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Original Research Article

In vitro antiplasmodial and HIV-1 neutralization activities of root and leaf extracts from *Berberis holstii*

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ABSTRACT

The *in vitro* antiplasmodial activities and HIV neutralizing activities of six crude extracts from the leaves and roots of *Berberis holstii* were evaluated. The antiplasmodial activities were assessed against a chloroquine sensitive 3D7 strain of the malaria parasite, *Plasmodium falciparum*, and the HIV neutralizing activity were assessed against HBX2 and ZM53 pseudovirions. Parasite lactate dehydrogenase (pLDH) assay was used to measure parasite viability and Luciferase Reporter Gene assay was used to measure HIV neutralizing activity. The crude alkaloid extract of the roots showed the most potent anti-parasitic activity (IC_{50} = 0.17 μg/mL) with a selectivity index (SI) of 701.2 while the DCM/MeOH extract also showed good activity (IC_{50} = 2.33 μg/mL). The aqueous and total alkaloid extracts of the roots displayed significant anti-parasitic activity (IC_{50} = 3.64 and 3.85 μg/mL, respectively). The aqueous extract of the leaves displayed good HIV neutralizing activity (IC_{50} = 5.84 μg/mL) against the HXB2 pseudo virus. All the extracts tested displayed low cytotoxicity against TZM-bl cells.

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1. Introduction

Malaria is one of the most prevalent health problems in the tropical and subtropical regions of the world (Muthaura et al., 2007a,b). It is responsible for over 1 million deaths each year and approximately 3.2 billion people living in 107 countries are at risk and over 80% of malaria deaths occur in Africa while 15% occurs in Asia (Dolabela et al., 2008). The disease is particularly devastating in sub-Saharan Africa where it is considered a major obstacle to socio-economic development (Kaur et al., 2007). Malaria continues to be the leading killer of the world's children in developing countries (Laufer et al., 2006) and is also the most frequent cause of morbidity and mortality (Muula et al., 2007). In Malawi, malaria is responsible for over 40% of deaths in children under the age of two (Ndawala et al., 2001). The malaria epidemic risk in Malawi stands at 12% and endemic at 88% with under-five

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mortality rate of 111/1000 (WHO, 2008). The cost of malaria to the average Malawian household has been estimated at US$35 annually or 7.2% of average household income (Ettling et al., 1994). Furthermore, it is estimated that treating 65–85% of cases would result in using 8.9–12.2% of the national health budget and up to 33.2% of the national drug budget (Muula et al., 2007). Malawi opted to use a combination of artesunate (AS) and amodiaquine (AQ) as a first line treatment and artemether/lumefantrine (AL) as a second line of treatment (Bosman and Mendis, 2007). Unfortunately, the implementation of artemesinin-based combination therapies (ACTs) has fallen behind due to various factors such as high costs and affordability (Muheki et al., 2004, WHO, 2010). At a current ex-manufacturer price of 1.35–2.40 US dollars (USD) per adult treatment course, ACTs are 20–40 times more expensive than conventional antimalarial medicines (Bosman and Mendis, 2007).

HIV/AIDS since the 1980s constitutes a devastating global epidemic, and over 40 million people are estimated to be living with the infection (UNAIDS, 2006). The sub-Saharan Africa is the center of this epidemic. The adult HIV prevalence ranges from less than 0.1% in Comoros to 32.4% in Swaziland (UNAIDS, 2006). In Malawi, 12% of the adult population is living with HIV or AIDS and the country has HIV prevalence rates of 25% and 11% in urban and rural areas, respectively (IMF, 2007). AIDS is reported to be the leading cause of death amongst the 15–49 year age group with about 80,000 AIDs related deaths every year (MoH, 2008). Antiretroviral therapy (ART) has been provided free since June 2004 (Makwiza et al., 2009). Despite the beneficial effects of these drugs in improving the quality of life of HIV and AIDS patients, prolonged use of these agents is limited due to the development of virus resistance, appreciable levels of toxicity, high cost, unavailability and above all, the lack of a curative effect (Sanchez-Palomino et al., 2002; Pomerantz and Horn, 2003). Due to these constraints, some communities use medicinal plants which are claimed to have therapeutic properties against malaria and HIV-virus including the management of HIV/AIDS related opportunistic infections. Natural resources therefore, are important sources of new medicines and leads besides tailored synthesis (Martin and Ernst, 2003). The aqueous extracts of the root bark of *Berberis holstii* are used by the communities surrounding Nyika National Park in Malawi for the treatment of malaria and management of HIV/AIDs opportunistic infections (Maliwichi-Nyirenda et al., 2011). This work was therefore undertaken to confirm the traditional use by evaluating the efficacy of *B. holstii* (Fig. 1) as a potential antimalarial and HIV inhibitor.



Fig. 1 – *Berberis holstii* plant (Nyika National Park, September 2010).

2. Materials and methods

2.1. Plant collection and preparation

Fresh plant materials of *B. holstii* were collected from Nyika National Park in Malawi and identified by Mr. A.J. Salubeni a trained taxonomist and a voucher specimen was deposited at the National Herbarium and Botanical Gardens of Malawi (NHBGW) voucher specimen number NHBG-Willis and Luhanga 109. The plant leaf and root materials were cut into smaller pieces and dried. The dried material was ground into fine powder using an electric grinder. The powdered samples were stored in air tight polyethylene bags until analysis.

2.2. Extraction and procedures

The powdered plant root (134 g) and leaf material (135 g) were each extracted with 800 mL of dichlormethane (DCM)/methanol (MeOH) (1:1) overnight at room temperature. Each extract was filtered and the extract concentrated by evaporation under reduced pressure using a Buchi rotary evaporator at 35 °C. Each of the remaining plant materials was then further extracted with 800 mL of purified water overnight, filtered and the aqueous extracts freeze dried. To extract the total alkaloids, the residues from the aqueous extractions were soaked in 800 mL of water acidified with 1 M HCl (pH 3) overnight, filtered, transferred to a 2 L separating funnel and basified with 600 mL of 6 N NH_4OH (pH 8–9). The basified solutions were extracted twice with DCM (2 × 600 mL) and the organic layer was decanted and evaporated *in vacuo* in a rotor evaporator at 35 °C. All the extracts were stored as reduced dry extracts in a cold room at −20 °C.

2.3. Culturing of Plasmodium falciparum

P. falciparum culture 3D7 asexual erythrocytic stages were cultivated continuously *in vitro* as described by Trager and Jensen (1976) at 37 °C and under an atmosphere of 3% CO_2, 5% O_2 and 92% N_2. The host cells were human red blood cells O+ supplied by the South African National Blood Service. The culture medium was RPMI 1640 (Gibco) containing 32 mM $NaHCO_3$,

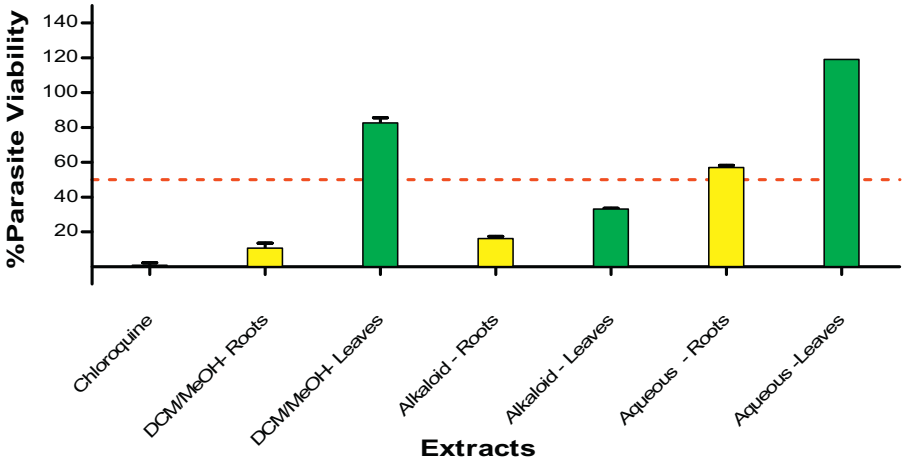


Fig. 2 – Antiplasmodial activities of the crude extracts at a single dose of 10 μg/mL.

25 mM HEPES and L-glutamine. The medium was supplemented with 1.76 g/L glucose (Sigma–Aldrich), 44 mg/mL hypoxanthine (Sigma–Aldrich), 100 mg/L gentamycin (Gibco) and 10% human pooled serum (final haematocrit 1%). Parasitaemia was maintained between 1% and 6%. Medium renewal and microscopic observation (100×) by blood smears fixed with methanol and stained with 10% Giemsa's stain were performed on a daily basis.

2.4. *In vitro test for antiplasmodial activity*

2.4.1. *Single dose concentration*

Stock solutions of the six crude extracts were made by dissolving each extract in DSMO followed by dilution with culture medium to a final working concentration of 10 μg/mL in 96-well flat bottom plates (Greiner Bio-one). Parasitized red blood cells were added to a final concentration of 1% haematocrit, 2% parasitaemia and the plates incubated for 48 h. Percentage parasite survival in each well was calculated relative to control wells that received nothing.

2.4.2. *Inhibitory concentration (IC_{50}) values*

Stock solutions (5 mg/mL) for the four most effective extracts from single dose antiplasmodial evaluation, were made by dissolving each extract in DMSO in a laminar flow hood followed by subsequent dilution to lower the concentration of DMSO to less than 1%. Reference drug chloroquine diphosphate (Sigma, 1 μg/mL) was prepared in a similar way. Parasite viability was measured using Parasite lactate dehydrogenase (pLDH). Inhibitory activity was determined by preparing serial dilutions of the four test extracts in a transparent 96-well flat bottom plate (Greiner Bio-one). Parasited red blood cells were added to a final concentration of 1% haematocrit, 2% parasitemia and the plate incubated for 48 h. Extracts were tested in 11 serial threefold dilutions (final concentration range 100–0.002 μg/mL for each test extract). All tests were performed in triplicate. The absorbance was determined using a multi-well spectrophotometer (Tecan Infinite F 5000) at 620 nm. The IC_{50} values were obtained from dose–response curves, using non-linear dose–response curve fitting analyses with GraphPad Prism v.3.00 software. Chloroquine diphosphate (Sigma) served as the positive control.

2.5. *Neutralizing activity on HIV-1 pseudovirions*

Stock solutions of the six crude extracts were made by dissolving 2 mg of each extract in DMSO (200 μL) and then diluted with Dulbecco's Modified Eagle's Medium (DMEM) to a final working concentration of 1 mg/mL. 60 μL of DMEM was added to all wells, and 85 μL to cell control (CC) wells. Each of the six crude extracts (15 μL of 1 mg/mL) was added to bottom row wells, in duplicate, then serial diluted to upper wells in 5-fold by transferring 15 μL media to upper wells. 15 μL of the top row wells were discarded. HXB2 pseudo virus (25 μL of approximately 20,000 RLU) was added to all wells, except CC followed by addition of TZM-bl cells (50 μL of 2×10^5 cells/mL) to all wells. The plate was incubated for 48 h at 37 °C and 5% CO_2. Supernatant (75 μL) was removed from all wells and discarded. Bright Glo luciferase substrate (50 μL (Promega)) was added to all wells and the plate incubated for 2 min. The cell supernatant (50 μL) from all wells was transferred to a black, flat bottom NUNC plate and luminescence was obtained using a luminometer read at 450 nm. The same procedure was used for assessing the activity of ZM53 pseudo virus. The results were analyzed using Excel and Graph pad Prism5. Positive controls T20 was prepared by using 1 mg/mL and 100 μg/mL working stock dissolved in PBS, and Tenofovir was prepared by using 1 mg/mL working stock dissolved in ddH_2O.

2.6. *Cytotoxicity assay*

DMEM (60 μL) was added to all wells followed by the six crude extracts (15 μL of 1 mg/mL) to the bottom row wells in duplicate and serial diluted to upper wells in 5-fold by transferring 15 μL media to the upper wells. The 15 μL of the top row wells were discarded. DMEM (25 μL) was added to all wells to replace the virus volume. TZM-bl cells (50 μL of approximately 2×10^5 cells/mL) were added to all wells, except the blank wells where 50 μL of media was added. The plate was incubated for 48 h, at 37 °C and 5% CO_2. PMS/MTS mix (28.5 μL) was added to all wells and the plates incubated for 45 min, at 37 °C and 5%

CO_2. Absorbance was obtained using a luminometer at 492 nm and the results analyzed using Excel and Graph pad Prism. The positive control Etoposide was prepared by using 10 mg/mL stock dissolved in 100% DMSO, diluted to 1 mg/mL working stock in DMEM.

3. Results and discussion

3.1. *Antiplasmodial activities of the extracts*

The antiplasmodial activities of all six crude extracts at a single dose concentration of 10 μg/mL are shown in Fig. 2. The results show that three extracts namely; DCM/MeOH of the roots and the alkaloid extracts of the roots and leaves had a significant effect on parasite viability (<50% parasite viability) while the aqueous extract of roots exhibited a moderate effect on parasite viability (>50% parasitic viability). This initial concentration was selected as a screen to eliminate less potent extracts. Inhibition of parasite growth at low concentrations of the extracts indicates selective activity as opposed to non-specific activity which is often observed at higher concentrations where host cells for the parasites are also affected through toxicity (Clarkson et al., 2004).

The root extracts exhibited better anti-parasitic activity than those from the leaves. This is consistent with the fact that the roots of this plant are traditionally used in the treatment of malaria. In some published results for the study of some Iranian plants, the aerial parts of *Berberis crataegina* did not show significant activity against *P. falciparum* (Esmaeili et al., 2009). The four most effective extracts (Fig. 2) were further evaluated to determine the inhibitory concentration. The results of the IC_{50} values of these extracts against the chloroquine sensitive 3D7 strain are presented in Table 1.

Antiplasmodial activities were classified as follows: good $IC_{50} \leq 5$ μg/mL, moderate $5 < IC_{50} \leq 10$ μg/mL and low $IC_{50} > 10$ μg/mL (Deharo et al., 2001). All the extracts exhibited good activities with the IC_{50} values all <5 μg/mL. Chloroquine had an IC_{50} value of 0.00483 μg/mL which is the normal range for the drug and this demonstrates that the bioassay with the appropriate controls was correctly executed. The crude alkaloid extract of the roots displayed the most effective antiplasmodial activity ($IC_{50} = 0.17$ μg/mL) followed by DCM/MeOH extract of the roots ($IC_{50} = 2.33$ μg/mL), aqueous extract of the roots ($IC_{50} = 3.64$ μg/mL) and the alkaloid extract of the leaves ($IC_{50} = 3.85$ μg/mL). Furthermore, the most active alkaloid extract, *i.e.* the root, exhibited the best selectivity (SI = 701.2) indicative of selective toxicity to the parasites. When the SI ≥ 10, the biological efficacy is considered not to be as a result of *in vitro* cytotoxicity (Vonthron-Senecheau et al., 2003). The observed activity of aqueous extracts is significant. Aqueous extracts rarely exhibit good antiplasmodial activities compared with other organic extracts. In a study of antiplasmodial activities of medicinal plants native to or naturalized in South Africa, the majority of the aqueous extracts did not show any activity (Clarkson et al., 2004). Similar results were reported in the study of antimalarial activities of some plants from Meru district in Kenya (Muthaura et al., 2007a,b). Secondly, aqueous extracts are the preferred method of preparing most herbal concoctions (Fennell et al., 2004). The results from the antiplasmodial activities of the crude extracts tested provide scientific evidence for the traditional use of the plant as an antimalarial.

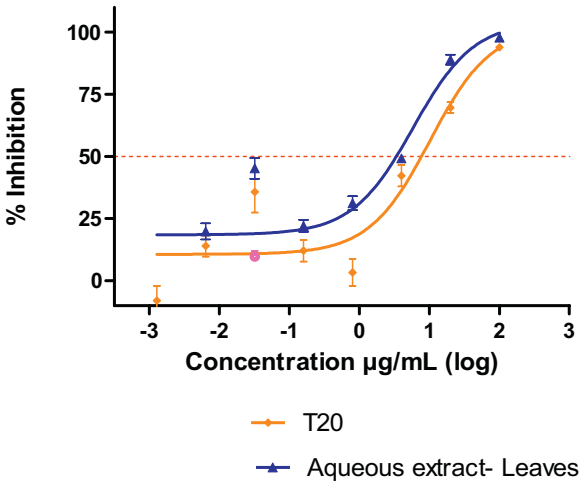


Fig. 3 – Dose–response curve for activity of leaf aqueous extract against HXB2 virus compared with T20 drug.

3.2. *In vitro HIV-1 neutralizing activities of the extracts*

The results of the neutralizing activity of the plant extracts against two HIV pseudovirions HXB2, an HIV-1 subtype B CXCR4 virus and ZM53, an HIV-1 subtype C CCR5 virus are presented in Table 2.

The aqueous extract of the leaves exhibited good neutralizing activity against the HXB2 virus ($IC_{50} = 5.84$ μg/mL). This activity was in the same order of magnitude to the fusion inhibitor T20 drug ($IC_{50} = 7.55$ μg/mL). Fig. 3 shows a dose–response curve comparison of the root aqueous extract and that of T20 drug against the HBX2 virus. A linear neutralizing activity is exhibited by the dose–response curves. All other extracts did not show neutralizing activities against both HXB2 and ZM53 pseudovirions ($IC_{50} > 50$ μg/mL). The reverse transcriptase inhibitor, Tenofovir had a much higher activity ($IC_{50} < 0.1$ μg/mL) against the HXB2 virus.

Although aqueous extracts contain few of the lipophilic compounds obtainable in organic extracts, some aqueous extracts have been reported to exhibit significant neutralizing activity against the HIV-1 virus. In an *in vitro* study of anti-HIV activity of some medicinal plant extracts used as folk medicines in Iberian Peninsula, antiviral activity was shown in the aqueous extracts rather than the ethanolic extracts (Bedoya et al., 2001). Furthermore, aqueous extracts from medicinal Thai plants showed higher percentage HIV-1 RT inhibition ratios than ethanol extracts from the same plants (Woradulayapinij et al., 2005). The aqueous extract of the leaves which showed potent activity was not cytotoxic ($CC_{50} = 349.5$ μg/mL) with the selectivity index of 59.8 against the HBX2 virus. The results are significant as they raise the prospect of obtaining potential antiviral compounds and remedies.

Table 1 – Inhibitory concentrations (IC_{50}) values of extracts against *P. falciparum* 3D7 strains.

Part of plant	Extract	IC_{50} (μg/mL)	CC_{50} (μg/mL)	SI[a]
Roots	DCM/MeOH	2.33 ± 0.23	61.7 ± 2.84	26.5
	Aqueous	3.64 ± 0.35	147.0 ± 7.67	40.4
	Alkaloid	0.17 ± 0.02	119.2 ± 5.13	701.2
Leaves	Alkaloid	3.85 ± 0.11	50.4 ± 3.16	14.1
	Chloroquine	0.00483 ± 0.0004		

[a] SI, selectivity index = CC_{50}/IC_{50}.

Table 2 – Summary of HIV-1 inhibitory concentrations (IC_{50}) and cytotoxicity (CC_{50}) values of extracts.

Part of plant	Extract	IC_{50} (μg/mL)		CC_{50} (μg/mL)	Selectivity index[b]	
		HXB2	ZM53		HXB2	ZM53
Roots	DCM/MeOH	369.3 ± 6.73	274.2 ± 7.65	61.7 ± 2.84	0.17	0.23
	Aqueous	107.3 ± 4.14	30.1 ± 2.28	147.0 ± 0.67	1.37	4.88
	Alkaloid	171.1 ± 3.71	418.9 ± 3.12	119.2 ± 5.13	0.70	0.28
Leaves	DCM/MeOH	62.3 ± 4.18	1086 ± 2.15	91.3 ± 6.43	1.47	0.08
	Aqueous	5.84 ± 1.89	50.83 ± 4.48	349.5 ± 9.42	59.85	6.88
	Alkaloid	115.2 ± 2.14	57.03 ± 1.17	50.4 ± 3.16	0.44	0.88
	T20	7.55 ± 1.22	1.04 ± 0.68			
	Tenofovir	0.068 ± 0.04	0.42 ± 0.12			
	Etoposide	–	–	15.36 ± 2.36		

[b] Selectivity index calculated as CC_{50}/IC_{50}.

It is worth mentioning that although the activities of the crude extracts in this work are compared to the positive controls, which are pure compounds, natural crude extracts contain complex mixtures of compounds. The good activities shown by the extracts could either be due to individual compounds or synergism. The compounds responsible for the activities once isolated may therefore exhibit better or comparable activities to the positive controls or indeed loose activity when fractionated (Houghton, 2000). This needs to be evaluated and this work is currently on-going.

4. Conclusion

The study has revealed for the first time, the *in vitro* antiplasmodial and HIV neutralizing potential of crude extracts of *B. holstii*. The crude alkaloid extract of the roots exhibited the most potent anti parasitic activity and the aqueous extract of the leaves showed good HIV-1 neutralizing ability. The different activity and safety studies performed in this work confirm the ethnopharmacological usefulness of the plant. It is envisaged that further studies of the active extracts may lead to developments of new and effective phytomedicines for treating HIV and malaria.

Conflict of interest statement

None declared.

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REFERENCES

Bedoya LM, Sanchez-Palomino S, Abad MJ, Bermejo P, Alcami J. Anti-HIV activity of medicinal plant extracts. J Ethnopharmacol 2001;77:113–6.

Bosman A, Mendis KN. A major transition in malaria treatment: the adoption and deployment of artemisinin-based combination therapies. Am J Trop Med Hyg 2007;77:193–7.

Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, et al. *In vitro* antiplasmodial activity of medicinal plants native to or naturalised in South Africa. J Ethnopharmacol 2004;92:177–91.

Deharo E, Bourdy G, Quenevo C, Munoz V, Ruiz G, Sauvain M. A search for natural bioactive compounds in Bolivia through a multidisciplinary approach: Part V. Evaluation of the antimalarial activity of plants used by the Tacana Indians. J Ethnopharmacol 2001;77:91–8.

Dolabela MF, Oliveira SG, Nascimento JM, Peres JM, Wagner H, Póvoa MM, et al. *In vitro* antiplasmodial activity of extract and constituents from *Esenbeckia febrifuga*, a plant traditionally used to treat malaria in the Brazilian Amazon. Phytomedicine 2008;15:367–72.

Esmaeili S, Naghibi F, Mosaddegh M, Sahranavard S, Ghafari S, Abdullah NR. Screening of antiplasmodial properties among some traditionally used Iranian plants. J Ethnopharmacol 2009;12:400–4.

Ettling M, Mcfarland DA, Schultz LJ, Chitsulo L. Economic impact of malaria in Malawian households. Trop Med Parasitol 1994;45:74–9.

Fennell CW, Lindsey KL, McGaw LJ, Sparg SG, Stafford GI, Elgorashi EE, et al. Assessing African medicinal plants for efficacy and safety: pharmacological screening and toxicology. J Ethnopharmacol 2004;94:205–17.

Houghton P. Use of small-scale bioassays in the discovery of novel drugs from natural sources. Phytother Res 2000;14:419–23.

International Monetary Fund, World Bank. Malawi: decision point document for the enhanced heavily indebted poor countries initiative. Washington: World Bank; 2007.

Kaur K, Patel SR, Patil P, Jain M, Khan SI, Jacob MR, et al. Synthesis, antimalarial, antileishmanial, antimicrobial, cytotoxicity, and methemoglobin (MetHB) formation activities of new 8-quinolinamines. Bioorg Med Chem 2007;15:915–30.

Laufer MK, Thesing PC, Eddington ND, Masonga R, Dzinjalamala FK, Takala SL. Return of chloroquine antimalarial efficacy in Malawi. N Engl J Med 2006;355:1959–66.

Makwiza I, Nyirenda L, Bongololo G, Banda T, Chimzizi R, Theobald S. Who has access to counseling and testing and anti-retroviral therapy in Malawi – an equity analysis. Int J Equity Health 2009;8:13.

Maliwichi-Nyirenda CP, Maliwichi LL, Franco M. Medicinal uses of *Berberis holstii* Engl. (Berberidaceae) in Malawi, the only African endemic barberry. J Med Plants 2011;5:1367–73.

Martin KW, Ernst E. Antiviral agents from plants and herbs: a systematic review. Antiviral Ther 2003;8:77–90.

Ministry of Health. HIV and syphilis sero-survey and National HIV prevalence and AIDS estimates report for 2007. Lilongwe, Malawi: MoH; 2008.

Muheki C, McIntyre D, Barnes KI. Artemisinin-based combination therapy reduces expenditure on malaria treatment in KwaZulu Natal, South Africa. Trop Med Int Health 2004;9:959–66.

Muthaura CN, Rukunga GM, Chhabra SC, Omar SA, Guantai AN, Gathirwa JW, et al. Antimalarial activity of some plants traditionally used in Meru district of Kenya. Phytother Res 2007a;21:860–7.

Muthaura CN, Rukunga GM, Chhabra SC, Omar SA, Guantai AN, Gathirwa JW, et al. Antimalarial activity of some plants traditionally used in treatment of malaria in Kwale district of Kenya. J Ethnopharmacol 2007b;112:545–51.

Muula AS, Rudatsikira E, Siziya S, Matiya RH. Estimated financial and human resources requirements for the treatment of malaria in Malawi. J Malaria 2007;6:168.

Ndawala J, Kalanda G, Mahy M. Malaria. In: Malawi demographic and health survey 2000 report. Zomba, Malawi: National Statistical Office; 2001.

Pomerantz RJ, Horn DL. Twenty years of therapy for HIV-1 infection. Nat Med 2003;9:867–73.

Sanchez-Palomino S, Abad MJ, Bedoya LM, García J, Gonzales E, Chiriboga X, et al. Screening of South American plants against human immunodeficiency virus: preliminary fractionation of aqueous extract from *Baccharis trinervis*. Biol Pharm Bull 2002;25:1147–50.

Trager W, Jensen JB. Human malaria parasites in continuous culture. Science 1976;193:673–5.

UNAIDS. Report on the global AIDS epidemic. Geneva: UNAIDS; 2006.

Vonthron-Senecheau C, Weniger B, Quattara M, Bi FT, Kamenan A, Lobstein A, et al. *In vitro* antiplasmodial activity and cytotoxicity of ethnobotanically selected Ivorian plants. J Ethnopharmacol 2003;87:221–5.

Woradulayapinij W, Soonthornchareonnon N, Wiwat C. *In vitro* HIV type 1 reverse transcriptase inhibitory activities of Thai medicinal plants and *Canna indica* L. rhizomes. J Ethnopharmacol 2005;101:84–9.

World Health Organisation. Guidelines for the treatment of malaria. Geneva, Switzerland: World Health Organisation; 2010.

World Health Organisation. WHO world malaria report 2008. Geneva, Switzerland: World Health Organisation; 2008.