another volumetric flask. Each of the standard solutions was analysed by an AAS (Model Perkin Elmer A Analyst 800, USA) and the standard calibration curve of each element was obtained. The concentration of Cd, Cr, As, Pb and Zn in the digested sample and blank was analysed in triplicates.

Results

A. Antimalarial activity of methanol and water extracts of P. sarmentosum
Figure 1A shows the antimalarial activity of methanol and water extracts of P. sarmentosum. The methanol and water extracts inhibited the parasite growth with an IC$_{50}$ value of 138.8 ± 0.1 µg/mL and 229.7 ± 0.1 µg/mL, respectively. A control drug artemisinin exhibited an IC$_{50}$ value of 4.0 ± 0.22 ng/mL (Figure 1B).

B. The toxicological activity of methanol and water extracts of P. sarmentosum
The toxicological activity of methanol and water extracts of P. sarmentosum is shown in Figure 2. The LC$_{50}$ values for methanol and water extracts were 894.9 ± 0.02 ppm and 2741.7 ± 0.02 ppm (>1000 ppm), respectively.

A dried leaves extract of P. sarmentosum was screened for the presence of heavy metals. The mean concentration of heavy metals such as Pb, Zn, As, Cd and Cr present in the crude extract is presented in Figure 3. Arsenic (As) was present with the highest concentration (0.94 ± 0.072 mg/L) in the crude extract followed by Zn (0.87 ± 0.019 mg/L), Cr (0.17 ± 0.000 mg/L), Pb (0.02 ± 0.003 mg/L) and Cd (0.003 ± 0.019 mg/L).
The percentage mortality of brine shrimps. The previous study also reported -50%, in which 50% of interest due to its, the most associated of the parasite to evade from various chemotherapies. The cytotoxic activity of P. sarmentosum methanol and water extracts was classified to have a highly active, a promising, a moderately active, and an inactive or undefined antimalarial activity, respectively. In the present study, the P. sarmentosum methanol and water extracts show an inactive activity against 3D7 parasites in contrast with the previous results that only emphasized on qualitative evaluation using Giemsa-stained thin blood films of the parasites. The previous study also reported an inactive activity of the methanol extract against the malaria parasite (FCR-3 strain) (IC<sub>50</sub> = 156 µg/mL). Although P. sarmentosum has frequently been used as a folk medicine to treat fever that is generally associated with malarial symptoms, but based on these results, this plant might probably act as an antipyretic agent or enhance the immune system due its properties to reduce inflammatory rather than having a direct antimalarial effect against the parasites. As expected, a control drug artemisinin exhibited a highly active activity and this result is similar to that reported previously for the same 3D7 strain.

P. sarmentosum has become of interest due to its medicinal values; however limited literature is available on the toxicity of this plant. Brine shrimp lethality test (BSLT), a rapid assessment of the toxic potential of plant extracts, was used in this present study. The test provides preliminary toxicological data for further experiments on mammalian animal models. The P. sarmentosum water extract was non-toxic to brine shrimps after 24 hours of exposure, while the methanol extract is shown to be toxic according to the Meyer’s toxicity index (LC<sub>50</sub> > 1000 ppm are considered as non-toxic). The toxicity of the methanol extract might be originated from contaminants such as high concentrations of heavy metals or from chemical compounds of the plant. A previous study suggested that the cytotoxic activity of Piper species was due to the presence of secondary metabolites such as piper alkaloids, piplartine compounds. Besides, another important factor for the toxicity is the solvent used in this assay, in which some organic compounds have been reported to enhance the toxic activity.

Since antimalarial drugs are mostly derived from plants, therefore many studies have been conducted to screen Malaysian medicinal plants such as P. sarmentosum to investigate their antimalarial activity. However, only one preliminary in vitro study was reported so far on the antimalarial activity of P. sarmentosum methanol and chloroform extracts against a chloroquine-sensitive (FCR-3) strain of P. falciparum. In the present study, a preliminary investigation on the antimalarial activity of P. sarmentosum methanol and water extracts was conducted against a chloroquine-sensitive strain (3D7) of P. falciparum by using a malaria SYBR Green I-based fluorescence (MSF) assay. The binding of SYBR Green I is specific for malarial DNA in the parasite development, making the MSF technique is more accurate for measuring the parasitemia of treated and untreated samples compared to the conventional microscopic method. Furthermore, this one-step technique is less expensive than the other methods that rely on radioisotopes and multistep procedures such as the radioactive substrate incorporation assay and the colorimetric enzyme-linked immunosorbent assay (ELISA).

According to Lekana-Douki et al., plant extracts showing an IC<sub>50</sub> value of ≤ 5 µg/mL, < 5 µg/mL and > 15 µg/mL, ≥ 10 µg/mL and ≤ 50 µg/mL, and > 50 µg/mL are classified to have a highly active, a promising, a moderately active, and an inactive or undefined antimalarial activity, respectively. In the present study, the P. sarmentosum methanol and water extracts show an inactive activity against 3D7 parasites in contrast with the previous results that only emphasized on qualitative evaluation using Giemsa-stained thin blood films of the parasites. The previous study also reported an inactive activity of the methanol extract against the malaria parasite (FCR-3 strain) (IC<sub>50</sub> = 156 µg/mL). Although P. sarmentosum has frequently been used as a folk medicine to treat fever that is generally associated with malarial symptoms, but based on these results, this plant might probably act as an antipyretic agent or enhance the immune system due its properties to reduce inflammatory rather than having a direct antimalarial effect against the parasites. As expected, a control drug artemisinin exhibited a highly active activity and this result is similar to that reported previously for the same 3D7 strain.

**Figure 2:** The percentage mortality of brine shrimps treated with various concentrations (0-1000 µg/mL) of methanol and water extracts of P. sarmentosum for 24 hours was plotted to estimate LC<sub>50</sub> values from three different occasions done in triplicates.

**Figure 3:** The mean concentration of heavy metals in the dried crude extract of P. sarmentosum from three different experiments done triplicates.

**Discussion**

Plants have constituted the basis of herbal medicines and been a good source of active compounds for drug development. Decades ago, quinine isolated from the Cinchona bark was active in killing malaria parasites and used as a template for the synthesis of commercial drugs such as chloroquine. Currently, artemisinin isolated from Artemisia annua, is currently used as an antimalarial drug replacing the less potent chloroquine. However, the resistance of P. falciparum, the most virulent malaria parasite to various antimalarial chemotherapy including artemisinin, which is currently the front-line treatment, has increased due to the capability of the parasite to evade from various antimalarial actions. Hence, new potent antimalarial agents are urgently needed especially from natural products such as plant.
solvents have high cytotoxicity in vivo. However, the final concentration of methanol solvent used in the present test did not exceed 0.05% and did not kill the brine shrimps, and therefore the solvent does not affect the toxicity of the result obtained.

The *P. sarmentosum* crude extract was further examined for the presence of Pb, Zn, Cd, Cr and As. These heavy metals are most commonly the subject of attention in manufacturing dietary supplements or herbal ingredients since health risks are usually associated with these heavy metals. National and international regulations on food quality have thus set up the maximum permissible levels of toxic heavy metals in human foods and products. Based on the WHO/FAO permissible level, the safe limits of Pb, Zn, Cd, Cr and As are 5.00 mg/L, 2.0 mg/L, 0.01 mg/L, 1.2 mg/L and 0.1 mg/L, respectively. In the present study, the concentrations of Pb, Zn, Cd and Cr present in the *P. sarmentosum* crude extract were below the safety limits recommended by WHO/FAO except for As. As is a metalloid element that occurs in a mineral-bound form in the earth’s crust. As and other heavy metals are neither created nor destroyed, but are simply redistributed such that some soils might contain higher amount of As either due to natural processes such as volcanic activity, weathering of minerals, and burning of coal or to pollution factors. As and other heavy metals can be absorbed into many plants as they grow. Because higher concentration of As can either be toxic, carcinogenic or have adverse reproductive effect, the toxicity of the methanol extract against the brine shrimps might probably be due to the As toxicity.

**Conclusion**

This study provided a basic information on the antimalarial and toxicological activities of *P. sarmentosum* methanol and water leaves extracts. Both water and methanol extracts were showed an inactive antimalarial activity against the malaria parasite. However, water extract was non-toxic while methanol extract was toxic. The crude leaves extract of the plant was detected with heavy metals (Pb, Zn, Cd and Cr), but below the maximum safe limits except for As. Further investigations are required to determine the toxicological profiles of *P. sarmentosum* extracts on mammalian models.

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