Journal of Medicinal Plants Research Vol. 5(8), pp. 1355-1360, 18 April, 2011
Available online at http://www.academicjournals.org/JMPR
ISSN 1996-0875 ©2011 Academic Journals

Full Length Research Paper

Synergism of artemisinin with abyssinone –V from *Erythrina abyssinica* (Lam. ex) against *Plasmodium falciparum* parasites: A potential anti-malarial combination therapy

J. S. Kebenei[1]*, P. K. Ndalut[2] and A. O. Sabah[3]

[1]Department of Chemistry, Kabarak University, P. O. Box 3270 Nakuru, Kenya.
[2]Department of Chemistry, Moi University, P. O. Box 1125 Eldoret, Kenya.
[3]Centre for Biotechnology Research and Development, Kenya medical Research Institute, P. O. Box 54840, Nairobi 00200, Kenya.

Accepted 2 January, 2011

A *Plasmodium falciparum in vitro* drug sensitive study was conducted in order to evaluate artemisinin in combination with potential anti-malarial drug from the stem bark of *Erythrina abyssinica.* Abyssinone-v was isolated and thus identified as a potential partner for a fixed combination therapy. Bioassay guided chromatographic separation of Ethyl acetate extract of *Erythrina abyssinica* led to the isolation of Abyssinone-v with IC_{50} value of 3.19 µg/ml against chloroquine-sensitive (D6) *P. falciparum* parasites. The structure of abyssinone-v was determined using nuclear magnetic resonance (NMR) and mass spectroscopic technique. The interaction of artemisinin and abyssinone-v was analyzed using combination ratios of 10:90 to 90:10 respectively against *P. falciparum* parasites. This led to the identification of anti-malarial combination therapy of artemisinin and abyssinone-v with sum of fraction inhibiting concentration (FIC) of 0.79 at a ratio of 2:3, respectively.

Key words: *Erythrina Abyssinica, Plasmodium falciparum,* abyssinone-v, artemisinin and combination therapy.

INTRODUCTION

Malaria is an infectious disease which causes about 275 to 350 million infections in human and approximately 1.3 million deaths annually (WHO, 2008). Sub- Saharan Africa accounts for 85% of these fatalities (Scott, 2005). Malaria mortality and morbidity are increasing in the Sub-Saharan region of Africa especially in the vulnerable groups such as children under five years and pregnant mothers (Snow et al., 2001), due to multi-drug resistance of *Plasmodium falciparum* parasites to available drugs. This has resulted in an economic constraint in malaria control (Bloland, 2001).Increase in the spread of multi-drug resistance of parasites to most of the available and affordable anti-malarial drugs is a major concern and requires innovative strategies in controlling it. There is an urgent need to discover and develop new anti-malarial drugs.

Traditional medicinal plants have been the main source of anti-malarial drugs with the two major drugs quinine and artemisinin drugs being used worldwide for treatment of malaria (Wright et al., 1990; Thomson, 1993). Quinine and artemisinin were obtained directly from *Cinchona Sp* and *Artemesia annua* plant respectively and were developed using plant-derived chemical structures as templates. Chemical modification of artemisinin has led to a series of potential anti-malarial drugs (artemether, artesunate and arteether) which are currently being used in combination with conventional drugs for treatment of malaria (Nicholas, 1994; Simon et al., 1990; Bai, 1993).

*Corresponding authors. E-mail: selkebenei@yahoo.com.

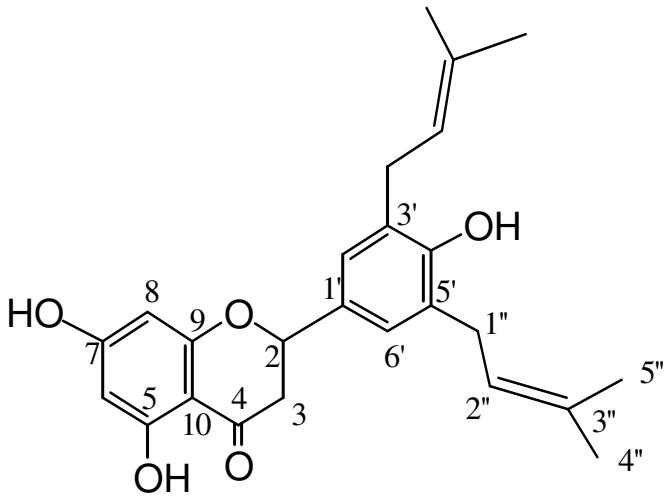

Figure 1. Abyssinone-v.

The use of combination chemotherapy is the current innovative strategy in controlling malaria disease; it involves the use of a short half-life acting anti-malarial agent of artemisinin drug in combination with long half-life conventional drug, example being artemether-lumifantrine and artesunate-melfoquine drugs (Nosten, 2000). The artemisinin drug (short half-life) kills the parasites and excreted rapidly, resulting in re-emerging of the parasites after a period of time. Therefore use in combination with longer half-life anti-malarial drug result in achieving full eradication of the parasites preventing the recrudescence that occur with use of artemisinin mono-therapy (Walsh et al., 2007).

Currently, the drugs being used in combination with artemisinin drugs are the conventional drugs (lumifantrin, melfoquine and amodiaquine), which have developed resistance to *P. falciparum* parasites when used in mono-therapy. The use of the conventional drugs in combination therapy might not solve the problem of multi-drug resistance of the parasites in the near future. There is an urgent need in discovering and developing new anti-malarial drugs from traditional medicinal plants that can be used in combination therapy with artemisinin drugs in order to reduce the development of resistance and the occurrence of recrudescence. Currently, the ongoing research is on developing artemisinin combination therapy from traditional medicinal drugs. Example, from recent research on artemisinin combination therapy with anti-malarial drugs from traditional medicinal plants was on artemisinin – flavanoids combination therapy. In this study, artemisinin –abyssinone-iv combination at ratio of 5:1 respectively showed synergism activity and artemisinin- abyssinone 4'-methyl ether combination showed additive activity against (D6 clone) *P. falciparum* parasites (Akala et al., 2010). In an effort to verify the efficacy of traditional anti-malarial drugs, a potential medicinal plant *Erythrina abyssinica* was screened for anti-malarial activity.

Phytochemical analysis of most active compound was done for identification of a natural product that has high potential of being used in combination therapy with artemisinin drug.

MATERIALS AND METHODS

Experimental

The melting point of compound 1 (Figure 1) was determined using an electro-thermal melting point apparatus with a thermometer range of 0 – 360°C and was uncorrected. An infrared spectrum was generated using IR-408 shimadzu spectrometer and the compound was isolated with KBr as discs. ^{1}H and ^{13}C NMR analysis were done using mercury Vx. (200 MHZ) NMR spectrometer in deuteriated chloroform with TMS as the internal standard. An electron impact mass measurement was recorded on a VG – 12 – 250 UPGRDE mass spectrometer machine. Silica gel (5 to 40 µm mesh) was used for dry column chromatography while 220 to 440 mesh silica was used for flash chromatography.

P. falciparum parasites (D6, Chlroquine-sensitive strain) from Sierra Leone were used for anti-malarial test. The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi, Kenya. The root bark of *E. abyssinica* was collected from Uasin-Gishu District, 320 Km West of Nairobi, Kenya. The staff of Botany Department Herbarium, Moi University, where a voucher specimen was deposited, identified the plant.

Collection, solvent extraction and chromatography

Following a survey on the ethno uses of medicinal plants in Uasin-Gishu District in the Rift-Valley province of Kenya through interviews with traditional health practitioners, *E. abyssinica* was identified to be the most commonly used medicinal plant for treatment of malaria. *E. abyssinica* was selected for the study. Botanical identification was done by a taxonomist and a voucher specimen deposited at the Botany Department Herbarium, Moi University, Eldoret - Kenya. The stem bark of *E. abyssinica* were chopped into small pieces, air dried at room temperature for two weeks, and grounded into powder form.

Two Kilogram of the powder was soaked in cold methanol for three days to extract the compounds. The resulting dark yellow filtrate was concentrated under reduced pressure using Buchii R110 rotatory evaporator to a dark brown semi-solid and was subjected to anti-malarial test as methanolic crude extract. Thirty grams of methanolic crude extract was subjected to solvent partitioning using n-hexane, ethyl acetate and the residue dissolved in methanol to obtain three solvent fractions, concentrated and subjected to anti-malarial test.

The Ethyl acetate fraction (5 g) was the most active and was chromatographed over silica gel, eluting under pressure with increasing amount of ethyl acetate in n-hexane. The eluted fractions were monitored by thin layer chromatography using n-hexane and ethyl acetate (6:4) mixture as a developing solvent. Four fractions were obtained and were subjected to anti-malarial test.

The most active fraction 3 (10 ml), was concentrated and further purified by flash chromatography using ethyl acetate in n-hexane as eluting solvent.

This led to the isolation of compound 1, which was re-crystallized using 10% of n- hexane in acetone.

Anti-malarial test

Stock solution

Stock solution (100 µg/ml) for in *vitro* assay of methanolic extract, solvent fractions and compound 1 were prepared in deionized autoclaved water and re-sterilized by passing through 0.22 µm micro-filters in a laminar flow hood. The water-insoluble extracts were dissolved in 100% DMSO (dimethylsulfoxide) from Sigma Chemical Co; St Louis, MO USA, followed by a subsequent dilution to lower the concentration of DMSO to less than 1% to avoid solvent carrying over solvent effect (Dominique et al., 2001). Stock solutions (1 µg/ml) of chloroquine diphosphate and artemisinin (MW = 282.332) were similarly prepared for use as reference drugs. Each drug was filter-sterilized with syringe adaptable 0.22 µml filters into sterile Bijoux bottles and was stored at -20°C until required for bioassay.

Parasite culture

Test samples were screened against *P. falciparum* D6 (CQ-sensitive) strain from Sierra Leone. The strains were obtained from the Division of Experimental Therapeutics, Walter Reed Army Institute of Research (WRAIR), Nairobi. The parasites were cultivated by a previously described *in vitro* technique (Trager et al., 1976). Complete culture medium (CMS) was prepared and consisted 10.4 g/l of RPMI 1640 (Rosewell Park Memorial Institute), 25 mM (5.94 g/l) HEPES buffer and 31.5 mM sodium bicarbonate supplemented with 10% human serum (Schlichtherle et al., 2000). Uninfected human blood group O Rh-positive erythrocytes less than 28 days old served as host cells. The cultures were incubated at 37°C in an atmosphere of 3% CO_2, 3% O_2 and 92% N_2 obtained from BOC gas Nairobi.

In vitro anti- plasmodium assay

The *in vitro* anti-malarial test was based on the inhibition of [G-^{3}H] – hypoxanthine (Amersham International, Buckinghamshire, UK) uptake by *P. falciparum* cultured in human blood (Desjardins, 1979). Twenty five microliters of aliquots of the complete culture medium (CMS) were added to all the wells of 96-well flat bottomed micro-culture plates (Falcon, Becton Dickinson, Franklin Lakes, NJ), followed by addition in duplicate of 25 µl of the test solution (plant extracts and compound 1) to the first row wells, a Titertek motorized hand diluter (Flow laboratories, Uxbridge, UK) was used to make serial twofold dilutions of each sample over a 64-fold concentration range. Negative controls treated with solvent (DMSO) alone were added to each set of experiment (Azas et al., 2001) to check for any solvent effects. The susceptibility tests were carried out with initial parasitemia (expressed as the percentage of erythrocytes infected) of 0.4% by applying 200 µl, 1.5% hematocrit, *P. falciparum* culture to each well.

Two hundred microliters of culture media without parasites was added into four wells on the last row of each plate to serve as a background (controls). Parasitized and non-parasitized erythrocytes were incubated at 37°C in a gas mixture containing 3% CO_2 , 5% O_2 and 92% N_2 for 48 h after which 25 µl of culture medium containing 0.5 µCi, of [G-^{3}H] – hypoxanthine was added to each well. The culture plates were further incubated for 18 h. At the end of incubation period, the radio-labeled cultures were harvested onto glass-fiber filters using a 96-well cell harvester. [G-^{3}H] hypoxanthine uptake was determined using a micro-beta trilux liquid scintillation and luminescence counter (Wallac MicroBeta Trilux). The drug concentration capable of inhibiting 50% of the *P .falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minute (cpm) using the formula:

IC_{50} = antilog (log X_1 +[(log Y_{50} – log Y_1) x (log X_2 – log X_1)]/ (log Y_2 – log Y_1)).

Where Y_{50} is the cpm value midway between parasitisized and non-parasitisized control cultures and X_1, Y_1, X_2, and Y_2 are the concentrations and cpm values for the data points above and below the cpm midpoints (Sixsmith et al., 1984). The analysis of IC_{50} values were done using the computer software called GraFit-4 database.

Drug combination bioassay

Template plates were used in preparation of drugs combinations. Test drugs were dispensed into an extra plate in various ratios of blends. The combined test samples were well mixed and transferred to multiple daughter plates or test plates, such that 2 daughter plates were made per two drugs pair. One daughter plate was used for the assay and the other kept at –20°C for running a repeat test on a different day at the end of which the average of the two experiments was given as the final result.

The method described by Canfield et al. (1995) was adopted. The solutions of intial concentrations 20 to 50 times the estimated IC_{50} values were combined in various ratios of various drugs. Thus fixed ratios of predetermined concentrations needed to inhibit parasite growth by 50% (IC_{50}) was used to determine the interaction of two drugs. Single and combined drug solutions were dispensed into the 96 well microtitre plates to give nine combinations ratios of 90:10 to 10:90 (extract A:extract B) (Fivelman et al., 1999). Incubation and subsequent procedures were followed as previously described in section of *in vitro* anti- plasmodium assay. Corresponding IC_{50} values were determined for each drug alone and in combination using the method of Sixsmith et al. (1984). The degree of synergy was evaluated according to the method of Berenbaum (1978). Fraction inhibition concentration (sum FIC) was calculated using the formula:

$A_c/A_e + B_c/B_e = K$ (sum FIC)

where A_c and B_c are the equally effective concentrations (IC_{50}) when used in combination, and A_e and B_e are the equally effective concentrations when used alone. When FICs < 1 it indicates synergism, sum FICs ≥1and < 2 it indicates additive interaction and sum FIC ≥ 1 it indicates antagonism (Gupta et al., 2002).

RESULTS AND DISCUSSION

Structural elucidation

Abyssinone-v (compound 1, Figure 1), a white powder re-crystallized from fraction 3 of ethyl acetate extract from *E. abyssinica* was the most active fraction against *P. falciparum* parasites, with a melting point of 168-9°C. The spectroscopic data (IR, ^{1}H-NMR, ^{13}C-NMR and mass spectra) were as follows: IR spectral data (Table 1) gave a broad absorptions at 3800 cm^{-1} corresponding to chelated hydroxyl groups, strong peak at 1700 cm^{-1} corresponding to conjugated carbonyl group, a medium peak at 1650 and 1610 cm^{-1} corresponding to C=C of alkenes and benzene ring respectively, and a C-O stretch peak at 950 cm^{-1}.

^{1}H-NMR spectral data (Table 2) indicate that the compound is a flavones with two prenyl groups. The

Table 1. IR parameters of abyssinone-v (Figure 1).

Wavenumbers (cm^{-1})	950	1650/1610	1700	2800-3000	3800
Functional groups	C-O	C=C	C=O	CH_3, or CH_2 or CH	O-H

Table 2. ^{1}H-NMR and ^{13}C-NMR parameters of abyssinone – v (compound 1).

Position of C	^{13}C-NMR δ (ppm)	^{1}H-NMR δ (ppm), *m, J* (Hz)
C-2	79.6	5.4*, d, J* =8.0
C-3	43.3	3.1 *dd,* (*J* = 13, 4.2 (axial)), 2.8 *dd*, (*J* = 14.2, 3.0 (eq))
C-4	196.6	
C-5	164.4	OH = 12.06 *s*
C-6	96.8	5.96 *s*
C-7	165.1	OH = 7.25 *s*
C-8	95.7	6.6 *s*
C-9	163.6	
C-10	103.3	
C-1’	127.8	
C-2’	121.8	7.04 *s*
C-3’	126.3	
C-4’	153.6	OH = 8.2 br, *s*
C-5’	126.3	
C-6’	121.8	7.04 *s*
C-1’’	29.9	3.3 *d*, *J* = 7.0
C-2’’	129.9	5.3 *m*
C-3’’	135.1.	
C-4’’	26.0	1.75 *s*
C-5’’	18.1	1.71 *s*

^{1}H-NMR spectrum of compound 1 showed resonance for two protons, H-3 (axial) at δ 3.1 *dd, J* = 13, 4.2 Hz and H-3 (equatorial) at δ = 2.8 *dd, J* = 14.2, 3.0 Hz, H-2 proton at δ 5.4 d, J = 8.0 Hz, a chelated 5-hydroxyl proton at δ = 12.06 (s). It also showed an aromatic singlet at δ 7.04 integrating for two protons assigned to H-2’ and H-6’ allowing the two prenyl groups at C-3’ and C-5’ in ring B. The prenyl group ware confirmed by a resonance H-1’’ proton exhibiting an AXY spin system at δ = 3.3 *d*, *J* = 7.0 Hz and a multiplet at δ = 5.3 corresponding to one alkene’s proton H-2’’.

The ^{13}C-NMR spectral data (Table 2) showed distinctive peaks at δ = 196.6 corresponding to a carbonyl carbon atom, the alkenes carbon atoms at δ = 129.9 and 135.1 corresponding to C-2’’ and C-3’’.

Compound 1 was identified as 5, 7, 4’-trihydroxy-3’, 5’-diprenylflavon (trivial name is abyssinone - v). The ^{1}H-NMR and ^{13}C-NMR values in Table 2 were comparable to reported literature values of Abyssinone-v (Kamat et al., 1981).

Compound 1 was verified to be Abyssinone - v with molecular formula $C_{25}H_{28}O_5$ by electron impact mass spectral data which showed a molecular ion peak at *m/z* 408.

Anti-plasmodium results

The anti-plasmodium activity criteria in the *in vitro* assay were defined as high when IC_{50} value was below 10 µg/ml, moderate when between 10 – 50 µg/ml and low when between 50 – 100 µg/ml. Drug samples with IC_{50} > 100 µg/ml was considered to be inactive. The anti-plasmodium activity of the methanolic crude extract of *E. abyssinica*, solvents extracts (n-hexane, ethyl acetate and residual methanolic extract) and compound 1 against CQ sensitive *P. falciparum* (D6 strain) are shown in Tables 3 and 4.

The Ethyl acetate fraction (Table 3) of the stem bark of *E. abyssinica* showed the highest anti-malarial activity of IC_{50} value = 1.00 µg/ml. This shows that most of the anti-malarial potency of *E. abyssinica* is concentrated in the ethyl acetate fraction. The chromatographic separation of ethyl acetate fraction led to isolation of the most active

Table 3. *In vitro* anti-plasmodium activity (IC_{50}) of methanolic extracts and solvent fractions of *E. abyssinica*.

Sample	IC_{50} (µg/ml)
Methanolic crude extract	3.50
n-Hexane fraction	59.00
Ethyl acetate fraction	1.00
Methananolic residual fraction	3.80
Chloroquine (CQ)	0.00759

Table 4. *In vitro* anti-plasmodium activity (IC_{50}) of chromatographic fractions of ethyl acetate extract of *E. abyssinica*.

Sample	IC_{50} (µg/ml)
Fraction 1	71.50
Fraction 2	44.30
Fraction 3 (compound 1)	3.20
Fraction 4	3.35
Chloroquine (CQ)	0.00759

Table 5. Interaction of abyssinone-v with ART (Artemisinin).

Combination ratios (%) of Abyssinone-v: artemisinin	90:10	80:20	70:30	60:40	50:50	40:60	30:70	20:80	10:90
Sum FIC	0.95[a]	0.95[a]	0.85[a]	0.79[a]	0.94[a]	0.97[a]	0.99[a]	0.96[a]	0.95[a]
Abyssinone -v IC_{50} IC_{50} (µg/ml)	3.35	3.07	2.78	2.46	2.72	2.16	2.21	1.92	1.02
Artemisinin IC_{50} (ng/ml)	0.09	0.3	0.24	0.33	0.54	1.05	1.06	1.21	1.84

a – synergistic.

compound 1 in fraction 3 with anti-malarial activity of IC_{50} value = 3.20 µg/ml. This shows that compound 1 has a lower activity than the ethyl acetate fraction due to loss in synergism with compounds separated out.

Anti-plasmodial activity of combination of drugs

Drug combination is one of the effective means to counter parasite resistance in anti-malarial chemotherapy (White and Olliaro, 1996). Combinations also help reduce risk of drug resistance development (Olliaro and Taylor, 2003; Anne et al., 2001). The abyssinone-v (compound 1) of *E. abyssinica* was further subjected to synergistic analysis with pure compound of artemisinin (ART) and the following results in Table 5 were obtained.

The interaction of abyssinone-v with the purified Artemisinin showed synergistic effect at all the combination ratios with highest synergism at IC_{50} = 2.46 and 0.33 µg/ml respectively. Therefore abyssinone-v drug has high potential to be used in combination with artemisinins drugs to help in the reduction of recrudescence that take place after use of artemisinins drugs only. Combination chemotherapy for malaria should take advantage of synergistic interactions, as these would enhance therapeutic efficacy and lower the risk of resistance emergence since the combined drugs protect each partner against emergence of resistance (Woodrow et al., 2005).

Conclusion

The most active compound abyssinone-v (Figure 1) isolated from *E. abyssinica* had anti-malarial activity of IC_{50} = 3.19 µg/ml. Although the compound abyssinone-v (1) showed lower activity than pure artemisinin (ART) drug with IC_{50} = 2.76 ng/ml, a combination of abyssinone-v and artemisinin showed a remarkable synergistic activity of sum FIC = 0.79 at a ratio of 2: 3 of the combination with each having IC_{50} = 2.46 µg/ml and 0.33 ng/ml respectively. Abyssinone-v/artemisinin combination has high potential of being developed into a combination therapy for treatment of malaria. This combination

justifies the use of natural products in developing combination drugs.

ACKNOWLEDGEMENTS

The authors acknowledge the Kenya Medical Research Institute (KEMRI), Malaria Unit section for allowing anti-malarial assays work done in their laboratory, Mr James W. Gathirwa of KEMRI, Malarial Unit section for assisting in the anti-malarial analysis and Kabarak University for the partial funding of research work at KEMRI.

REFERENCES

Akala HM, Waters CN, Yenesew A, Wanjala Akenga TA (2010). *In vitro* antiplasmodial and cycli- dependent protein kinase (pfmrk) inhibitory activities of selected flavanoids in combination with chloroquine (CQ) and artemisinin. Res. Pharm. Biotech., 2(4): 40-45.

Ann R, Francoise B, Odile D, Bernard M (2001). From classical anti-malarial drugs to new compounds based on the mechanism of action of artemisinin. Pure Appl. Chem., 73: 1173-1188.

Azas N, Laurencin N, Delmas F, Giorgio C, Gasquet M, Laget M, Timon-David P (2001). Synergistic *in vitro* anti-malarial activity of plant extracts used as traditional herbal remedies in Mali. Parasitol Res., 88: 165-171.

Bai D (1993). Traditional Chinese medicines and new drug development. Pure Appl. Chem., 65: 1103-1112.

Berenbaum MC (1978). A method for testing for synergy with any number of agents. J. Infect. Dis., 137: 122-130.

Bloland PB (2001). Drug resistance in malaria, World Health Organization, GENEVA, Report No. WHO/CDS/CSR/DRS/2001.4.

Canfield CJ, Pudney M, Gutteridge WE (1995). Exp. Parasitol., 80: 373-381.

Dominique D, Karine LR, Pina S, Pietro A, Daniel P, Patrick P, Laurent M, Christian D (2001). Pfnek-1, a NIMA – related kinase from the human malaria parasite Plasmodium falciparum; biochemical properties and possible involvement in MAPK regulation. Eur. J. Biochem., 268: 2600-2608.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD (1979). Quantitative assessment of anti-malarial activity *in vitro* by an automated dilution technique. Antimicrob. Agents Chem., 16: 379.

Fivelman QL, Walden JC, Smith PJ, Folb PI, Barnes KI (1999). The effect of Artesunate combined with standard anti-malarials against chloroquine sensitive and chloroquine resistant strains of *Plasmodium falciparum in vitro.* T. Roy. Soc. Trop. Med. H., 93: 429-432.

Gupta S, Thapar MM, Wernsdorfer WH, Björkman A (2002). *In vitro* interactions of artemisinin with atovaquone, quinine, and mefloquine against *Plasmodium falciparum.* Antimicrob. Agents Ch., 46: 1510-1515.

Kamat VS, Chuo FY, Kubo I, Nakanishi K (1981). Anti-microbial agents from an East African medicinal plant Erythrina abyssinica. Heterocycles, 15: 1163.

Nicholas JW (1994). Artemisinin, current status. T. Roy. Soc. Trop.Med. H., 88(1): 3-4.

Nosten F, van Vugt M, Price R (2000). Effects of artesunate-melfloquine combination on incidence of *Plasmdium falciparum* malaria and melfloquine resistance in Western Thailand: A prospective study. Lancet., 356: 297-302.

Olliaro P, Taylor WRJ (2003). Anti-malarial compounds: from bench to bedside. J. Exp. Biol., 206: 3753-3759.

Scott PL (2005). Principles of Infectious Disease Epidemiology / EPI 220 (htt:// www.ph/ucla.edu/epi/layne/Epidemiology%20220/07.malaria.pdf).

Schlichtherle M, Wahlgren M, Perlmann H, Scherf A (2000). Methods in malaria research, 3rd edn. MR4/ATCC, Manassas, pp. 1-74.

Simon JE, Charles D, Cebert E, Grant L, Janick J, Whipkey A (1990). Artemisia Annua: A promising aromatic and medicinal, 6: 522-526

Snow RW, Trape JF, Marsh K (2001). The past, present and future of childwood malaria mortality in Africa. Trends Parasitol., 17: 593-597.

Sixsmith DG, Watkins WM, Chulay JD, Spencer HC (1984). *In vitro* anti-malarial activity of tetrahydrofolate dehydrogenase inhibitors. Am. J. Trop. Hyg., 33: 772-776.

Thomson RH (1993). The Chemistry of natural products, 2nd ed. pp. 117-121.

Trager W, Jensen JB (1976). Human malaria parasites in continuous culture. Science, 193: 673-675.

Walsh JJ, Coughlan D, Heneghan N, Gaynor C, Bell A (2007). A novel artemisinin – quinine hybrid with potent antimalarial activity. Bioorg. Med. Chem. Lett., 17: 3599-3602.

White NJ, Olliaro PK (1996). Strategies for the prevention of anti-malarial drug resistance: rationale for combination chemotherapy for malaria. Parasitol. Today, 12: 399-401.

WHO (2008). World malaria report. (htt:/www.who.int/malaria/mediacentre/wmr2008/)

Woodrow CJ, Haynes RK, Krishna S (2005). Postgrad. Med. J., 81: 71-78.

Wright CW, Phillipson JD (1990). Natural products and development of selective antiprotozoal drugs. Phytother. Res., 4: 127-129.