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In vitro and *in vivo* Activity of *Mist Amen Fevermix* and *Edhec Malacure,* Polyherbal Antimalarial Products on Field Isolates of *Plasmodium falciparum and Plasmodium berghei*

Bernard K Turkson,[1*] Merlin L.K Mensah,[2] Isaac K Amponsah,[3] Abraham Y Mensah,[3] Emmanuel Achaab,[4] Richard Boateng Mensah,[4] Emmanuel Atakorah,[4] Ebenezer Ofori Attah,[5] Felix Zoiku[5]

ABSTRACT

Background: Malaria is a life-threatening infectious disease, which pose a public health challenge. Currently, reports of parasites resistance to artemisinin-based combination therapies is widespread. This has renewed calls for new antimalarial medications including herbal products for the treatment of malaria. This study was undertaken to scientifically assess the antiplasmodial potentials of *Mist Amen Fevermix* and *Edhec Malacure*, two polyherbal antimalarial products used for the management of uncomplicated malaria, and establish their phytochemical constituents.

Methods: Qualitative phytochemical screening based on standard analytical methods. The antiplasmodial activity was assessed *in vitro* by using *Plasmodium falciparum*. The SYBR® Green assay was used to measure parasite growth inhibition. *In vivo* activity was assessed with *Plasmodium berghei* parasites using the Rane's curative method with artesunate as positive control.

Results: The phytochemical screening of the products revealed the presence of alkaloids, flavonoids, tannin, steroid and saponin. In the *in vitro* studies, the IC_{50} values for *Edhec Malacure* was 70.89 ng/mL and 112.5ng/mL for *Mist Amen Fevrmix*. Artesunate exhibited an IC_{50} value of 1.571ng/mL. *Edhec Malacure* suppressed parasitemia by 76.17% (at 4.25mg/kg^{-1}) and *Mist Amen Fevermix* by 69.03% (at 1.56 mg/kg^{-1}) in vivo.

Conclusion: *Mist Amen Fevrmix* and *Edhec Malacure Mixture* demonstrated antiplasmodial activity and may be useful alternative antimalarial agents.

Keywords: antiplasmodial, alternative, antimalarial, phytochemical, uncomplicated malaria and parasites.

*Correspondence to:
Bernard K Turkson, Institute of Traditional and Alternative Medicine, University of Health and Allied Sciences, Ho, Ghana.
bentsi2000@yahoo.com, bkturkson@uhas.edu.gh

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INTRODUCTION

Herbal products have been used for the treatment of diseases since time immemorial, and plant based remedies have formed the major part of traditional medical systems. They have been relied upon to prevent illness, support, promote and maintain human health and are used in every country of the world (Li *et al*., 2009; Shi *et al*., 2010). The world is home to diversified herbal products most of which have been widely used and marketed as antimalarial products and for other diseases (Soelberg *et al*., 2015). Nevertheless, most of them have not been scientifically validated in terms of safety and efficacy.

The increasing resistance to conventional antimalarial agents in clinical use has renewed the need for new therapies to mitigate the threat to life from malaria. Alternative efficacious drugs are urgently required to counter the threat posed by the malaria parasite. The emergence of drug resistance is a serious threat to global malaria control and eradication efforts; thus warrants the introduction of potent new therapies against the malaria parasite (Muregi *et al*., 2007). This situation urshered in the era of artemisinin-based combination therapy (ACT) as first-line treatment for uncomplicated *falciparum* malaria in all endemic areas (WHO, 2015) to replace older antimalarial drugs due to resistance. However, recent evidence suggests that parasites are becoming resistant to the artemisinins in the Greater Mekong sub-region of Southeast Asia (Dondorp *et al*., 2009; Phyo *et al*., 2016; Pluijm *et al*., 2019). There is, therefore, the urgent need to carry out systematic investigations using modern scientific methods to establish the quality and

[1]Institute of Traditional and Alternative Medicine, University of Health and Allied Sciences, Ho, Ghana.
[2]Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[3]Department of Pharmacognosy, Faculty of Pharmacy and Pharmaceutical Sciences, Kwame Nkrumah University of Science and Technology, Kumasi, Ghana.
[4]Tafo Government Hospital, Ghana Health Service, Accra, Ghana.
[5]Noguchi Memorial Institute for Medical Research, University of Ghana, Accra.

efficacy of available alternatives products, such as *Mist Amen Fevrmix* and *Edhec Malacure*, herbal prodcuts used in Ghana for the treatment of uncomplicated malaria.

Mist Amen Fevrmix is a finished herbal product prepared from the stem barks of *Morinda lucida* Benth (Family: Rubiaceae) and *Parinari robusta* Oliv. (Family: Chrysobalanaceae). The product, a decoction on the recommended Essential Herbal Medicines List (EHML) of the Ministry of Health and used in the Herbal Medicine Units in Ghana. It is approved by the Food and Drugs Authority (FDA) for public use *Edhec Malacure* is a finished herbal product prepared from the stem bark of *Morinda lucida* Benth (Family: Rubiaceae), leaves of *Cleistopholis patens* Benth. Engl. and Diels (Family: Annonaceae), and stem bark of *Mangifera indica* Linn. (Family: Anacardiaceae); it is not on the EHML, however, it is approved by the FDA also for public use.

The present study aimed at validating the quality and efficacy of *Mist Amen Fevrmix* and *Edhec Malacure* as alternatives for the management of uncomplicated malaria.

MATERIALS AND METHODS (Drugs, chemicals, solvents)

Five bottles each of *Mist Amen Fevermix* (330 mL) and *Edhec Malacure* (500 mL) were procured from the Pharmacy Department, Tafo Government Hospital, Kumasi, Ghana. SYBR Green, artesunate and Chloroquine powder, 5% O_2, 5% CO_2 and 90% Nitrogen were obtained from the Department of Pharmacology, KNUST, Kumasi. The following solvents Methanol, 1% lead acetate, ammoniacal alcohol, 1% H_2SO_4, 20% NaOH, dilute NH_3, HCl, Fehling's solution A and B, Dragendorff's reagent, chloroform and ethanol, were obtained from the Department of Pharmacognosy, KNUST, Kumasi. RBC (O^-, Rhesus positive) Falcon and ACD tubes were obtained from the Tafo Government Hospital, Kumasi, Ghana.

Processing of Herbal Products and Preparation of Solutions

About 330mL of *Mist Amen Fevermix* and 500mL of *Edhec Malacure* were freeze-dried to powder form (3.1175 g and 2.6067 g) respectively. About 25 mg of weighed powders (*Mist Amen Fevermix* and *Edhec Malacure*) were transferred into 15 mL Falcon tubes containing 5 mL of 70% ethanol to yield a stock concentration of 3000 µg/mL. An initial concentration of the study products was prepared by transferring 1.7 mL each into 15 mL Falcon tubes. The initially prepared concentration was serially diluted 8 fold to obtain 1000 µg/mL, 500 µg/mL, 250 ug/mL, 125 µg/mL, 62.5 µg/mL, 31.3 µg/mL, 15.6, 7.8 µg/mL and 3.9 µg/mL.

Ethical Approval

The study was carried out after ethical approval from the Ethics Committee on Animal Work, Department of Pharmacology, KNUST, Kumasi, Ghana.

Parasite Sample Collection and Culturing.

In vitro susceptibility assays of *Mist Amen Fevermix* and *Edhec Malacure Mixture* were performed on *P. falciparum* field isolate obtained from study participants at the Tafo Government Hospital. About 2.5 mL of blood samples containing *P. falciparum* field isolatewere collected aseptically from a venous puncture using the vacutainer system from six patients into ACD tubes and then stored in the liquid nitrogen. The parasites were then cultured based on a method previously described by (Hout *et al*., 2006) with some modifications. Parasite vials obtained from a liquid nitrogen tank were appropriately thawed in a water bath at a temperature of 37°C. The vials (cell culture) were centrifuged at 2000 rpm for 10 minutes, and the resultant supernatant was discarded. An equal amount of a mixture of 3.5% NaCl in distilled water was added to each of the pellet, which was centrifuged at 2000 rpm for 10minutes. The pellets were gently disengaged and a 1 mL aliquot of complete parasite medium (5mL of L-glutamine, 2.5 mL of 10 mg/mL and 50 mL Albumax in 500 mL of RPMI 1640) was added and centrifuged again at 2000 rpm for 10 minutes (Jensen and Trager 1980). This procedure was duplicated, and the parasites were then suspended in 25mL BD Falcon tubes (culture flask) containing 200 µL freshly prepared pack of RBC (O^-, Rhesus positive) and 5mL of complete parasite medium to have a haematocrit of 4%. A 2% Oxygen, 5.5% Carbon dioxide and 92.5% Nitrogen was used to gas the culture for 30 seconds for 25 mL culture flask. The flasks were quickly closed and put into an incubator (RS Biotech) at a temperature of 37°C in 5 percent O_2, 5 percent CO_2 and 90 percent Nitrogen. Parasites were allowed to grow for 3 days before the assay.

Parasite Preparation and *In vitro* Antiplasmodial Assay

After few days of adaption and growth of the parasites in the culture, they were harvested at the ring stage (trohpozoites) and initial parasitemia estimated for each sample concentration using Giemsa stained slides and light microscope with 100X magnification. Samples were then processed and

2% hematocrit with 1% parasitemia was prepared using uninfected blood to make a total of 14 mL parasite mixture in a complete culture medium. One hundred microliters of each of the nine dilutions (1000 µg/mL, 500 µg/mL, 250 ug/mL, 125 µg/mL, 62.5 µg/mL, 31.3 µg/mL, 15.6, 7.8 µg/mL and 3.9 µg/mL) were plated in duplicates 96 wells coastal plate. Test control drugs, 200 ng/mL Artesunate and 1000 ng/mL of Chloroquine were plated alongside with the *Mist Amen Fevermix* and *Edhec Malacure*. One hundred microliters of the parasite were mixed with 2 percent hematocrit and 1 percent parasitemia was added to each treated well starting from the 2nd well to the tenth well. One hundred microliters of parasite mixture were added to the 11th wells as a negative control and the procedure was repeated for the five other samples and the plates were arranged in a modular Chamber under an atmosphere of 5% Oxygen, 5% Carbon dioxide and 90% Nitrogen and then kept at 37°C for 72 h. The assay was paused by adding 100 uL lysing buffer containing SYBR Green to each 96-well micro-titre plates well and was thoroughly and gently spun to avoid the production of bubbles (Izumiyama *et al*., 2009). The *In vitro* activities on strains of *P. falciparum* were then determined (Izumiyama *et al*., 2009). A thin blood smear was prepared on microscope slides, fixed in absolute methanol, stained with 10% Giemsa in phosphate buffer under sterile conditions in a laminar flow safety cabinet (Hitachi Clean Bench, Japan) for 10 minutes. The slides were dried and observed under a compound light microscope using 100X oil immersion objective lens and also using FLUOstar OPTIMA Fluorometer plate reader with control software version 2.20 at 470 nm and 520 nm wavelengths (Hout *et al*., 2006; Lambros and Vanderberg, 1979). Various IC_{50} values were then determined.

The level of parasitaemia was estimated by measuring lactate dehydrogenase activity, based on a method as previously described (Kenmogne *et al*., 2006). The results were expressed as the mean IC_{50} (the concentration of a drug that reduced the level of parasitaemia to 50%).

In Vivo Assay

The experimental animals, Swiss albino mice of both sexes used for the study were obtained from the Noguchi Memorial Research Institute for Medical Research, Legon, Accra, Ghana. All the animals were allowed to acclimatize; housed for 7days and fed with mice feed and distilled water at ambient temperature. The parasite used for the inoculation, *P.berghei,* maintained in liquid nitrogen and stored at a temperature of -18°C, was obtained from the Department of Pharmacology, Kwame Nkrumah University of Science and Technology (KNUST).

Phytochemical analysis

Phytochemical analysis of the polyherbal products were performed for selected phytoconstituents including: alkaloids, steroids, flavonoids, terpenoids, tannins and saponins using standard methods (Trease and Evans, 2009).

Experimental Design

Parasite Inoculation

The test animals were quarantined for 7 days to acclimatize before infection. Standard inoculums of 1×10^7 *P. berghei* infected erythrocytes in 0.2mL of blood were prepared by diluting infected blood with 0.9% (w/v) normal saline. Each mouse was inoculated by intraperitoneal injection with a blood suspension (0.2 mL) containing 1×10^7 parasitized erythrocytes (Moll *et al*., 2008).

Curative Test

The curative action of *Mist Amen Fevermix* and *Edhec Malacure* in Swiss albino mice were tested for using the methods described by Ryley and Peters (1970). Twenty mice were used for the evaluation of each product. Infected animals were divided into 4 groups (positive, negative and two test groups (n = 5). *Edhech Malacure* was tested at one dose level of 4.25 $mgkg^{-1}$ and *Mist Amen Fevermix* at a dose of 1.56 $mgkg^{-1}$/day). The doses were calculated based on the dose on the label of the products for humans. Two control groups (n = 5) were used, namely, positive control (infected and treated with 4 $mgkg^{-1}$ of artesunate) and negative control (infected and treated with distilled water). The treatments for the study samples lasted for 7consecutive days, the standard treatment lasted for three days. Blood samples were collected from the tip of the tails of the animals (Waako *et al*., 2005) on days 4, 7 and 10 post- 4-day treatment.

Assessment and Monitoring of Parasitemia

Parasitemia was assessed based on a method by (Tarkang *et al*.,(2015). Blood samples were collected from the tip of the tails of the animals on day 4. The blood samples were placed on a microscope slide, spread, dried, and fixed using methanol, and subsequently stained with 10% Giemsa stain for about 30minutes. The stained film was washed off using phosphate buffer, pH 7.2 and allowed to dry. The film was immersed in oil and viewed at x100 magnification. The parasitaemia level was determined by counting the number of parasitized erythrocytes out of 100 erythrocytes in random fields of the

microscope (Toma *et al*., 2015). Average percentage parasitemia was calculated using the formula:

$$\% \text{ Parasitaemia} = \frac{\text{Total number of parasitized erythrocytes}}{\text{Total number of erythrocytes counted}} \times 100$$

The average percentage of chemo suppression was calculated using the formula:

$$\% \text{ Suppression} = \frac{\text{Parasitemia in Negative Control - Parasitemia in Test Group}}{\text{Parasitemia in Negative Control}} \times 100$$

Statistical Analysis

Each product (*Mist Amen Fevermix* and *Edhec Malacure*) was tested in duplicate and the product concentration that inhibits asexual *Plasmodium falciparum* parasite by 50% (IC_{50}) were estimated from dose-response curves by non-linear regression analysis using Graph Pad Prism version 7.0 Software (Graph Pad Software, San Diego, CA, USA)

RESULTS AND DISCUSSION

The results of the phytochemical screening of *Mist Amen Fevermix* and *Edhec Malacure* showed the presence of alkaloids, flavonoids, tannins, phytosterols and saponins.The results is as summarized in Table 1.

Table 1 **Phytochemicals present in *Mist Amen Fevermix* and *Edhec Malacure***

Phytoconstituents	MAF	MEM
Alkaloids	+	+
Tannins	+	+
Saponins	+	+
Flavonoids	+	+
Phytosterols	+	+
Terpenoids	+	+

Key: MAF- ***Mist Amen Fevermix,*** MEM- ***Edhec Malacure***

Table 2 **IC_{50} of *Mist Amen Fevermix* and *Edhec Malacure* in *P. falciparum*-Infected Mice on Day 4 against Standard Drugs**

Drugs	IC_{50}(ng/mL)
AS	1.571ng/mL
MAF	112.5ng/mL
MEM	70.89ng/mL

Key: MAF- ***Mist Amen Fevermix,*** MEM- ***Edhec Malacure,*** AS-artesunate

In Vitro and *In Vivo* Antiplasmodial Activity

The results for the *in vitro* antiplasmodial tests on *Mist Amen Fevermix* and *Edhec Malacure* against standard drugs are summarized in Table 2. Among the study products, the IC_{50} of *Edhec Malacure* was 70.89 µg/mL as compared to 112.5 µg/mL exhibited by *Mist Amen Fevermix* Table 2. The standard drug artesunate exhibited an IC_{50} of 1.571 ug/mL.

In the *in vivo* study, there was an increase in weight in the positive control group of mice from16.98 ± 1.44 g to 18.98 ± 1.37 g (P = 0.0546) compared to *Mist Amen Fevermix* whose administration resulted in a decrease in weight from 14.033 ± 4.69g to 13.767 ± 4.71g (P = 0.9308). The weight increase in *Edhec Malacure* was also not significant (p =0.95) Table 3.

For the *in vivo* antiplasmodial study, *Edhec Malacure* suppressed parasitemia by 76.17%, by *Mist Amen Fevermix* 69.03% with the positive control (artesunate) recording 98.06% suppression (Table 4).

Harnessing the potential clinical use of herbal products as alternative therapies to conventional drugs has many benefits to the population who rely on herbal products for their primary health care needs. This may enhance the safety and improve the quality of life of consumers. *Mist Amen Fevermix* and *Edhec Malacure* have been use in clinical practice in Ghana since the year 2011, for the treatment of uncomplicated malaria. The present study sought to validate their use by employing *in vitro* and *in vivo* antiplasmodial assays to evaluate the activity of *Mist Amen Fevermix* and *Edhec Malacure.*

The *in vitro* anti-plasmodial assay of *Mist Amen Fevermix* and *Edhec Malacure* revealed that the highest antiplasmodial activity with an IC_{50} value of 70.89 ng/mL with *Mist Amen Fevrmix* showing (IC_{50} value of 112.5 ng/mL). Artesunate, used as reference anti-malarial, exhibited a much higher activity with an IC_{50} value of 1.571 ng/mL than the test samples. This may be in agreement with previous reports on some herbal antimalaria products which recorded considerable activities with IC_{50} values in the range of 81.59 ± 1.48 ng/mL to 82.25 ± 1.91 ng/mL (Amoah et al., 2015).

In vivo antiplasmodial activity of *Mist Amen Fevermix* and *Edhec Malacure* was investigated using the curative model on *P. berghei* strain. *Mist Amen Fevermix* suppressed parasitaemia by 69.03% at a dose of 4.25 $mgkg^{-1}$ bodyweight with *Edhec Malacure* suppressing parasitaemia by 76.17% at a dose of 1.56 $mgkg^{1}$ body weight. The reference drug artesunate (at 4 $mgkg^{-1}$ bodyweight) suppressed parasitaemia by 98.06%. The considerable anti-plasmodial activity of *Mist Amen Fevermix* and *Edhec Malacure* in established infection puts these products in the spotlight for malaria treatment in

Table 3 **Effect of Test Samples on Weight of Mice**

Drugs	Initial Weight/g	Final Weight /g
NC	22.40 ± 1.38	21.55 ± 1.75
PC	16.98 ± 1.44	18.98 ± 1.37
MAF	14.033 ± 4.69	13.767 ± 4.71
MEM	12.32 ± 3.93	12.47 ± 3.97

Key: NC-Negative control, PC-Positive control, MAF- ***Mist Amen Fevermix***, MEM- ***Edhec Malacure***.

Table 4 **Curative Effect of *Mist Amen Fevermix* and *Edhec Malacure* (Rane's Test)**

	% Parasitaemia			
Drugs	Day 3	Day 7	Day 10	% Suppression
NC	63.90 ± 7.09	75.90 ± 4.93		--------
MAF	74.74 ± 4.75	23.51 ± 13.23	20.38 ± 0.89	69.03
MEM	72.95 ± 10.23	18.09 ± 6.21	15.23 ± 8.20	76.17
PC	69.30 ± 1.72	1.51 ± 0.21		98.06

Key: NC-Negative control, Artesunate, MAF- ***Mist Amen Fevermix***, MEM- ***Edhec Malacure***.

developing countries. The reduction in parasitaemia is indicative of the curative potential of *Mist Amen Fevermix and Edhec Malacure*, antimalaria products that are already widely in use clinically.

The low sensitivity of the test products on parasite growth *in vitro* (as shown by the IC_{50} values) was supplemented by the more potent antiplasmodial activity *in vivo*, which confirms the limited relevance of *in vitro* testing (Rasoanaivo *et al.,* 2004).

The phytoconstituents observed in the test products were; alkaloids, saponins, tannins and flavonoids. The antiplasmodial effects of *Mist Amen Fevrmix* and *Edhec Malacure* could be due to the presence of these phytochemicals. It has been established that, several classes of phytoconstituents present in herbal products are responsible for their antimalarial activity. Alkaloids are considered as an important phytoconstituent exhibiting diverse biological activities, particularly antimalarial activity (Kaur and S. Arora., (2015). Saponins are also known to posses antiprotozoal activity (Newbold *et al*., 1997).

The body weights of test animals were not significantly affected Table 3.

CONCLUSION

Mist Amen Fevermix and Edhec Malacure possess antiplasmodial properties in vitro and in vivo, thus validating their clinical use in the management of uncomplicated malaria and as alternative antimalarial agents. Their activities could be due to their phytochemical constituents; alkaloids, tannins, saponins, flavonoids and Phytosterols.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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