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In vitro Anti-Plasmodial Activity of Aqueous and Ethanolic Extracts of *Moringa oleifera* and *Phyllanthus amarus*

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ABSTRACT

Many herbal medicinal products contribute to the treatment of malaria in endemic areas. In Ghana, there is documented evidence of the use of several plant species in the management of both infectious and non-infectious diseases. This study sought to validate the activity of extracts from two such plants, *Phyllanthus amarus* and *Moringa oleifera*, against *Plasmodium falciparum*. Anti-plasmodial activities of aqueous and ethanolic extracts of *M. oleifera* (whole plant and twig) and *P. amarus* (whole plant and stem) were assessed against the 3D7 laboratory strain of *P. falciparum*. Extracts were evaluated *in vitro* at concentrations of 12.5, 25, 50, 100 $\mu g\ mL^{-1}$ and the level of potency in each case was expressed as the concentration of the extract that inhibited 50% of the parasites (IC_{50}) relative to negative controls (100% parasitaemia). Artesunate was used as a positive standard in all assays. All extracts showed significant inhibition of parasite growth, with (IC_0) less than 50 $\mu g\ mL^{-1}$, except the aqueous extract of the whole plant of *P. amarus* which showed a relatively high IC_{50} of 115.43 $\mu g\ mL^{-1}$. Interestingly, increasing concentration of ethanolic extract of combined twig and leaves of *Moringa oleifera* reduced inhibition of the parasite growth while a decreasing extract concentration resulted in increased parasite inhibition. The extracts of *M. oleifera* and *P. amarus* demonstrated potential anti-plasmodial activity which can be explored in malaria therapy. It is of interest to identify, isolate and characterize the active anti-plasmodial bioactive compounds from the plants.

Key words: Plant extracts, *Plasmodium falciparum*, parasitaemia, IC_{50}, percent inhibition

INTRODUCTION

Malaria is one of the most important infectious diseases that afflict vulnerable groups such as pregnant women and children under five years in the developing world (WHO., 2012). The disease is characterized by fever, headache, chills, tiredness, nausea and general malaise. Disease

complications such as anaemia, convulsion and cerebral malaria could arise in severe cases. Malaria is caused by protozoan parasites of the genus *Plasmodium*, with five species known to cause disease in humans (*Falciparum*, *Vivax*, *Ovale*, *Malariae* and *Knowlesi*). Malaria due to *Plasmodium falciparum* is the most deadly and predominates in Africa. In 2012 there were about 207 million malaria cases across the globe with an estimated 627,000 deaths. According to World Health Organization (WHO., 2012) in 2013, Africa was the most affected continent, accounting for about 90% of all deaths worldwide.

Malaria affects productivity and economic development. Global malaria control efforts have employed a myriad of strategies, including the prophylactic and therapeutic use of anti-malaria drugs. These control measures are however hampered by the development of resistant strains of the disease vectors and parasites, respectively. There are reported cases of *P. falciparum* resistance to artemisinin based combination therapies (ACT's), which are WHO's recommended first line drugs for malaria treatment in South East Asia (Noedl *et al*., 2009). These resistance problems underlie the need to continually identify new, low cost anti-malarial drugs in order to sustain the gains made in the fight against malaria.

Local plants preparations contribute to the traditional treatment of malaria in most malaria-endemic areas. In many places in Ghana, herbal medicines play an important role in the treatment of malaria and many other diseases due to their perceived less side-effects, cost-effectiveness and availability (Asase *et al*., 2005; Sam *et al*., 2011; Mensah and Gyasi, 2012). *Phyllanthus amarus* and *Moringa oleifera* are two medicinal plants that have been used for many years (Divya *et al*., 2011; Adebayo and Krettli, 2011; Caceres *et al*., 1992) for the treatment of malaria.

Phyllanthus amarus, a perennial herb widely used in Ayuverdic medicines, belongs to the family Euphorbiaceae. It grows in moist, shady and sunny places and is widely distributed in the tropics (Ajala *et al*., 2011). Formulated capsules of both the aqueous and ethanol extracts of the plant have demonstrated chemotherapeutic effects on *Plasmodium yoelii* in Swiss albino rats with results comparable to chloroquine (Ajala *et al*., 2011). *Moringa oleifera* Lam is an important plant source for bioactive compounds and is used by in countries around the world because of its multipurpose medicinal and nutritional properties. Extracts from the plant are believed to have antitumor, antipyretic, antiepileptic, antioxidant, antibacterial, antifungal, diuretic and antihypertensive activities (Caceres *et al*., 1992; Nikkon *et al*., 2003; Morton, 1991; Dahot, 1988). Extracts of *M. oleifera* seeds have shown marked potential against the larvae, pupae and adult insect stages of mosquitoes (Leite *et al*., 2005; Prabhu *et al*., 2011; Ravindran *et al*., 2012).

Extracts of *P. amarus* and *M. oleifera* have been shown to contain phytochemicals such as glycosides, flavonoids, steroids, terpenoids, saponins, tannins and anthraquinones (Sinha, 2012; Anwar *et al*., 2007). The potency of different solvent extracts of various parts of these plants against *P. falciparum* has not been comprehensively investigated. The aim of this study was to assess the *in vitro* activity of aqueous and ethanolic extracts of *P. amarus* and *M. oleifera* against the *P. falciparum* parasite.

MATERIALS AND METHODS

Collection of plant materials: Plant parts of *Phyllanthus amarus* and *Moringa oleifera* were collected from different areas in Navrongoin the Upper East Region of Ghana between September and October 2013. The plants were first identified in the field using standard keys and descriptions (Dalziel, 1956; Keay, 1989). The botanical identities were further confirmed and authenticated by Dr. Isaac Sackey of the Department of Applied Biology, University for Development Studies.

Extraction of plant material: Samples of *P. amarus* were thoroughly washed with distilled water and air-dried for two weeks. Using a mortar and pestle, different parts of the plant (leaves/fruits, stem/roots or whole plant) were ground to fine powder (passed through a 50 mesh sieve). Aqueous extracts of the different parts were separately prepared by soaking 200 g of each ground sample in 2 L distilled water for 48 h. The resulting mixtures were filtered using Whatman No. 1 filter paper and freeze dried in the laboratories of the Centre for Scientific Research into Plant Medicine at Akuapim Mampong in the Eastern Region of Ghana. Ethanolic extracts of the whole plant was prepared by soaking 200 g of the powdered plant sample in 1 L of ethanol for 48 h. The resulting mixture was filtered and concentrated by means of a rotary evaporator at 30°C to produce a gel-like extract that was stored at -20°C until use.

The leaves and twigs of *M. oleifera* were dried and processed separately into powdered form as described above. Powdered samples (150 g) of each plant part were weighed, macerated in 0.4 L distilled water in separate conical flasks and left to stand for 72 h with occasional shaking. The mixtures were filtered through Whatman No. 1 filter paper and the filtrates were concentrated to dryness using a rotary evaporator. Dried samples were stored in the refrigerator at -20°C until use. Ethanolic extracts were obtained starting with 150 g each of the powdered samples macerated in 400 mL absolute ethanol and allowed to stand for 72 h. The extracts were filtered and the resulting filtrates were concentrated using a rotary evaporator at temperatures up to a maximum of 40°C. The concentrated extract was stored at -20°C until use.

Culturing of *Plasmodium falciparum* parasites: The 3D7 strain of *P. falciparum* was kept in culture with complete parasite medium, CPM (RPMI 1640 supplemented with 10% Albumax II, 50 μg mL^{-1} gentamycin, 1% L-glutamine). Parasites were cultured with O^+ RBCs at 4% haematocrit. For periodic maintenance, parasitaemia was checked by Giemsa staining and the culture replenished by addition of an appropriate amount of fresh O^+ RBCs, flushed with a gentle flow of mixed gas (5.5% CO_2, 2.5% O_2 in N_2) for at least 30 sec and kept in a 37°C incubator.

Sorbitol synchronization of the parasites: Sorbitol treatment was used to enrich the ring stages of the parasite for assay purposes and was performed when most of the parasites were at the ring stage. The culture was transferred into a 15 mL falcon tube and centrifuged for 10 min at 350×g after which the medium (supernatant) was removed. To the pellet was added 5 mL of a 5% sorbitol solution with gentle shaking and the mixture allowed to stand for 10 min at 37°C. The mixture was subsequently spun at 350×g for 10 min and the supernatant removed. The pellet was then washed twice with parasite wash medium (RPMI 1640 with 50 μg mL^{-1} gentamycin, 1% L-glutamine) and finally with CPM. A thin smear was later prepared to check for the effectiveness of the synchronization procedure. The pellet was used to inoculate a T-25 culture flask at 4% haematocrit, gassed for about 30 sec and kept in an incubator at 37°C for at least one schizogonic cycle before use in an assay.

***In vitro* anti-plasmodial assay:** The assays were performed in triplicate for each plant extract. A stock solution of 100 mg mL^{-1} of each extract was prepared in Phosphate Buffered Saline (PBS) and sterilized by filtration through a 0.22 μm membrane (Millipore). The filtered extracts were diluted to 1000 μg mL^{-1} in CPM and this was serially diluted to finally yield four concentrations of extracts for the assays (1000, 500, 250 and 125 μg mL^{-1}). These dilutions were added in duplicate to a 48-well tissue culture plate containing *P. falciparum* ring stages at 0.7% parasitaemia and at

3% haematocrit. The final concentrations of extracts were 100, 50, 25 and 12.5 μg mL^{-1} in the respective wells (50 μL of extract plus 450 μL of culture). Negative control wells had parasites without any extract while positive controls were made with artesunate at concentrations of 400, 200, 100 and 50 ng mL^{-1}, respectively.

After 48 h of incubation at 37°C, the contents of the wells were harvested and a thin blood film prepared for parasitaemia estimation. The films were fixed with methanol, stained for 10 min in 10% Giemsa and viewed under a light microscope after washing and drying. The percentage parasite inhibition was calculated using the formula:

$$\text{Inhibition (\%)} = \frac{\text{Mean control parasitaemia} - \text{Mean test parasitaemia}}{\text{Mean control parasitaemia}} \times 100$$

Data analysis: The anti-plasmodial activities of the extracts were expressed by the concentrations of the drug that inhibited 50% parasites (IC_{50}) relative to the negative control. These IC_{50} values were calculated by plotting the inhibition (%) against the logarithm to the base 10 of the concentrations of the extracts (log_{10} concentration) using Microsoft Excel. The antiplasmodial activity was analyzed as described by Inbaneson *et al*. (2012). The paired t-test was used to analyze the statistical significance of the results obtained from the ethanolic extract while ANOVA was used to analyze results obtained from the different aqueous extracts, followed by Dunnett *post-hoc* tests where necessary. Statistical analyses were performed using the MINITAB® 14 statistical software (Minitab Inc.) and differences were deemed significant when p values were less than 0.05.

RESULTS AND DISCUSSION

The *in vitro* anti-plasmodial activities of eight different extracts from *M. oleifera* and *P. amarus* were assessed against the chloroquine-sensitive (CQS) 3D7 strain of *P. falciparum* together with the standard anti-malarial drug, artesunate. Selection of plant parts for use in this study paralleled parts that are included in supposedly efficacious traditional herbal preparations. Table 1-3 present

Table 1: Effect of various concentrations of *Phyllanthus amarus* extracts on parasitaemia

Plant extracts and concentration of extracts (μg mL^{-1})	Mean parasitaemia (%)	Inhibition (Mean parasitaemia %)
Aqueous whole plant		
12.5	1.20±0.16	37.31
25	1.21±0.02	36.21
50	1.18±0.42	38.58
100	0.88±0.24	53.38
Aqueous stem with root		
12.5	1.27±0.06	32.94
25	0.50±0.02	68.17
50	0.79±0.18	59.15
100	0.55±0.04	70.61
Aqueous leaves		
12.5	0.98±0.26	48.66
25	0.80±0.08	58.83
50	0.72±0.02	62.33
100	0.45±0.01	75.92
Ethanol whole plant		
12.5	0.91±0.03	52.64
25	0.68±0.02	63.88
50	0.75±0.04	60.47
100	0.38±0.08	80.09
Control (No. extract)	1.91±0.06	0.00

Results shown represent the mean parasitaemia produced at the different concentrations of the extracts at two separate experiments

Table 2: Effect of various concentrations of the ethanolic extracts of *Moringa oleifera* on parasitaemia

Plant extracts and concentration of extracts (μg mL^{-1})	Parasitaemia (%)	Inhibition (%)
Ethanolic whole leaves		
12.5	0.46±0.14	69.33
25	0.75±0.01	50.00
50	0.94±0.48	37.33
100	0.90±0.22	40.00
Ethanolic leaves		
12.5	0.76±0.09	49.33
25	0.77±0.04	48.67
50	1.16±0.15	22.67
100	1.32±0.01	12.00
Control (No. extract)		
0.00	1.50±0.13	0.00

Results shown represent the mean parasitaemia produced at the different concentrations of the extracts at two separate experiments

Table 3: Percentage parasitaemia and percentage inhibition measurements of aqueous extracts of *Moringa oleifera* at variable concentrations

Plant extracts and concentration of extracts (μg mL^{-1})	Parasitaemia (%)	Inhibition (%)
Aqueous whole leaves		
12.5	1.21±0.13	19.33
25.0	0.97±0.10	35.33
50.0	0.93±0.09	38.00
100	0.56±0.00	62.67
Aqueous leaves		
12.5	1.01±0.01	32.67
25	0.99±0.00	34.00
50	0.66±0.11	56.00
100	0.55±0.01	63.33
Control (No. extract)		
0.00	1.50±0.13	0.00

Results shown represent the mean parasitaemia produced at the different concentrations of the extracts at two separate experiments

Table 4: Minimal inhibition concentrations of the various plant extracts

Plant extracts	IC_{50} (μg mL^{-1})
Phyllanthus amarus	
Aqueous whole plant	115.43
Aqueous stem with roots	21.14
Aqueous leaves with fruits	13.96
Ethanol whole plant	10.10
Moringa oleifera	
Aqueous twig	63.36
Aqueous leaves	43.65
Ethanol twig	33.11
Ethanol leaves	15.13
Standard drug: Artesunate	8.75×10^{-3}

IC_{50} represent the concentrations of the extracts that inhibited fifty percent (50%) of parasites relative to the standard drug artesunate

the concentrations and the corresponding parasitaemia/inhibition relating to various extracts of the two plants. The IC_{50} values of the extracts against *P. falciparum* (3D7 strain) are presented in Table 4. It was revealed that the whole plant ethanolic extract of *P. amarus* showed the highest anti-plasmodial activity (IC_{50} = 10.10 μg mL^{-1}) while, the aqueous extract of the whole plant gave the lowest anti-plasmodial activity of IC_{50} = 115.43 μg mL^{-1}. Artesunate used as positive standard in the study gave an IC_{50} value of 8.75 ng mL^{-1} (data not shown).

The aqueous extract of the leaves with fruits of *P. amarus* gave an IC_{50} value of 13.96 μg mL^{-1}. The anti-plasmodial activity of the leaves of *P. amarus* has been reported by Inbaneson *et al*. (2012) against *P. falciparum*. Their results indicated that the ethanolic leaf extract showed an IC_{50} of 94.19 μg mL^{-1}. Here, the ethanolic extract of the whole plant of *P. amarus* showed an IC_{50} value

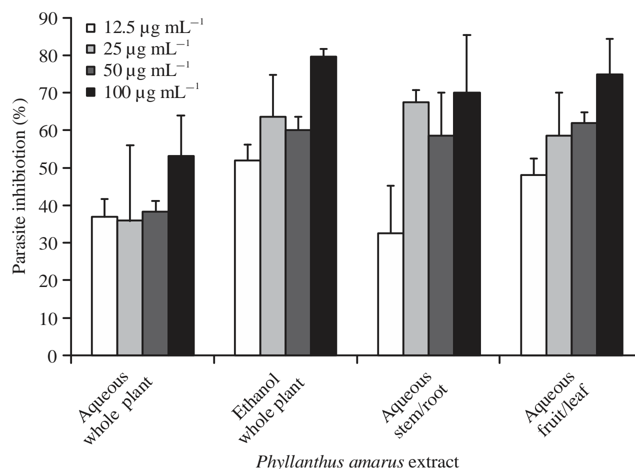

Fig. 1: Antiplasmodial effects of both aqueous and ethanolic extracts of *Phyllanthus amarus* on *Plasmodium falciparum* at the various concentrations and the parasitaemia induced. The data shown represent the average of three wells treated at the same day. The experiment was repeated 3 times and day-to-day variation was found to be within one fold of the presented data

of 10.10 µg mL^{-1} (Table 4). This value was also found to be less than the value of 11.2 µg mL^{-1} reported by Sha'a *et al*. (2011) in their study on the *in vitro* anti-plasmodial activity of extracts of *Vernonia amygdalina*, a plant of parallel medicinal use in Nigeria.

Anti-plasmodial activity of stem/root aqueous extract of *P. amarus* was not as high as the whole plant ethanolic extract (Fig. 1). The IC_{50} value (21.14 µg mL^{-1}) of the stem/root aqueous extract was found to be lower, thus was more active against *P. falciparum* relative to the root (IC_{50}>100 µg mL^{-1}) and stem (IC_{50} = 83.53 µg mL^{-1}) separately as reported by Inbaneson *et al*. (2012). This could be due to the combined effects of the phytochemicals present in the roots and those in the stem. The aqueous extract of the whole plant of *P. amarus* gave a very high IC_{50} value of 115.43 µg mL^{-1} and was therefore considered to have relatively lower levels of bioactive compounds with anti-plasmodial activity. However, results from *in vivo* studies by Ajala *et al*. (2011) indicated that the aqueous extract of the whole plant of *P. amarus* was effective against *P. falciparum* and had a chemotherapeutic effect similar to that of chloroquine. According to Dapper *et al*. (2007), *P. amarus* had significant antimalarial properties even though the effects were not sustained compared to effects of the combination of pyrimethamine and sulfadoxine. The differences in the level of anti-plasmodial activity exhibited by whole plant extracts might be due to the amount of the bioactive compounds extracted by the two different solvents. Since, different parts of the plant exhibited different levels of anti-plasmodial activity from the same solvent extracts, we speculate also that the phytochemicals responsible for the antimalarial activity were not evenly distributed in the plant in terms of concentration or variety.

The anti-plasmodial activities of the crude aqueous and ethanolic extracts of *Moringa oleifera* are represented in Fig. 2. The four extracts obtained from the different parts of *M. oleifera* showed inhibitory effects with their respective IC_{50} values of 15.18 µg mL^{-1} (leaf extract from ethanol), 33.11 µg mL^{-1} (twig extract from ethanol), 43.65 µg mL^{-1} (aqueous leaf extract) and 63. 36 µg mL^{-1} (aqueous twig extract) (Table 2 and 3). For the ethanolic whole leaf and leaf extract of *M. oleifera*, inhibition by the extracts was found to increase as concentration of the extracts decreased (Fig. 2).

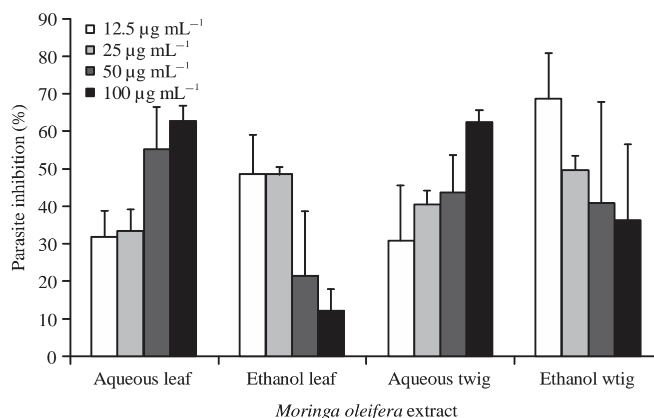

Fig. 2: Representative percentage inhibition of *Plasmodium falciparum* growth applying aqueous and ethanolic extracts of various plant parts of *Moringa oleifera*

In contrast, the aqueous extract indicated decrease in inhibition as concentration of extracts was decreased. It is thought that the ethanol extracted at least two different bioactive components and that whereas one seems to promote growth at high enough concentrations, the other causes inhibition even at concentrations where the former has no significant activity. This is an exciting area for further study. Ethanol at 95% used as negative control in the assays for the *M. oleifera* ethanolic extracts showed no significant effect on the growth of the parasite (data not shown).

CONCLUSION

All the extracts of the various parts of *P. amarus* and *M. oleifera* used in this study were found to possess some antimalarial activity, justifying the traditional use of the plant in treatment of malaria. The ethanolic extract of the whole plant of *Phyllanthus amarus* exhibited the highest antimalarial activity. In addition, the aqueous extract of the leaves and fruits gave better antimalarial activity *in vitro* than the aqueous extract of the whole plant as well as the stem and roots. The *Moringa oleifera* ethanolic whole leaf and the leaf extracts activity against the 3D7 strain showed that the inhibitory trend of extracts (whole leaf and leaves) was found to increase as concentration of the extracts decreased. Further study into this interesting finding is recommended.

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