ANTIMALARIAL ACTIVITY OF CROTON MACROSTACHYUS EXTRACTS AGAINST PLASMODIUM SP.

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Abstract

The use of medicinal plants to cure parasitic infections has been practiced for time immemorial. Malaria control using plant extracts can be targeted against the parasites both *in vitro* and *in vivo* or against the female anopheline vectors. Some chemical constituents in plants with antimalarial activity include the alkaloids, terpenes, terpenoids, epoxides, quinones and various steroidal compounds.The overall aim of this study is to isolate and test antimalarial compounds from the extracts of C. *macrostachyus in vivo*. The data for this study was obtained by an experimental case-control procedure using inbred Balb/c mice for treatment and control groups. Mice in groups of six were infected with Plasmodium berghei (ANKA) and treated with various concentrations of *C. macrostachyus* extracts, while artemether was used as positive control and Tween-80 as a negative control. An in vivo treatment assay of *C. macrostachyus* against P. *berghei* showed percent suppression of parasitaemia for ethyl acetate extract positive control (87.64±1.264), 500mg/kg(81.71±0.728), 250mg/kg(81.71±0.728) and 100mg/kg (61.82±0.571). The methanol extract showed percent suppression of parasitaemia for positive control (97.22±0.225), 500mg/kg(68.14±0.670), 250mg/kg (33.61±0.609) and 100mg/kg (27.44±0.443). The aqueous extract results were for positive control as 99.20±0.156, 500mg/kg, 71.85±0.447, 250mg/kg, 44.23±0.064 and 100mg/kg 24.36±0.447. The butanol extract give results for positive control as 100±0.000, 500mg/kg as 80.44±1.259, 250mg/kg as 60.66±0.445 and 100mg/kg as 72.69±0.306. Analysis of variance showed significant differences in the suppression of parasitaemia p(<0.001).
The study showed that it is possible to control the growth of parasites by various extracts of *C. macrostachyus in vivo* in Balb/c mice. *C.* macrostachyus extracts can be further studied and purified for possible incorporation into antimalarial drug production.

Key words: *Croton macrostachyus, Plasmodium falciparum D6, Plasmodium berghei* ANKA, parasitaemia

Introduction and Literature Review

Medicinal plants have been used to cure parasitic infections from time immemorial. It is estimated that the number of medicinal plants vary between 30,000 – 75,000 (Norman et. al., 1985). For the past two decades there has be increased use of medicinal plants but little research has been carried out on them (WHO, 2009). The World Health Organization has compiled a list which contains 20,000 medicinal plants that are used all over the world. It has also been stated that about 4000 plant drugs have been widely used and that in Western Europe, about 400 plant drugs are widely traded (Husnu, 1996). Less than 30% of these

registered plant species have been analyzed for potential medicinal properties.

A world health organization study has shown that 80% of the world's population relies solely upon medicinal plant as a source of remedies for the treatment of diseases, (Geoffrey, 1996). In China, India, Africa and Latin America, modern drugs are simply not available, or, if they are, they often prove to be too expensive, unavailable or inaccessible. Furthermore social factors may also make the application of "conventional" medical treatment difficult. The majority of drugs that are active against infectious agents are in fact developed from natural products ("leads"). As a source of novel drugs, plants remain grossly understudied and under-used, especially in the developed world. Quinine, which was obtained from species of cinchona originating in South America, remains a vital drug in the treatment of malaria. Except for antifolate antimalarial drugs, all the other commonly used antimalarial molecules are based upon plant-derived compounds (Geoffrey, 1996).

There is a new trend in the world today to turn back to natural substances due to the various side effects by some synthetic drugs (Huang et al., 1992). Production of unrefined drugs for export is also becoming very common in some countries. Examples include morphine from the opium poppy (*Papaver sominferum*), one of the best known pain killers. Coca leaves are also known to contain a strong stimulant (cocaine). Some steroid products with medicinal value have been isolated from plants; for example, diosgenin from *Dioscorea* species (Bergner, 2001) and stigmasterol from the soya bean (Carter et. al., 2007). The study for herbal remedies in modern medicinal and agricultural practices is also being enhanced by pharmaceutical companies and research institutions in various countries where exploration of the medicinal plants is taking place (Hodgkin, 1991; Kofi-TseKpo, 1993a and 1993b). This will help in the management of forests and generating some foreign exchange to these countries. Plants also provide useful active compounds used to make insecticides, fungicides (pyrethrum plant) and industrial raw materials. The antimalarial activity of Ajuja remota has also been determined in vivo (Kuria et. al., 2011).

Malaria is one of the major parasitic infections in many tropical and subtropical regions, (Leonardo et al., 1994). . *Plasmodium falciparum* is the most important agent of human malaria transmitted by the *Anopheles* mosquito in the human blood. It is a major cause of morbidity and mortality, claiming an estimated one to two million lives year in Africa alone (Geoffrey, 1996). The resistance of P. *falciparum* to 4-aminoquinones (chloroquines, amodiaquine), antifols, and in some areas of Southeast Asia, the emergence of *in vitro* and *in vivo* resistance to aminoalcohols (quinine, mefloquine, halofantrine) have been reported (Leonardo et al., 1995). Problems associated with malaria control are mostly associated with growing drug resistance by the parasite. Some plant extracts used for the cure of malaria include *Enantia chlorantha* and *Rauwalfia vomitaria* (Agbaje and Elueze, 2006). The plant to be used in this study against *Plasmodium spp*. is *Croton macrostachyus*. It has been used traditionally to treat some diseases in Kenya (Kokwaro, 2009).

Members of the genus *Croton* have also been widely used for treatment against malaria and other microbial diseases. There is need for more drug discovery for use in both complicated and uncomplicated cases of the disease and for prophylaxis purposes.

Materials and Methods

C. macrostachyus bark was obtained from Baraton area in Nandi County of Kenya. Baraton is 10 km from Kapsabet, the headquarters of Nandi Central District. It is located in the highlands of Kenya and has a cool climate. Tea is grown as a cash crop and maize on a small scale and subsistence level of farming. Highland malaria is seasonal, with epidemic cases observed mainly from May to August. This is when the rainy season is at its peak and hence malaria transmission is optimum. Sample of plant were deposited at the Herbarium in the Department of Biological Sciences, University of Eastern Africa, Baraton and The National Museums of Kenya for identification. Assays were carried out at the Kenya Medical Research Institute (KEMRI) department of malaria. Chemical analysis was done at the University of Eastern Africa Baraton, Department of Chemistry and the University of Illinois at Chicago.

The study design was a quantitative case control study in which infected mice were treated with various extract concentrations and control groups consisted of a positive control treated with artemisin and an untreated negative control.

An acute toxicity test was carried out to ascertain the safety of the extract in clean, uninfected BALB/c mice. Five groups of six clean uninfected

BALB/c mice each were used in the toxicity test. The groups of six mice were given an oral dose 0.2ml per animal of either 500mg/kg, 250mg/kg or 100mg/kg body weight doses of the extract. One group was given 0.2ml per animal of 10% Tween and another group was given artemether positive control. The weights of all animals were taken before and after the experiment. The animals in each group were observed for any change in physical activity and signs of abnormal growth or disease condition also. These included observations of mortality, hair erection, tremors, lacrimation, convulsions, salivation, diarrhea, and abnormal features in organs and blood.

Consent was obtained from the ethical committee of the medical research institute to use the experimental animals in the study. BALB/c male mice 6-8 weeks old weighing 20 ± 2g were selected for the in vivo study. Each group of 30 BALB/c mice was infected by injecting 2 x 107 erythrocytes parasitized with *Plasmodium berghei* strain ANKA intraperitoneally. For each extract, 6 animals were selected to be tested as positive controls, negative controls, 500, 250 and 100 ug/kg body weight respectively. One group of mice was infected but not treated and served as untreated controls (NC). Another group was treated with artemether, and therefore served as treated or positive control group (PC). The other groups were treated with the crude ethyl acetate, butanol, methanol and aqueous extracts at 500, 250, and 100 mg/kg body weight. The plant fractions were dissolved in 10% w/v Tween 80 with the aid of ultrasonication and was further diluted with distilled water to achieve an end concentration of 500 mg/kg body weight (Gessler et. al., 1995). This concentration was then used to make 250 and 100mg/kg body weight for each of the extract. The animals were treated orally once on days 0, 1, 2, and 3 with a volume of 0.02 ml/g body weight. The mice received NAFAG pellets (9009 PAB – 45) as diet and were held at room temperature (Peters et al., 1995). The survival of the mice in all the groups was checked twice a day. Parasitized erythrocytes was counted in Giemsa stained thin films from tail blood on day 4. The percentage suppression of parasitaemia for each plant extract or fraction will be calculated as:

$$PSP = \frac{100 - 100x\ (\text{mean\% parasitaemia in treated mice})}{\text{mean\% parasitaemia in control mice}}$$ (Gessler et al., 1995).

The percent parasitaemia and percent suppression of parasitaemia was analysed using analysis of variance (ANOVA (SPSS 20.0 for Windows) to compare variation among the treatment group and the untreated control groups. A Tukey's honestly significant difference test was conducted to make a pairwise comparison between different treatment groups and control.

Results

Acute Toxicity

The results from the toxicity experiment showed that all animals of the ethyl acetate group were normal at the end of the study period. No adverse effect was observed in this group. The animals were normal during observations and at the end of the study period. A similar observation was made for the methanol and water extract groups. The animals in the butanol extract treatment group however showed some signs of acute toxicity. The animals showed signs of tremor on the third day. On a closer observation, the fur seemed thin and slightly colored unlike the usual white fur and were erected. No animal experienced salivation, lacrimation, diarrhea or convulsions. Two animals that received butanol extract died before the end of the treatment period.

Table 1

Mean ± S.E. of percent parasitaemia for the in vivo experiment after infected BALB/c mice were treated with different extract concentrations and a positive control

Extracts / Treatments	EtOAc	MeOH	Aqueous	BuOH
PC	2.15±0.22	0.51± 0.23	0.15±0.09	0.00±0.00
NC	17.39±0.27	18.33±0.38	18.72±0.90	18.20±1.30
500	3.18±0.42	5.84±0.45	5.27±0.38	3.56±0.57
250	7.26±0.31	12.17±0.66	10.44±0.56	7.16±1.28
100	6.64±0.54	13.30±0.67	14.16±0.56	4.97±1.59

The results of treatment with ethyl acetate showed that the 500mg/kg dose give an average percent parasitaemia of2.15±0.22, the500mg/kg 3.18±0.42, the 250mg/kg 7.26±0.31 and the 100mg/kg 6.64±0.54. The methanol extract give percent parasitaemia of chloroquine control as 0.1±0.23, 500mg/kg as 5.84±0.45. This was the best result because the other concentrations showed higher values(Table 13). The methanol extract also showed that 500mg/ml had an average percent parasitaemia of 5.84±0.45, 12.17±0.66 and 100mg/kg 13.30±0.67. The aqueous extract experiment had a chloroquine control group of 0.15±0.09, a 500mg/kg group of 5.27±0.38, 250mg/kg of 10.44±0.56 and 100mg/kg of 14.16±0.56. The butanol extract had parasitaemia for the positive control group as 0.00±0.00, 500mg/kg as 3.56±0.57, 250mg/kg as 7.16±1.28 and 100mg/kg as 4.97±1.59. For all the negative control groups, percent parasitaemia were not less than 17 % (Table 1).

Table 2

Tukey's multiple comparison test for the effect of extracts of C. macrostachyus on P. berghei parasitaemia in BALB/c mice

Comparison	EtOAc		MeOH		Aqueous		BuOH	
	p-value	sig	p-value	sig.	p-value	sig	p-value	sig
PC vs NC	0.000	S	0.000	S	0.000	S	0.000	S
PC vs 500	0.346	NS	0.000	S	0.000	S	0.246	NS
PC vs 250	0.000	S	0.000	S	0.000	S	0.110	NS
PC vs 100	0.000	S	0.000	S	0.000	S	0.046	NS
NC vs 500	0.000	S	0.000	S	0.000	S	0.000	S
NC vs 250	0.000	S	0.000	S	0.000	S	0.000	S
NC vs 100	0.000	S	0.000	S	0.000	S	0.000	S
500 vs 250	0.000	S	0.000	S	0.000	S	0.998	NS
500 vs 100	0.000	S	0.000	S	0.000	S	0.977	NS
250 vs 100	0.797	NS	0.519	NS	0.001	S	0.998	NS
ANOVA	F=262.258		F=187.620		F=168.168		F=32.243	

Key: PC=positive artemether control; NC=negative tween 80 control; EtOAc=ethylacetate extract; MeOH= methanol extract; Aqueous = aqueous extract; BuOH= butanol extract; S=significant; NS=not significant

The Tukey honestly significant difference test showed that there were significant differences in percent parasitaemia between the positive control and negative control for all the in vivo extract treatement experiments, $p<0.001$. The percent parasitaemia for the positive controls were significantly lower than that of the 500mg/kg treatment groups in the methanol and aqueous treatment groups, $p<0.001$, while not significantly different ($p>0.05$) for the ethyl acetate and butanol treatment groups. The percent parasitaemia for the positive controls were all significantly lower than the 250 mg/kg group in the ethyl acetate, methanol and aqueous extract groups, but was not significantly different for the butanol extract treatment group, $p>0.05$. The mean percent parasitaemia of the artemeter positive control was also significantly lower than that of the 100mg/kg dose group for the ethyl acetate, methanol and aqueous extract treatment groups ($p<0.001$), but not significantly different for the butanol group ($p>0.05$). The mean percent parasitaemia of the negative control group was significantly higher than the 500mg/kg, 250mg/kg and 100mg/kg group for all the extract treatments ($p<0.001$). The mean percent parasitaemia for the 500mg/kg dose treated animals was significantly lower than the 250mg/kg dose in the ethyl acetate, methanol and butanol extract treatment groups ($p<0.001$) and not significantly different in the butanol extract treatment group. The percent parasitaemia of the 250mg/kg dose was not significantly different from the 100mg/kg dose in the ethyl acetate, methanol and butanol extract treatment groups but they were significantly different in the aqueous extract treatment groups ($p<0.05$) (Table 2).

Discussion and Recommendations

Several mechanisms have been proposed for the activity of the extracts from *C. macrostachyus* against *P. berhei in vivo*. According to Kokwaro, (2009), *C. macrostachyus* crude extract has been used traditionally to treat malaria, mumps, diarrhea and other ailments. The *in vivo* treatment assay showed a decrease in parasite density for all the extract groups after treatment. The best result obtained for the percent suppression of parasitaemia was for the aqueous extract (99.20±0.156). The activity of *C. macrostachyus* extracts in vivo is comparable to results from studies in which antiplasmodial activity has been related to a range of several classes of secondary plant metabolites including alkaloids and sesquiterpenes, triterpenes, flavonoids, inonoids, quassinoids (Salatino, 2007). These compounds are mostly amphiphiles and are said to protect erythrocytes against hypotonic hemolysis (Hagerstrand and Isomaa, 1994).

In this study using C. *macrostachyus* crude extracts, it was found that the best result for percent suppression of parasitaemia was obtained by the 500mg/kg dose of the ethyl acetate extract (99.88±0.058). Immunoprophylaxis study has been conducted on *Mastomys coucha* using Withanferin A isolated from *Withania somnifera* and this protected the rodents from infection with *Brugia malayi*, a filarial worm (Kushwahaa et. al., 2011). The results from C. *macrostachus* using BALB/c mice proved that the mice developed immunity to P. berghei and were able to destroy the parasites *in vivo*. Stomatocytes were observed in the blood film and parasitaemia decrease was probably due to membrane curvature changes that were unsuitable for the parasite survival (Ziegler, 2002).

Conclusion

The *in vivo* assay showed that the extracts of C. *macrostachyus* were able to significantly suppress parasites in infected Balb/c mice. The tukey's test showed that some of the extract suppression of parasitaemia were comparable to artemether, the control drug.

The methanol, aqueous and ethyl acetate extracts of *Croton macrostachyus* have shown potential as antimalarial agents. They are able to indirectly control Plasmodium parasites multiplication through reversible erythrocyte membrane modification to form stomatocytes that will destroy malaria parasites and later on transform to normal red blood cells.

The aqueous extract suppressed parasitaemia the highest in the immunoprophylaxis study. The butanol extract proved to be very effective in the treatment of mouse malaria, but was lethal in high doses. It should be prepared in extremely low doses if it is to be considered as a potential antimalarial drug. At 100mg/kg body weight, all the extracts were potent as immunoprophylactic agent. All animals given the doses before infection and treatment survived and had low or no parasitaemia level at the end of the study period. *Croton macrostachyus* extracts had active immunoprophylactic properties against *P. berghei.* It can therefore be concluded that the extracts of *C. macrostachyus* should be tested on a

larger sample in animals for possible clinical trials to be conducted for approval for human use.

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References

Agbaje, E.O. and Elueze, N.R. (2006). Antimalarial activities of *enantia chlorantha* and *rauwalfia vomitaria* extracts in rodents malaria. *Tropical Journal of Medical Research, 10*(1), 11-14.

Bergner, P. (2001). Discorea: Wild yam and hormonal synthesis, *Medical Herbalism, Journal for the Clinical Practioner, 4*(4), 9.

Carter, B. A., Taylor, O. A., Prendergast, D. R., Zimmerman, T. L., Von Furstenberg, R., Moore, D. D., & Karpen, S. J. (2007). Stigmasterol, a soy lipid-derived phytosterol, is an antagonist of the bile acid nuclear receptor FXR. *Pediatric Research, 62*, 301-306.

Gessler, M. C., Tanner, M., Chollet, J., Nkunya, M. H. H., & Heinrick, M. (1995). Tanzanian medicinal plants used traditionally for the treatment of malaria: *In vitro* anti malarial and *in vitro* cytotoxic activities. *Phytotherapy Research, 9*(7), 504-508.

Gurib-Fakim, A. (2006). Medicinal Plants: Traditions of yesterday and drugs of Tomorrow, *Molecular Aspects of Medicine, 27*, 1-93.

Hägerstrand, H. & Isomaa, B. (1994). Lipid and protein composition of exovesicles released from human erythrocytes following treatment with amphiphiles. *Biochem. Biophys. Acta, 1190*, 409-415.

Hodgkin, D. (1991). Seeking cures in the Jungle. *Insight, 7*, 30.

Huang, P. L., Huang, H. I., & Lee-Huang, S. (1992). Developing drugs from traditional medicinal plants, *Chemistry and Industry, 8*, 290-293.

Kokwaro, J. O. (2009). *Medicinal plants of East Africa*. Nairobi: University of Nairobi Press.

Kuria, K. A., De Coster, S., Muriuki, G., Masengo, W., Kibwage, I., Hoogmartens J., &Laekeman, G.M. (2001). antimalarial activity of *ajuga remota* benth (labiatae) and *caesalpinia volkensii* harms (caesalpiniaceae): *In vitro* confirmation of ethnopharmacological use. J*ournal of Ethnopharmacology, 4*(2), 141-148.

Kushwahaa, S., Sonia, V. S., Singha, P. K., Banoa, N, Kumarb, A., Sangwanb, R. S., & Bhattacha ryaa, S. M. (2011). *Withania somnifera chemotypes NMITLI 101R, NMITLI 118R, NMITLI 128R and Withaferin A protect Mastomys coucha from Brugia malayi infection.* Oxford: Blackwell Publishing Ltd.

Leonardo, K. B., Ramiliarisoa,)., & Le Bras, J. (1995). *In vitro* activity of atovaquone against the African isolates and clones of *plasmodium falciparum. American Journal of Tropical Medicine and Hygiene, 53*(4), 388-391.

Salatino, A. (2007). Traditional uses, chemistry and pharmacology of croton species. *Journal of Brazillian Chemistry Society, 18*(1).

Ziegler, H. (2002). *In vitro plasmodium falciparum* drug sensitivity assay: Inhibition of parasite growth by incorporation of stomatocytogenic amphililes in erythrocyte membrane. *Antimicrobial Agents and Chemotherapy, 46*(5), 1441-1446.