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Antimalarial Anthrone and Chromone from the Leaf Latex of *Aloe debrana* Chrstian

Worku Gemechu[1], Daniel Bisrat[1] and Kaleab Asres[1*]

[1] Department of Pharmaceutical Chemistry and Pharmacognosy, School of Pharmacy, College of Health Sciences, Addis Ababa University, Addis Ababa, Ethiopia

In Ethiopian traditional medicine, the leaf latex of *Aloe debranan* Chrstian is used for the treatment of several diseases including malaria. In an ongoing search for effective, safe and cheap antimalarial agents from plants, the leaf latex of *A. debrana* was tested for its *in vivo* antimalarial activity, in a 4-day suppressive assay against *Plasmodium berghei*. Activity-guided fractionation of this latex which showed good antiplasmodial activity resulted in the isolation of two compounds identified as 10-C-*β*-D-glucopyranosyl-1,8-dihydroxy-3-(hydroxymethyl)-9(10H)-anthracenone, commonly known as aloin, and (*E*)-2-(1-hydroxy-2-methylpropyl)-8-(6'-*O*-cinnamoyl)-*β*-D-glucopyranosyl-7-methoxy-5-methylchromone (HCGMM). Aloin displayed a significant ($p<0.05$) antimalarial activity at doses of 25, 50 and 100 mg/kg with chemo-suppression values of 48.38, 69.66 and 78.31%, respectively, while the effect of HCGMM was slightly less than that of aloin inhibiting growth of the parasite by 35.49, 47.02 and 63.13%, at the same doses. Acute toxicity studies revealed that the latex possesses no toxicity in mice up to a maximum dose of 5000 mg/kg suggesting the relative safety of the plant when administered orally. The results of the present study indicate that aloin and HCGMM are among the antimalarial principles in this medicinal plant, and further support claims for the traditional medicinal use of the plant for the treatment of malaria.

Keywords: leaf latex, *Aloe debrana*, antimalarial, aloin, (*E*)-2-(1-hydroxy-2-methylpropyl)-8-(6'-*O*-cinnamoyl)-*β*-D-glucopyranosyl-7-methoxy-5-methylchromone

INTRODUCTION

The genus *Aloe* that contains 600 species and subspecies belongs to the sub-family Alooideae (Aloaceae) of the family Asphodelaceae (Kawai *et al.*, 1993). Asphodelaceae is one of the six smaller families found in the large and heterogeneous family Liliaceae, which consists of two subfamilies, namely, Asphodeloideae and Alooideae (Dagne and Yenesew, 1994; Yagi *et al.,* 2006). Aloes are native to sub-Saharan Africa, the Saudi Arabian Peninsula, and to many islands of the western Indian Ocean, including Madagascar. It has been suggested that the center of diversity for this genus is the highland of southeast Africa (Demissew *et al*., 2001). In South Africa about 160 species of *Aloe* are found (Steenkamp and Stewart, 2007). Some have been introduced to Asia (*A. chinensis* Bak), Barbados Islands in Central America (*A. barbadensis* Miller) and Europe (*A. arborescens* Miller) (Capasso *et al*., 1998). There are about 75 species of *Aloe* found in northeast Africa, of which, about twenty species have been reported to be endemic to Ethiopia (Demissew *et al*., 2001). *Aloe debrana* is one of the endemic species which grows mostly in Showa, central part of Ethiopia and rarely found in other part of the country (Demissew and Gilbert, 1997). People who live in the different parts of Ethiopia use this plant as a laxative in order to clean the digestive system from parasites, as antidiabetic, as a wound healing agent, for cleansing the blood, as antimalarial agent, against nausea and gastric problem (Beyene, 2008; Lulekal *et al*., 2008; Giday, 2009).

Malaria remains one of the most important diseases of the developing world, killing 1-3 million people and causing disease in 300-500 million people annually worldwide. In Ethiopia, it is estimated that three-fourths of the land below 2000 m is malarious with two-thirds of the country's population at risk (Negash, 2004). This makes malaria the number one health

* Correspondence: kaleab.asres@aau.edu.et

problem in the country with an average of 5 million cases a year (Senay and Verdin, 2005) and 9.5 million cases per year between 2001 and 2005 (PMI, 2008). It has further been reported that the disease claims the lives of 70,000 Ethiopians, mainly children, each year, and accounts for 17% of outpatient visits to health institutions with *Plasmodium falciparum* and *P. vivax* comprising 40% and 60% of infections, respectively (PMI, 2008). In the past several years, the burden of malaria has increased due to a combination of mixed infections of *P. falciparum* and *P. vivax* on one hand, and increasing parasite resistance to malaria drugs on the other. This has highlighted the urgent need to develop new antimalarial agents, preferably inexpensive drugs that are affordable for developing countries, where malaria is prevalent. One of the areas for the search of new antimalarials is traditionally claimed antimalarial plants that may lead to the discovery of potentially active lead compounds. The aim of this paper is therefore to evaluate the *in vivo* schizontocidal activity of the leaves of *A. debrana* against early *Plasmodium berghei* infection.

MATERIALS AND METHODS

Materials:

Chemicals and solvents:

$CHCl_3$ (E. Merck, Stockholm), EtOAc (Research-Lab-Fine, India), MeOH (Reagent Chemical Limited, UK), silica gel G6 F_{254} (E. Merck, Darmstadt), Giemsa and Tween 80 (BDH Ltd, England), and chloroquine phosphate (EPHARM, Addis Ababa) have all been used as received.

Instruments:

NMR spectra (^1H, ^{13}C, DEPT 135) were recorded on Bruker Avance DMX 400 FT-NMR spectrometer operating at 400 MHz for ^1H and 100 MHz for ^{13}C at room temperature using DMSO-d_6 as a solvent. A region from 0 to 13 ppm for ^1H and 0 to 205 ppm for ^{13}C was employed for scanning. Signals were referred to an internal standard tetramethylsilane (TMS). Chemical shifts are reported in δ units. Multiplicities of ^1H NMR signals are indicated as *s* (singlet), *d* (doublet), *dd* (doublet of doublets), *m* (multiplet) and *nr* (not well resolved). ESI-MS were recorded on Ultimate 3000 LC-MS. The measurement was carried out by an electrospray ionization method with negative and positive modes. The source voltage and temperature were fixed at 3kV and 250 ºC.

Plant material:

The leaf latex of *A. debrana* was collected in February 2010 from Agarfa district, Bale zone, Oromia region, south eastern Ethiopia. The authenticity of the plant material was confirmed by Prof. Sebsibe Demissew, the National Herbarium, Department of Biology (DOB), Addis Ababa University (AAU), where voucher specimen (collection number WG001) was deposited.

Experimental animals and parasite:

Healthy adult Swiss albino mice of either sex (22-28 g and 6-8 weeks of age) bred and maintained at the Ethiopian Health Nutrition and Research Institute (EHNRI) were used. They were maintained under standard condition (temperature of 22 ± 3°C, relative humidity of 40-50% and 12 h light/12 h dark cycle), with food and water *ad libitum* in the animal house of the DOB, College of Natural Sciences, AAU. Animals were acclimatized for one week to the experimental environment, and each animal was housed individually in a cage under standard environmental conditions. *P. berghei* ANKA strain (chloroquine sensitive) was obtained from Aklilu Lemma Institute of Pathobiology, AAU. It was subsequently maintained in the

laboratory by serial blood passage from mouse to mouse on weekly bases. At the end of each experiment the animals were euthanized with diethyl ether. All procedures complied with the Guide for the Care and Use of Laboratory Animals (ILAR, 1996) and approved by the Institutional Review Board of the School of Pharmacy, AAU.

Methods:

Collection of the latex:

Latex was collected from the leaves of *A. debrana* by arranging the leaves concentrically around a depression in the soil, which was covered with a plastic sheet. It was then left in open air for 3 days to allow evaporation of water, which yielded a dark brown powder.

Isolation of compounds:

The latex was dissolved in methanol and directly applied to preparative thin layer chromatographic (PTLC) plates over silica gel. PTLC developed in a solvent system of chloroform and methanol mixture (4:1), afforded two major compounds with R_f values of 0.65 (**1**; anthrone) and 0.79 (**2;** chromone), respectively. Chromatograms were visualized under UV light at 254 and 366 nm.

Acute toxicity test:

Acute toxicity tests were carried out on nine groups of mice, each containing three mice. The mice in each group were fasted over night. The weight of each mouse was measured. Mice in the first group were treated with the vehicle (control group) while groups II and III were given, 2000 and 5000 mg/kg of the latex, respectively. Mice in groups IV, V, and VI were given 100, 250 and 500 mg/kg/day of the isolated compound **1**, respectively. Similarly, mice in groups VII, VIII and IX were provided with 100, 250 and 500 mg/kg/day, respectively, of the isolated compound **2**. Animals were observed for any signs of toxicity including death, gross behavioural and physical changes like, lacrimation, muscle weakness, sedation, urination and convulsion every four h post treatment in the first day and then every twelve h for the next 72 h (experimental period), and observation continued for seven days (Dikasso *et al*., 2006; OECD, 2001).

Subacute toxicity test:

Subacute toxicity tests were carried out on male Swiss albino mice. The mice were randomly divided into four groups of three animals per cage. The mice in the first three groups were treated with test latex in three different doses (500, 700 and 1000 mg/kg), while the mice in the fourth group received 0.3 ml of vehicle for four consecutive days. Data on weight and packed cell volume (PCV) of the mice were obtained on day 0 and day 4. In order to assess other effects of the latex, the mice were kept for 14 days post treatment. The subacute toxicity of the latex was evaluated in terms of gross behavioural changes, mortality, weight loss and reduction in PCV. PCV was calculated as follows:

$$\text{PCV}=\frac{\text{Vol.of erytrocytes in a given volume of blood}}{\text{Total blood volume}}$$

***In vivo* antimalarial activity test:**

In vivo antimalarial activity test was performed using a 4-day suppressive standard test against chloroquine sensitive *P. berghei* infection in male mice. The mice were divided into 11 groups of 5 mice per cage. Mice in group I were given the vehicle (negative control), groups II, III and IV received the latex at doses of 200, 400, and 600 mg/kg/day, respectively. Groups V, VI and VII were given doses of 25, 50, and 100 mg/kg/day of compound **1**, respectively.

Similarly, groups VIII, IX and X were treated with doses of 25, 50, and 100 mg/kg/ day of compound **2**, respectively**.** Mice in group XI (positive control) were treated with chloroquine at a dose of 25 mg/kg/day in a volume of 0.3 ml. All the test substances were administered through oral route by using oral gavages. Treatment was started 3 h after infection on day 0 and then continued daily for four days (i.e. from day 0 to day 3). On the fifth day (D_4) thin blood smears were prepared fixed in methanol and stained with 10% Geimsa solution to be examined under the microscope with an oil immersion objective of 100x magnification power to evaluate the percent suppression (Dikasso *et al*., 2006; Akuodor *et al*., 2010; Okokon *et al*., 2011). Percentage growth inhibition of the parasites was calculated by the following formula:

$$\text{Growth inhibition (\%)} = \frac{\text{PNC - PSG}}{\text{PSG}}$$

where, PNC is parasitaemia in negative control, and PSG represents parasitaemia in study group.

RESULTS AND DISCUSSION

Acute and subacute toxicity

No signs of toxicity or mortality were observed in mice after oral administration of the latex of *A. debrana*, even at doses as high as 5000 mg/kg signifying that the oral LD_{50} was greater than 5000 mg/kg. The latex showed no significant difference (p>0.05) in the weight of mice at dose levels 500, 700 and 1000 mg/kg compared to the control group on day 0 and also on day 4 (Table 1). Comparison made between the pre-treatment (day 0) and post-treatment (day 4) weight of the mice showed no significant difference (p>0.05) at all dose levels. Although not statistically significant, the weight of the mice treated with the latex at dose levels of 500 and 700 mg/kg showed a slight decrease of weight on day 4. PCV values of mice treated with the latex showed no significant difference (p>0.05) when compared with the control group on day 0 and day 4. Similarly, no significant difference has been observed in PCV values between pre-treatment and post-treatment at all dose levels (500, 700, and 1000 mg/kg). It was observed that, although statistically insignificant, PCV values of mice treated with the latex at doses of 700 and 1000 mg/kg were slightly decreased on day 4. Similarly, the control group showed no significant difference in weight and PCV values between day 0 and day 4. Moreover, no significant difference (p >0.05) was observed in PCV values between control and treated groups on day 0 and day 4. Although not statistically significant, the PCV values of mice treated with the latex at all dose levels was relatively lower on day 4 than on day 0. At all dose levels (500, 700

Table 1. Subacute toxicity test results of the leaf latex of *Aloe debrana* in male Swiss albino mice.

Group	Dose (mg/kg/day)	Parameter	Pre-treatment(D_0)	Post-treatment(D_4)
I	500	Weight (g)	31.9 ± 1.02	30.15 ± 0.83
		PCV (%)	53.9 ± 0.04	54.50 ± 0.79
II	700	Weight (g)	30.9 ± 1.07	30.17 ± 0.95
		PCV (%)	56.0 ± 1.20	53.20 ± 1.13
III	1000	Weight (g)	29.7 ± 0.65	29.30 ± 0.55
		PCV (%)	56.7 ± 1.78	51.10 ± 2.35
IV	NC	Weight (g)	31.3 ± 0.44	32.15 ± 0.46
		PCV (%)	55.1 ± 0.56	52.70 ± 1.48

Values are presented as mean ± SEM; NC (negative control): 0.3 ml of 3% Tween 80; D_0: day 0 (the day treatment started); D_4: day 4 (the fifth day of treatment); PCV = packed cell volume.

and 1000 mg/kg) no significant difference (p>0.05) has been observed in PCV values between pre-treatment and post-treatment (Table 1).

In this study, the leaf latex of *A. debrana* showed no significant difference (p>0.05) in weight and PCV values between treated and control groups. There were also no significant difference in weight and PCV values between pre-treatment and post-treatment at all dose levels. In addition, no toxicity manifestations like hair erection, reduction in motor activity, change in feeding and drinking activities and death of mice were observed within 14 days after treatment. These results suggest that the latex is practically free of toxicity in experimental mice (Chunlaratthanaphorn *et al*., 2007; Jimoh *et al*., 2008; Adak and Kumar, 2009).

Similarly, at a dose of 500 mg/kg, neither of the isolated compounds showed signs of toxicity or mortality, signifying that the oral LD_{50} was greater than 500 mg/kg. Also, there were no gross behavioral changes such as loss of appetite, hair erection, tremors, lacrimation, convulsions, salivation etc. on the test animals suggesting the absence of toxicity of the test compounds. This fulfills the criteria set by Center for Drug Evaluation and Research (CDER 1996; OECD, 2001) for lack of acute toxicity. Therefore, these compounds were regarded as non-toxic to mice, up to a dose of 500 mg/kg body weight.

Structural elucidation

The leaf latex of *A. debrana* subjected to repeated preparative TLC (silica gel) using chloroform: methanol (4:1) as a solvent system afforded two major compounds with R_f values of 0.65 (**1**) and 0.79 (**2**).

Compound **1** was isolated as a pale yellow amorphous substance. The positive ion ESI-MS of compound **1** gave pseudomolecular ions at m/z = 441 $[M+Na]^+$ and 419 $[M+H]^+$, while a negative pseudomolecular ion was obseved at m/z = 417 $[M-H]^-$ in the negative mode, indicating a relative molecular weight (M_r) of 418. A molecular formula $C_{21}H_{22}O_9$ was deduced for compound **1** by combining the information obtained from ESI-MS, ^{1}H, and ^{13}C NMR including DEPT-135 spectral data. Thus, compound **1** was unequivocally identified as aloin (10-C-β-D-glucopyranosyl -1,8-dihydroxy-3-(hydroxymethyl)-9(10H)-anthracenone) by comparing its ^{1}H and ^{13}C NMR data with those reported for the same compound (Coopoosamy and Magwa, 2006).

Compound **2** was also obtained as a pale yellow amorphous substance with R_f value of 0.79 in $CHCl_3$/MeOH (4:1). The negative-mode ESI MS of compound **2** gave a pseudomolecular ion at m/z = 553 $[M-H]^-$, indicating a relative molecular weight (M_r) of 554. A molecular formula of $C_{30}H_{34}O_{10}$ was deduced based on the mass spectrum, which was also consistent with the ^{1}H and ^{13}C NMR data.

Analysis of the ^{1}H NMR signals for protons assignable to two *trans*-vinyl H-2" (1H, δ 6.1, *d*) and H-3" (1H, δ 7.3, *d,*) and five aromatic protons H-5"/H-9" (2H, δ 7.3, *nr*), H-6"/H-8" (2H, δ 7.4, *nr*) and H-7" (1H, δ7.3, *nr*) led to the identification of a *trans*-cinnamoyl residue as a part of the structure of compound **2**. The presence of two singlets each integrating for one proton at 6.1 and 6.9 ppm in the ^{1}H NMR spectrum, corresponding to H-3 and H-6, respectively, was also noted. The presence of 30 carbons in compound **2** was evident from the ^{13}C NMR data (Table 2). The DEPT experiment revealed three methyl (δ 19.8, 2xC-11; δ 22.1, C-12), one oxymethylene (δ 62.0), one methoxyl (δ 56.9), 16 methines and 9 quaternary carbons including two carbonyl carbons (Table 2).

The placement of a *trans*-cinnamoyl residue at C-6′ hydroxyl of the glucose was based on the downfield shift effect of an ester group on the nearby two C-6′ protons (5.0 *dd*; 5.1 *dd*). Complete assignments of protons and carbons are given in Table 2.

Table 2: ^{1}H and ^{13}C NMR spectral data of compound **2** isolated from the leaf latex of *A. debrana*.

Assignments	^{1}H (δ, ppm)	^{13}C (δ, ppm)
2	-	163.1
3	6.1 *s*	112.6
4	-	179.4
4a	-	115.1
5	-	143.3
6	6.9 *s*	111.0
7	-	160.5
8	-	112.0
1a	-	158.8
9	3.9 *s*	69.2
10	2.0 *d*	30.2
11	1.1 *m*	19.8
12	2.7 *s*	22.1
7-MeO	3.8 *s*	56.9
1′	5.5 *dd*	71.1
2′	3.6 *dd*	72.2
3′	3.7 *m*	76.1
4′	3.6 *m*	71.1
5′	3.5 *m*	82.0
$6'_a$	5.1 *dd*	62.0
$6'_b$	5.0 *dd*	62.0
1″	-	168.2
2″	6.1 *d*	118.5
3″	7.3 *d*	146.0
4″	-	135.1
5″, 9″	7.3 *nr*	128.3
6″, 8″	7.4 *nr*	129.2
7″	7.3 *nr*	131.7

s: singlet, *d*: doublet, *dd*: doublet of doublets, *m*: multiplet and *nr*: not well resolved

Therefore, the structure of compound **2** was established as (*E*)-2-(1-hydroxy-2-methyl-propyl)-8-(6'-*O*-cinnamoyl)-*β*-D-gluco-pyranosyl-7-methoxy-5-methyl-chromone (**2**) (Figure 1).

In vivo antimalarial activity

The leaf latex of *A. debrana* showed significant suppression ($p<0.05$) of *P. berghei* parasites at all dose levels tested compared to the mice in the negative control group (Table 3). After 4 days of treatment, the mean parsitaemia of the test group ranged from 3.55 ± 0.72% to 8.86 ± 1.72%, while that of the negative control group was 14.21 ± 6.22%. *P. berghei* parasites in mice treated with chloroquine were reduced to non detectable level on day 4. The latex produced a dose dependent chemosuppressive effect at various doses given orally with the highest suppression of parasitaemia (75.02%) observed at a dose of 600 mg/kg/day. Chemosuppression of the latex was dose dependent, increasing with an increase in concentration of the latex. These findings are in agreement with the previous studies on the antimalarial activity of the water and methanol leaf extracts of *A. debrana* (Deressa *et al*., 2010).

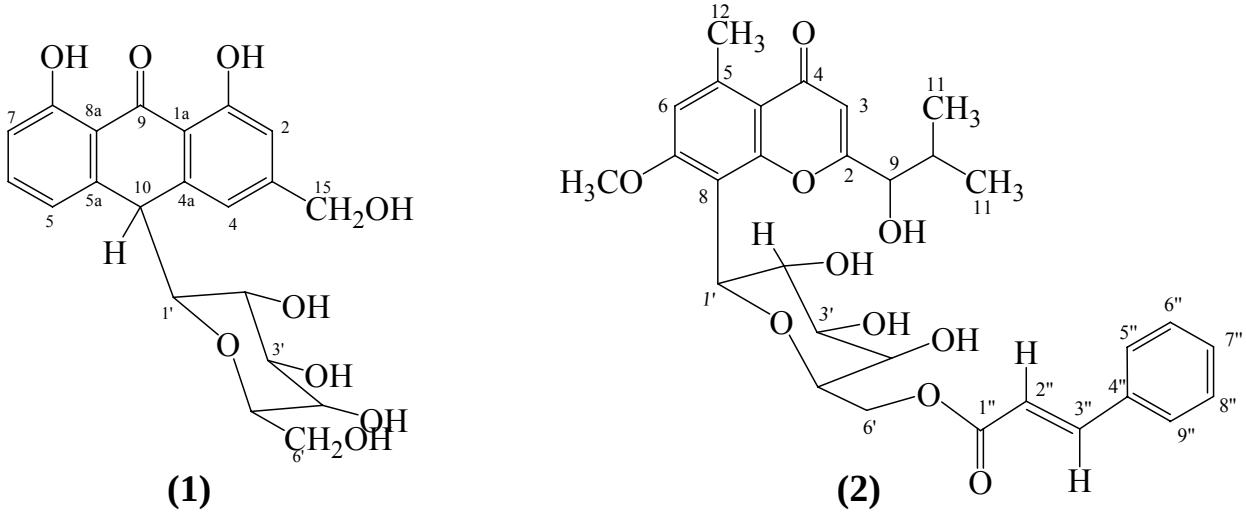

Figure 1. The structures of isolated compounds **1** and **2.**

Table 3. Percentage suppression of *Plasmodium berghei* in mice after treatment with the leaf latex, and isolated compounds (**1** and **2**).

Test substances	Dose (mg/kg/day)	% Parasitaemia ± SEM	% Suppression
NC	0.3 ml	14.21 ± 6.22	
Latex	200	8.86 ± 1.72*[a]	38.27
Latex	400	5.71 ± 1.13*[a]	59.82
Latex	600	3.55 ± 0.72*[a]	75.02
NC	0.3 ml	14.08 ± 6.26	
Aloin (**1**)	25	7.27 ± 1.44*[a]	48.38
Aloin (**1**)	50	4.26 ± 0.86*[a]	69.66
Aloin (**1**)	100	3.05 ± 1.15*[a]	78.31
NC	0.3 ml	10.65 ± 0.86	
HCGMM (**2**)	25	6.86 ± 0.47*[a]	35.49
HCGMM (**2**)	50	5.64 ± 0.89*[a]	47.02
HCGMM (**2**)	100	3.93 ± 0.40*[a]	63.13
Chloroquine	25	0.00*[a]	100

Values are presented as M ± SEM; n =5; NC (negative control): 3% Tween 80 [a] = compared to negative control; *($p< 0.05$); HCGMM: (E)-2-(1-hydroxy-2-methylpropyl)-8-(6'-*O*-cinnamoyl)-β-D-glucopyranosyl-7-methoxy-5-methylchromone.

The leaf latex of some *Aloe* species has been reported to have antimalarial activity against mice infected with *P. berghei* (Minale *et al*., 2014). Although the exact mechanism of action of the latex has not been elucidated, some plant extracts are known to exert antiplasmodial activity either by causing red blood cell oxidation or by inhibiting protein synthesis depending on their phytochemical constituents. The latex could have exerted its action through either of the two mechanisms mentioned above or by some other mechanisms as the it may contain different compounds that either cause or enhance production of O_2 radicals inside cells. Moreover, Bhatia *et al*. (2012) reported that some plant latex improve immune function of a host that speeds up its recovery from infectious disease.

Natural products play an important role in the fight against malaria. The final products would be antimalarial chemical entities, potential new drugs or templates for new drugs development, and/or standardized antimalarial extracts which are required for pre-clinical and clinical studies when the aim is the development of effective and safe phythomedicines (Oliveira *et al*., 2009).

Thus, in the present study, aloin isolated from the leaf latex of *A. debrana* showed significant suppression ($p<0.05$) against *P. berghei* at dose levels of 25, 50, and100 mg/ kg/day compared to the mice in the negative control group. After 4 days of treatment with aloin, the mean parasitaemia of the test groups ranged from 7.27 ± 1.44% to 3.05 ± 1.15%, while that of the negative control group was 14.08 ± 6.26%. Comparison of the chemosupperessive effect of aloin at dose levels of 25, 50, and 100 mg/kg/day has showed significant difference ($p<0.05$) compared to the negative control. Aloin showed 48.38% parasitaemia suppression against *P. berghei* at a dose of 25 mg/kg/day (Table 3), which is about half of that of chloroquine at the same dose. Aloin displayed

highest suppression of parasitaemia (78.31%) at a dose of 100 mg/kg.

HCGMM also showed significant suppression ($p<0.05$) against *P. berghei* at dose levels of 25, 50, and 100 mg/kg/day compared to the mice in the negative control group. The antimalarial activity displayed by HCGMM was statistically significant ($p<0.05$) at all dose levels compared to the negative control group. The reduction of parasitaemia was also dose dependant. As shown in Table 3, the antimalarial activity of aloin which has an anthrone moiety is better than that of HCGMM which contains a chromone nucleus.

Generally, alkaloids are one of the major classes of compounds possessing antimalarial activity. However, a number of investigations showed that many naturally occurring non-alkaloidal compounds such as terpenes, limonoids, chromones, xanthones, flavonoids and anthraquinones possess antimalarial activity when tested in different malarial models (Saxena *et al.*, 2003). By targeting medicinal plants, it is hoped that structural novelty with the required bioactivity will be achieved more efficiently.

In this study, it was observed that neither of the isolated compounds is as active as chloroquine phosphate since they failed to suppress parasitemia to undetectable level. However, they have the potential to be used as a scaffold for the development of safe and cost effective antiplasmodial drugs that can be useful in the continuing fight against malaria.

CONCLUSION

In conclusion, the present study has demonstrated that the leaf latex of *A. debrana* and the compounds isolated thereof namely, aloin and HCGMM possess genuine antimalarial activity in mice infected with *P. berghei* in a dose dependent manner. Moreover, the test substances were found to be safe at the dose levels used in the study. Thus, the results obtained in this work provide a scientific evidence for the traditional use of *A. debrana* in the treatment of malaria.

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