

Research Article

Synergistic Antiplasmodial Activity of *Artemisia annua* fractions against *in vitro* cultures of *Plasmodium falciparum*

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Background: *Artemisia annua* has a very rich phytochemistry comprising several classes of compounds, mainly monoterpenes, sesquiterpenes, and flavonoids. It has been used in China for about 2000 years in the treatment of fever.

Objective: The aim was to determine if there is any synergistic effect on the *Artemisia annua* phytochemicals.

Materials and methods: *Artemisia annua* used in this study was obtained from a hybrid of the plant grown in the Tanzania highlands (2000-2200 m altitude) in Arusha by Natural Uwemba System for Health (N.U.S.Ag). The dried leaves were ground, and sequentially extracted with hexane, dichloromethane (DCM), methanol and water and the extracts were then combined. The extract was then fractionated using high performance liquid chromatography (HPLC). The effect of the combined crude extract was tested at different doses on *in-vitro* cultures (a CQ sensitive isolate D6 and CQ resistant isolate W2) of *Plasmodium falciparum*. The fractions and different blends of these were tested at different doses to determine their role, if any, on the activity of the full blend of the plant.

Results: Of nine fractions thus tested against D6 and W2, four had activities of less than 3.9 µg/ml, three fractions had activities of between 4.77-14.76 µg/ml and the remaining two had activities above 250g/µml. The seven more active fractions were re-evaluated in a subtractive bioassay procedure, in which one of each fraction was excluded at a time from the full 7-component blend. The activity of the combined seven active compounds was 10.40±0.50 µg/ml against W2. Of these, one showed IC₅₀ of less than 3.9 µg/ml and all blends showed IC₅₀ at below 27 µg/ml.

Conclusion: The results show that different components of *A. annua* contribute to the synergistic anti-*Plasmodium* activity. The results constitute a useful basis for identifying the components of the plant other than artemisinin that contribute to the activity of herb.

Key words- *Artemisia annua*, malaria, *Plasmodium falciparum*, artemisinin, synergy.

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1. Introduction

Malaria is one of the most debilitating tropical diseases causing fever and morbidity in the tropics and a significant source of mortality especially among infants and young children. In 2012, there were an estimated 207 million cases world-wide, causing an estimated 627 000 deaths, mainly among children under 5 years of age in Africa. Ninety percent of all deaths occur in Sub Sahara Africa (WHO 2013). More than a third of the world's population (about 2 billion people) live in malaria endemic areas and 1 billion people are estimated to carry parasites at any one time. In Africa alone, there are an estimated 200-450 million cases of fever in children infected with malaria each year (Breman *et al.*, 2001).

The resistance of *P. falciparum* to more available, safe, and easily administered drugs, especially chloroquine and pyrimethamine-sulfadoxine (SP), has become a serious obstacle to the control of malaria. Artemisinin combination therapy (ACT), using carefully matched drugs, is now the recommended strategy both for clinical care and for the avoidance of drug resistance. However studies at the Thai-Cambodian border, a historical epicenter of multidrug resistance, have detected reduced susceptibility to artemisinins as manifested by prolonged parasite-clearance times, raising considerable concerns on resistance development (O'Neill, 2010).

Artemisinin was first isolated from *Artemisia annua* L., (sweet annie, sweet wormwood,) in China in 1972. The highest artemisinin compound is found in plant *A. annua* plants aged 12 to 13 weeks (Bouwmeester 2006). The total amount of artemisinin found in different varieties of *A. annua* varies between 0.01 to 1.4% by weight based on weight of dry leaves. This plant is used as a traditional medicine that has been consumed as anti-malarial drug and after analyzing the compound, artemisinin and its derivatives have been found to inhibit the growth of *P. falciparum* both *in vitro* and *in vivo* (Casteel 1997). Artemisinin, is a sesquiterpene trioxane lactone containing a peroxide bridge, and was shown to be effective in killing malaria parasites (Mueller *et al.*, 2000). Besides artemisinin itself, some derivatives such as artemether, dihydroartemisinin, arteether, and artesunate have anti-plasmodial activity (Wang *et al.* 2005). The endoperoxide bridge in artemisinin reacts with ferrous iron atom to form free radicals (Zhang *et al.*, 1992, Posner *et al.*, 2000). Artemisinin becomes toxic to malaria parasites as they contain a high amount of iron in the form of heme molecules, and generation of free radicals leads to macromolecular damages and cell death (Anderson *et al.*, 1999).

A. annua has a very rich phytochemistry comprising several classes of compounds, mainly monoterpenes, sesquiterpenes (including artemisinin), and flavonoids (Bhakuni *et al.*, 2002). The composition of each category of these 'secondary' metabolites is variable, both qualitatively and quantitatively. The principal monoterpenoid constituents of *A. annua* from different regions are artemisia ketone (30-80%), artemisia alcohol (7-56%), camphor (3-22%), myrcene (~5%), β -guainene (<5%), germacrane D (0-18%) and 1,8-cineole (~3-13%) (Woerdenbag *et al.*, 1993; Hethelyi *et al.*, 1995; Ahmad and Mishra, 1994). Of the sesquiterpenes,

in addition to artemisinin, a series (up to 30) of compounds related to artemisinin occur in variable amounts in different chemotypes. However, only some of these (including arteannuin B and artemisinic acid) occur in consistently higher relative amounts (Bhakuni *et al.*, 2002). Likewise, a large number of closely related non-glycosidic flavonoids and some glycosidic ones have been isolated from different *A. annua* varieties.

Indirect evidence from studies by Yao-De (Yao-De *et al.* 1992) suggests strongly that other constituents contribute to anti-plasmodium activity of the crude extracts of products derived from *A. annua*. Chloroform extracts of cell suspension cultures developed from *A. annua*, with no significant amounts of artemisinin, has been found to have moderate antimalarial activity *in vitro* ($IC_{50} \sim 10^{-5}$ M, compared to 10^{-8} - 10^{-7} M for artemisinin) attributable to methoxy flavonoids (Liu *et al.*, 1992). At lower concentrations, these flavonoids had a marked and selective potentiating effect on the antiplasmodial activity of artemisinin (Elford *et al.*, 1987; Yang *et al.*, 1989; Liu *et al.*, 1992). Of the sesquiterpenes, so far, one (arteannuin B) has been tested and shown to synergise the activity of artemisinin (Chang and But, 1986).

A major concern on the use of *A. annua* for malaria is that ingestion of sub-lethal doses of artemisinin may accelerate the development of resistance to this drug. Indeed, some degree of resistance to artemisinin may have already occurred in China. The IC_{50} of pure artemisinin varies according to the strain of *P. falciparum* and can be as low as 6nM (Wongsrichanalai *et al.*, 1997). Interestingly, Chinese strains ($IC_{50} = 630nM$) are much less sensitive than African ones ($IC_{50} = 25nM$).

Plant derived medicines continue to play an essential role in health care. It is estimated that approximately 80 % of the world inhabitants in developing countries rely heavily on traditional medicines for primary health care (Arvigo and Balick 1993, Farnsworth *et al.* 1985). Traditional medicine has been used to treat malaria for many years and *Artemisia annua* has been an important herb in Chinese treatment of malaria ((WHO, 2003). In Kenya plant extracts are still widely used in the treatment of malaria and other ailments (Kokwaro 1993).

Artemisinin and its derivatives are safe and well tolerated antimalarial drugs. They should be administered with combination with another effective blood schizontide to reduce recrudescence and to slow the development of resistance. At present drug of choice for the combined therapy is mefloquine in the dose of 12-25 mg base/kg (WHO/MAL, 1998), however the combined regimens of mefloquine plus an artemisinin derivative have been associated with more side effects than those with an artemisinin derivative alone (Price *et al.*, 1999).

Artemisinin derivatives, artesunate, artemether and arteether, are all metabolised into dihydroartemisinin (DHA) (Balint, 2001) which is just as potent as its parent compounds, but has a longer half-life of approximately 4- 11 h. Artemisinin on the other hand is not metabolised into DHA but rather into inactive metabolites deoxyartemisinin and dihydroxydeoxyartemisinin (Balint, 2001). Artemisinin-

based combination treatments (ACTs) are now generally accepted as the best treatments for uncomplicated falciparum malaria. They are rapidly and reliably effective. Efficacy is determined by the drug partnering the artemisinin derivative and, for artesunate-mefloquine, artemether-lumefantrine, and dihydroartemisinin-piperaquine, this usually exceeds 95%. Artesunate-sulfadoxine-pyrimethamine and artesunate-amodiaquine (Nosten and White 2007).

Parasite resistance to existing anti-malarial drugs has already reached alarmingly high levels in Southeast Asia and on the African continent, and therefore there is a dire need for new drugs in the prophylaxis and treatment of malaria (WHO, 1998).

In our efforts to determine if other phytochemicals in *Artemisia annua* are also effective in malaria treatment the fractions were blend and tested against in-vitro cultures of *Plasmodium falciparum* D6 (CQ sensitive strain) and W2 (CQ resistant strain).

2. Materials and Methods

2.1 Collection of plant material

Uppermost foliar portions of *Artemisia annua* were collected in November 2006, from Arusha Tanzania on onset of flowering. A voucher specimen of the plant (**No NMK/BOT/CTX/1/2**) was deposited at the National Museums of Kenya Nairobi.

The collected plant parts were air dried under shade for two weeks and ground using a laboratory mill.

2.2 Preparation of Extracts

Air dried and ground plant materials were extracted using hexane, dichloromethane methanol and water. The solvents were filtered and then the organic extracts were removed using rotary evaporation. The dry extract was then fractionated using high performance liquid chromatography (HPLC). The crude and the fractioned extract were tested on *in-vitro* cultures of *Plasmodium falciparum*.

For bioassays test samples were first dissolved in dimethyl-sulphoxide (DMSO) solvent concentration of less than 0.02% and then in water and filtered in a 0.44 mesh size filter and then stored at 4°C.

2.3 Parasite cultivation

Laboratory adapted *Plasmodium falciparum* cultures of D6 originally from Sierra Leone and W2 strain originally from Indochina were used in the study. The strains were cultured and maintained in the malaria laboratory at Kenya Medical Research Institute (KEMRI) Nairobi.

The culture medium was a variation of that described by Trager and Jensen (Trager and Jensen 1976) and consisted of RPMI 1640 supplemented with 10% human serum 25 mM N-2-hydroxyethyl/piperazine-N-2-ethanesulfonic acid (HEPES) and 25 mM NaHCO₃. Human type O+ erythrocytes (<28 days old) served as host cells and the cultures were incubated at 37°C in an

atmosphere of 3%CO₂, 5% O₂ and 92% N₂ (Trager and Jensen 1976).

2.4. Drugs Assay

The in-vitro semi-automated micro-dilution assay technique that measured the ability of the extract to inhibit the incorporation of (G-³H) hypoxanthine into malaria parasite was used (Des Jardins *et al* 1979, Muregi *et al* 2003). Aliquots (25µl) of culture medium were added to all 96 wells of the flat bottomed micro-culture plate (Costar Glass Works, Cambridge, UK).

Aliquots (25µl) of the tests solutions were added in triplicate to the first wells and by using a tite-tek motorized hand dilutic (flow laboratories, Uxbridge, UK) serial 2_fold dilutions of each sample was added over a 64_fold concentration range .

Aliquots (200 µl) of 1.5 % (vol/vol) suspension of parasitized erythrocytes in culture medium (0.4 % parasitemia) and a growth rate of >3 fold per 48 hours were added to all test wells.

Parasitized and non-parasitized erythrocytes were incorporated into all tests. The plates were incubated at 37°C in a gas mixture of 3% CO₂, 5 %O₂ and 92% N₂.

After 48hrs each well was pulsed with 25µl of culture medium containing 0.5µci of (G-³H) hypoxanthine and the plates incubated for a further 18hrs.

The contents of each well were then harvested with a beta cell harvester (Wallac Zurich) onto glass fibre filters and washed thoroughly with distilled water, dried and the filters were inserted into plastic bags with liquid scintillant and the radioactivity measured in counts per minute (cpm) per well at each concentration.

2.5 Data analysis

Computation of the concentration of drug causing 50% inhibition of (G-³H) hypoxanthine uptake (IC₅₀) was carried out by interpolation after logarithmic transformation of both concentration and cpm values using the formula,

$$IC_{50} = \text{antilog} \left(\log X_1 + \frac{(\log Y_{50} - \log Y_1) (\log X_2 - \log X_1)}{(\log Y_2 - \log Y_1)} \right)$$

Where Y₅₀=cpm value midway between parasitized and non-parasitized control cultures and X₁, Y₁, X₂, and Y₂ are the concentration and cpm values for the data points above and below the cpm midpoints (Sixsmith *et al* 1984).

3. Results and Discussion

Nine fractions were obtained and used in the bio-assays; these were labeled as F1, F2, F3, F4, F5, F6, F7, F8, and F9. The full blend (F10) indicated the mixed crude extract. The asterick (*) indicated the fractions that had an activity of less than 3.9 µg /ml.

In the preliminary studies the crude extract and fractions were screened against *P. falciparum* D6 (a Chloroquine sensitive isolate). Nine fractions (F1-F9)

were used in the bioassay and the results were summarized in **Table 1**. Only two fractions showed an IC₅₀ of above 250 µg /ml (L3 and L4) and this showed that these fractions were not active *in-vitro* against *P. falciparum* D6. The crude extract (full blend) gave a IC₅₀ of 10.40±0.50µg /ml against D6, however the crude extract was not very soluble as the material was very gummy, this probably led to a high IC₅₀.

Table 1: *in vitro* activity for the nine fractions against *P. falciparum* D6 and W2 strains

FRACTIONS	IC ₅₀ for D6 (µg /ml)	IC ₅₀ for W2 (µg /ml)
F1	4.77±1.49	6.59±0.36
F2	14.763	ND
F3	>250	ND
F4	>250	ND
F5*	<3.9	ND
F6	6.95±4.34	ND
F7*	<3.9	ND
F8*	<3.9	2.21±0.01
F9*	<3.9	13.13±0.20
F10	10.40±0.50	ND
Dihydroartemisinin	2.025 ng/mL	ND

ND: not determined

Table 2: *in vitro* activity for the various blends of the fractions against *P. falciparum* D6 and W2 strains

BLEND	IC ₅₀ for D6 (µg /ml)	IC ₅₀ for W2 (µg /ml)
Full blend -F1	16.19±1.74	
Full blend -F2	9.19±0.31	
Full blend- F5	10.52±0.35	
Full blend -F6	10.12±0.70	
Full blend -F7	26.75±8.43	
Full blend -F8	10.40±0.50	
Full blend- F9	9.72±0.17	
F18=F1+F2		7.95±0.08
F19=F2+F6		15.96±0.31
F20=F1+F6		<3.9
F7+F8+F9		7.07±0.09
F5+F8+F9	6.332	
F5+F7+F9		11.87±1.95
F5+F7+F8	5.063	7.74±0.09
F5+F7+F8+F9	5.039	
Dihydroartemisinin	2.025 ng/mL	

Key: Full blend - F1 = F2+F3+F4+F5+F6+F7+F8+F9
 Full blend - F2 = F1+F3+F4+F5+F6+F7+F8+F9
 Full blend - F5 = F1+F2+F3+F4+F6+F7+F8+F9
 Full blend - F6 = F1+F2+F3+F4+F5 +F7+F8+F9
 Full blend - F7 = F1+F2+F3+F4+F5+F6+F8+F9
 Full blend - F8 = F1+F2+F3+F4+F5+F6+F7+F9
 Full blend - F9 = F1+F2+F3+F4+F5+F6+F7+F8

Four fractions (F5, F7, F8, and F9) showed an IC₅₀ of below 3.9 µg/ml meaning that these fractions had notable activity against *P. falciparum* D6. These were assayed further with isolate *P. falciparum* W2 (CQ resistant isolate). Fraction F1's activity was also tested against the CQ resistant isolate W2. Three of these fractions had an IC₅₀ below 14µg /ml, which reflected notable activity against this strain (**Table 1**). The results also indicated that higher concentrations of the fractions were generally required for a CQ resistant isolate.

When the fractions were further blended, all the blends gave an IC₅₀ of less than 27 µg/ml. This indicated possible additive effects. Two blends showed an activity of less than 10 µg /ml against the D6 strain (Full blend - F2, Full blend - F9) and had an activity of 9.19µg /ml and 9.72µg /ml respectively. Three blends had an activity of between 10 and 11. Full blend - F5 (10.52µg /ml), Full blend - F6 (10.12µg /ml), and Full blend - F8 had an activity of 10.40µg /ml with D6. The lowest activity (IC₅₀= 26.75 µg /ml) of the blends occurred when one of the more active fractions (F5) was absent; this indicated the contribution of the omitted fraction which probably contained an active compound.

After blending some of the most active fractions very high activity was observed against the W2 strain i.e. F18 had an IC₅₀ of 7.95µg /ml and F19 had an IC₅₀ of 15.96µg /ml. One blend (F20) had an activity of <3.9µg /ml and this indicated notable activity that calls for further research (**Table 2**).

4. Conclusion

Biochemical investigations on some of the fractions and blends of *Artemisia annua* compounds may provide useful templates for the development of novel antimalarial drugs that are effective against CQ resistant *P. falciparum*.

Conflict of Interest Declaration

The authors declare no conflict of interest.

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