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The *in vitro* Anti-plasmodial Activity of 22*β*-[(S)-2-methyl-2-butenoyloxy]-3-oxoolean12-en-28-oic acid on Chloroquine Resistant Indochina W-2 Parasite Clones

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Bioassay guided chromatographic separation of the methanolic extract from *Lantana camara* root bark – used by the Luo tribe in Nyanza Province of Kenya to control malaria led to isolation of 22β-[(S)-2-Methyl-2-butenoyloxy]-3-oxoolean-12-en-28-oic acid (**1**) whose structure was elucidated using physical and spectroscopic (IR, ^{1}H-NMR and ^{13}C-NMR) data. A regression analysis on larval mean mortality at different concentrations revealed a high larvicidal activity (LC_{50} = 41.34 μM) for the compound against *Anopheles gambiae* larvae at 2nd instar stage. However, the activity was lower than 1.12 μM exhibited by pyrethroids against the larvae. The compound also showed a remarkably anti-plasmodial activity (IC_{50} = 21.79 $\pm$ 0.59 μM) against the Chloroquine resistant Indochina (W-2) *Plasmodium falciparum* parasite clones. However, the crude methanolic extract showed a higher activity (IC_{50} = 1.95 μM) against *Plasmodium falciparum* parasite clones compared to compound **1** (IC_{50}= 21.79 μM). Both the methanol extract and compound 1 were more active against the parasite than chloroquine diphosphate (IC_{50}= 53.75 μM) but less active than the anti-malarial drug, artemisinin (IC_{50} =0.44 μM).

Key words: *Lantana camara*, 22β-[(S)-2-Methyl-2-butenoyloxy]-3-oxoolean-12-en-28-oic acid, *Anopheles gambiae, Plasmodium falciparum* and malaria

Introduction

Due to its high morbidity and mortality, human malaria is an Infectious disease of enormous importance in tropical countries.

Despite the eradication programs that started more than 100 years ago, malaria is still a threat to over 2 billion people living in areas of high incidence (Asimeng, 2000). Although statistics vary widely, it is estimated that there are 200 million infected humans, along with 150 million new cases every year (Nosten *et al.,* 2004). It is also estimated to cause more than 2 million deaths annually, among which half are children under five years old. Recent investigations have reported alarming deterioration in the effectiveness of the conventional antimalarial drugs (Kevin, 1994). Furthermore, resistance to artemisinin, which appears to be the most effective and most promising actual antimalarial drug, has been induced in a rodent malaria model and hence may occur naturally (Mayunga, 2002). If complete resistance to artemisinin emerges, no drug will be available to offer protection against malaria in all regions of the world. Therefore, the need for novel chemotherapeutic agents

is acute. Treatment of malaria started with quinine, a botanical derivative that has been used for more than 100 years. After several years of incursion into synthetic drugs, the final compound to replace quinine appears to be another botanical derivative, artemisinin, isolated from Chinese medicinal herb *Artemisia annua* (Esamai, 2000).

As part of phytochemical and pharmacological investigations of antimalarial plants, a bioactivity guided isolation of compounds from a methanolic extract of *Lantana camara* unveiled 22β-[(S)-2-Methyl-2-butenoyloxy]-3-oxoolean-12-en-28-oic acid. The compound showed significant activity against drug-resistant W-2 *Plasmodium falciparum* parasite clones.

Lantana camara

The plant is commonly known by the Luo tribe living in Nyanza province of Kenya as Magwagwa, Obengle or Nyabende and by the Iraqw tribe as Xaslaslaamo (Kokwaro, 1976). Ash from its burnt leaf together with a little salt acts as a good remedy for coughs, sore throat and conjunctivitis. For the treatment of toothache, the leaves are simply chewed. The leaves are also used as an inhalant for the treatment of colds and headaches. For this kind of treatment, pounded leaves or roots are put in boiling water, transferred into container with a small opening like a bottle and the escaping vapour inhaled continually. The medicine should be changed or prepared everyday until the patient is cured. Smoke from green leaves is very effective as a mosquito repellent when used indoors. A water extract form the root-bark is also used as a remedy for malaria and is sometimes mixed with a water extract of lemon grass. This is taken in a glass; three times a day until the patient recovers (Kokwaro, 1976).

Materials and Methods

Collection of plant parts

Fresh root bark of *Lantana camara* was collected in February 2005 from Migori District which is about 300 km West of Nairobi, Kenya and identified at Moi University Herbarium where a voucher specimen (No. Mig 02 06 001) was deposited. The collected plant parts were air-dried for 5 d and then chopped into small pieces.

Extraction and isolation of compounds

The dried pieces were ground into a fine powder using an electric grinder. A 1 kg of the material was soaked in ethyl acetate at room temperature for 48 h. The mixture was then filtered and the solvent evaporated under reduced pressure using a rotary evaporator to afford 50 g of a dark brown gummy solid.

A weight of 70 g of the crude extract was adsorbed onto a dry deactivated silica gel (mesh size 0.063 - 0.2 mm) and subjected to column chromatography in a column (packed under *n*-hexane with 140 g of the de-activated silica gel).

The column was first eluted with pure *n*-hexane followed by a mixture of *n*-hexane/ethyl acetate with increasing polarity. Elution of the column with an *n*-hexane/ethyl acetate mixture (2:3 v/v) led to isolation of a white powdery compound (**1**).

The column was finally washed with methanol. The eluants were collected in portions of 20 ml. The compound was characterized on the basis of spectroscopic (MS, IR and NMR) data and melting point.

Larval mortality test

Stock solution of 100 µg both compound 1 and the crude methanolic extract were dissolved separately in 1 ml of distilled water to make 100 µM solutions. Commercially available pyrethroid used, as a standard, was prepared as per the manufacturer's specification - dissolving 10ml in 20 litres. A total of twenty (20) Larvae of *Anopheles gambiae*, at their second instar stage of development were introduced into each test vial containing various concentrations ranging from 100 ppm- 0 ppm (control). Besides, a positive control containing a standard larvicide (pyrethroid) made as per the manufacturers specification was also set-up. To each test viol, three granules of yeast food were added. The experiments were done in triplicate. The vials were left in an incubator while maintaining a temperature range of between 37°C and 40°C. Dead larvae were counted and removed out of the solution after every 24 hrs interval until the first pupa was noticed in any of the dishes. Adding distilled and de-ionized water to the test vials maintaining the original mark maintained the drug concentration. Data was recorded as cumulative mortality for each of the concentrations. The % mean mortalities for the three test viols were calculated as follows;

% Mean Mortality = Average of dead larvae in each conc. X 100

The data, recorded in form of tables, were used to draw graphs of % mean mortalities verses concentration of the crude and any isolated compounds. From these graphs LC_{50} were got from the equation of the resulting best line of fit.

Anti-malarial screening

The invasion of erythrocytes by *P. falciparum* merozoites is a key step in the pathogenesis of the malaria disease since it is only after that phase of the parasite's life cycle that the first symptoms appear. In the first step, the merozoite attaches reversibly to the erythrocyte surface followed by apical reorientation and formation of an irreversible junction. This is followed by a parasitophorous vacuole stage and, finally, entry into the vacuole by movement of the junction and resealing of the vacuolar and erythrocytic membranes (Desjardins. *et al*., 1979). .

The *in vitro* assays on compounds **1,** the crude methanolic extract, and the reference drugs were performed by using a modification of the semiautomated microdilution technique described by Desjardins. (Desjardins *et al*., 1979). *P. falciparum* malaria parasite clones, designed as chloroquine resistant Indochina (W-2) parasite clones were utilized in susceptibility testing. The W-2 clone is resistant to chloroquine, pyrimethamine, and sulfadoxine (Desjardins. *et al*., 1979). The tested extracts were dissolved in Dimethylsulfoxide (DMSO) and serially diluted with media. The uptake of [^{3}H]-hypoxanthine monohydrochloride was used as an index of inhibition of parasite growth.

Chloroquine and artemisinin were used as reference drugs. The results of the *in vitro* screening of various extracts of **1** are shown in Table II.

Drug samples, artemisinin and chloroquine were tested against W-2 *Plasmodium falciparum* parasite clones resistant to chloroquine. The drug concentrations capable of inhibiting 50% of the *P. falciparum* were determined by computation after logarithmic transformation of both concentration and counts per minute (cpm) values as described by Desjardins and group, (Desjardins *et al*., 1979). This can also be done using the formula;

$$IC_{50} = \text{antilog}\ (\log X_1 + (\log Y_{50} - \log Y_1) \quad (\log X_2 - \log X_1) \div (\log Y_2 - \log Y_1)$$

Where Y_{50} is the counts per minute (cpm) value midway between parasitized and non-parasitized control cultures and $X_1$and Y_1 are the concentrations for the data points above and below the cpm midpoints respectively while X_2 and Y_2 are cpm values for the data points above and below the cpm midpoints respectively

Parasite culture and growth inhibition assays

The anti-malarial screening using cultures of the chloroquine resistant W-2 *P. falciparum* parasite clones was performed at the Kenya Medicinal Research Institute (KEMRI), Nairobi-Kenya. The *in vitro* efficacies of the crude extract and compounds **1** against the chloroquine resistant W-2 *P. falciparum* parasite clones were determined by assessing [^{3}H]-hypoxanthine incorporation.

Plasmodium falciparum isolated with a known parasitaemia (ranging from 0.5 to 1%) was maintained in culture in a specific medium composed of Rosewell Park Memorial Institute (RPMI) 1640 + 10% human serum, with uninfected human blood cells at a haematocrit value of 2.5%. The cell suspension was then distributed into a flat-bottomed well plate containing serial concentrations of the plant extracts or pure compounds at an optimal volume of 0.7ml, together with untreated controls. The culture was then incubated at 37°C under N_2 containing 2-5% CO_2 and 3-8% O_2 gases being microaerophilic in culture. *P. falciparum* exhibits optimal growth under these conditions and should reach the mature trophozoites stage after 18hrs.

A radio-labelled DNA precursor, namely tritiated hypoxanthine (G-^{3}H), was then added to each well and incubation continued at 37°C in required atmosphere for further 24 hr. This enabled the parasites to complete their cycle of maturation (mature blood schizonts). The contents of the well were then frozen at −30°C for 2 hr and unfrozen at 50°C for 1 hr to lyse the cells. This was then harvested by filtration and finally washed several times with water then dried, added to toluene scintilator in vials and radioactivity incorporated into parasite nucleic acid from each well estimated in a liquid scintillation counter.

Values obtained are inversely proportional to the percentage inhibition of *P. falciparum* by the plant extract. Concentrations of plant extracts inhibiting growth of over 50% of the parasite indicate the biological activity of the plant extract (Phillippe and Suzanne, 1993).

Effects of increasing concentrations of drugs on the *in vitro* growth of chloroquine-resistant (W-2) strains of *Plasmodium falciparum* were expressed as % survival as compared to control-in the absence of added drugs.

Results and Discussions

Larval mortality activity

Graphical and regression analysis for mean mortality for concentrations ranging 100 µM-0 µM (negative control) for compound 1, methanol extract and pyrethroids (for positive control) were done. Graphs of % mean mortality against respective concentrations (ppm) were plotted using Microsoft excel database for both the crude and the pure compounds. Best lines of fit as well as the equations of these lines were obtained using the same database. The equations of the lines were obtained using the general equation y = mx + c, where y is the % mean mortality, m is the gradient of the line, x is the concentration of the crude as well as the pure compounds and finally, c is a constant. LC_{50} values were obtained from the respective equations of straight lines that resulted from each graph. The table below shows the LC_{50}, R^2 (where R is Pearson's correlation coefficient) and *p*-values obtained.

Table I. Relative larvicidal activities 22β-[(S)-2-Methyl-2-butenoyloxy]-3-oxoolean-12-en-28-oic acid, pyrethroids and methanol extracts from *Lantana camara*

Sample	LC_{50} values	R^2 value	P-values
Methanol extract	15.06 µM	0.9718	4.554×10^{-5}
Compound **1**	41.34 µM	0.9959	1.0×10^{-4}
Pyrethroids	1.12 µM	0.9400	3.7×10^{-4}

The crude extract exhibited highest larvicidal activity (15.06ppm) than compound **1 (**LC_{50} = 41.34 µM), possibly due synergistic effect between compounds. However, these activities were lower than 1.12 µM exhibited by pyrethroids. A regression analysis showed that the effect of an increase of the drug concentration on mortality was very significant for all the test drugs $p<0.05$ as well as R-square values output being very close to unity

Anti-plasmodial screening

The *in vitro* antimalarial test was based on the inhibition of (G-^{3}H) – hypoxanthine uptake by *Plasmodium falciparum* cultured in human blood.
The table below gives a representation of cpm for the drugs samples tested on W-2 *Plasmodium falciparum* clones versus their concentrations

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Table II: *In vitro* anti-malarial Activity of *Lantana camara* isolates against W-2 *P. falciparum* parasite clones

Test Drugs	**IC_{50}(µg/ml)±SD**	**Comment**
Methanol extract	1.95 µM	Test drug
Compound **1**	21.79 µM	Test drug
Chloroquine diphosphate	53.75 µM	Standard
Artemisinin	0.44 µM	Standard

The methanolic crude showed the highest antiplasmodial activity towards the chloroquine-resistant (W-2) strains of *Plasmodium falciparum.*

There was also a clear correlation between the IC_{50} value and the membrane curvature caused by compound **1** at different concentrations. In this investigation, preincubation of erythrocytes with compound **1**, followed by extensive washing, made the cells unsuitable for the parasite growth, suggesting that the compound incorporates into the erythrocyte membrane irreversibly. On the other hand, the parasite culture continued to grow well in untreated erythrocytes. Relative results were obtained with chloroquine reference drugs.

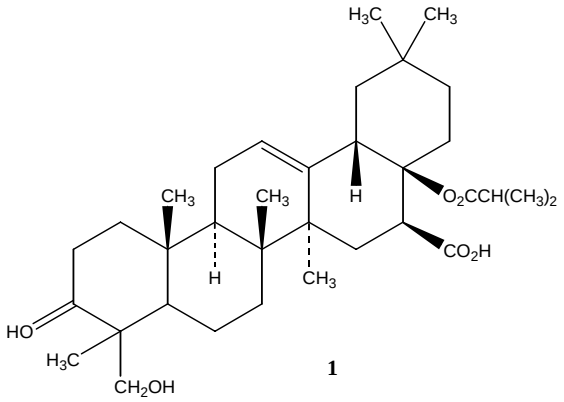
The anti-plasmodial activity of this type of compounds could be oblique, exclusively due to stomatocytic transformation of the host cell membrane and not to toxic effects via action on a drug target within the parasite. This activity should therefore be due to the incorporation of compound **1** into the lipid bilayer of erythrocytes that may cause the modification of cholesterol-rich membrane rafts, to be important in parasite vacuolization.

Compound 1: White powder: M.P; 260ºC. IR (KBr) *λ*max 3400-2600, 2900, 1700, 1690, 1650, 1450, 1230. ^{1}H NMR; MeOH δ 0.72 Ac, 5.153OH, 17.638, 25.092 6-CH_3 steroid. ^{13}C NMR; MeOH δ 209 (C3), 180.86 (C17), 144.179 (C13), 122.633 (C12), 78.683 (C22), 55.743 (C5), 47.495-48.516, 41.872 (C4) and 14.912-46.619 (C1-C21).

Its IR spectrum gave a characteristic broad band with a frequency range of 3400-2600 cm^{-1}, suggesting the presence of an -OH functional group in the molecule. Other peaks appeared at frequencies of 1700 cm^{-1} for a C=O stretch, 2900 cm^{-1} for C-H stretch, 1650 cm^{-1} for C=C stretch and an ester at 1230 cm^{-1}. The presence of an O-H was confirmed by ^{1}H NMR absorption peak at 5.153 ppm and the presence of -CO_2H at 180.80 ppm for ^{13}C NMR. C=O was also confirmed by the presence of a ^{13}C NMR peak at 209 ppm (C-3). A more critical observation was the observation -CH_2OH at 55.743 ppm (C-5). More peaks were observed as follows: ^{13}C NMR: C-steroid; 14.912-46.619 ppm (C1-22), C=C; 144.179 ppm (C-13) and an ester; 78.683 ppm (C-22). The presence of acetate was observed at 0.762 ppm (C-29) in ^{1}H NMR spectra.

Its ^{13}C NMR spectra showed 22 distinguished peaks at different chemical environments. There were four main chemical environments in the compound, aliphatic C-atoms, mostly in the steroid structure at higher field from 14.912-47.834 ppm, ester and alcohol C- atoms from 55.743-78.682 ppm in the middle field, 122.633, alkene and carboxylic acid C- atoms at low field from 122.633-180.086 ppm. Finally carbonyl C- at very low field of 209.201 ppm due to high de-shielding effect of oxygen atom.

Based on the physical and spectroscopic data, compound **1** was identified as 22-β-[(S)-2-Methyl-2-butenoyloxy]-3-oxoolean-12-en-28-oic acid.



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