

Antiplasmodial and Phytochemical Investigations of Leaf Extract of *Anthocleista vogelii* (Planch)

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ABSTRACT

Anthocleista vogelii Planch (*Loganiaceae*) is a medicinal plant used widely in Nigeria for management of malaria and other ailments. The aim of this present study was to investigate the anti-plasmodial activities of the leaf extract against residual infection in chloroquine sensitive *Plasmodium berghei* infected mice. Phytochemical analysis and oral acute toxicity in mice were evaluated. Iron chelating ability of the extract and isolated compound were also determined. The extract was found to be safe at up to 2000 mg/kg dose. The extract produced a dose dependent reduction in parasite density compared to the control group when given intraperitoneally. There was no reduction when the extract was administered orally. The phytochemical investigations of extract resulted in isolation of four compounds whose structures were elucidated using IR, GC-MS, LC-MS, 1D and 2D NMR spectroscopy as decussatin (1-hydroxy-3,7,8-trimethoxyxanthone), stigmaterol (stigmasta-5,22-dien-3-beta-ol), swertiaperennin (1,8-dihydroxy-3,7-dimethoxy-xanthone), and hexadecanoic acid. Decussatin demonstrated very weak reduction in parasite density at 10mg/kg. The extract and decussatin demonstrated good iron chelating ability at the tested concentration (1mg/ml) which may be involved in its antiplasmodial activities.

Keywords: *Anthocleista vogelii*; Ferrous chelating ability; Antiplasmodial; Parasite density.

INTRODUCTION

Malaria is a disease condition most peculiar to Sub-Sahara Africa and still remains a challenge till today as a result of the resistant sensitive protozoa - *Plasmodium species* to currently existing drugs; therefore the search for newer and effective antimalaria remains a continuous scientific exercise. According to the World Health Organisation's latest global malaria report, though good progress has been made over

the past decade, with deaths estimated to have dropped to 781,000 in 2009 from nearly a million in 2000 but Africa still accounts for nine out of 10 deaths, mainly children under five (Kelland, 2011). Plants no doubt have been and are still a veritable source of medicine to mankind and will continue to provide leads and template to drug discovery.

Anthocleista vogelii Planch (Family-*Loganiaceae*) is a tree 6-20m high. The bark is noted for its anti-pyretic, tonic and purgative properties and a decoction of the leaves is known to prevent malaria and alleviate symptoms of malaria such as fever. It is also used to treat jaundice and as haemostatic (Burkill, 1995). We investigated antiplasmodial activity of extract of *Anthocleista vogelii* and also performed fractionation of the extract in order to isolate and characterize compounds from it.

MATERIALS AND METHODS

Plant material: Leaves of *A. vogelii* were collected in August, 2007 from uncultivated land in University of Lagos, Akoka, identified and authenticated by Mr. M. O. Onadeji, Herbarium Officer at the Forest Research Institute of Nigeria (FRIN), Ibadan and voucher specimen (FHI NO 107844) was deposited at same institute. The leaves were air dried at 35°C, pulverised using electric grinder and kept in airtight containers at room temperature prior to use.

Extraction, Isolation and Structure Elucidation: The dried pulverized leaves of *A. vogelii* (350g) were extracted with petroleum ether for 72h at room temperature. The pet ether fraction (18.50g) was chromatographed on a column of silica gel (200g) and eluted with hexane-EtOAc (10:1; 1–10) to give fourteen fractions A-N of 20 ml each. The fractions thus obtained were compared using TLC (fluorescence silica gel plates using hex- EtOAc {8:2}) as solvent. Those giving similar spots were combined. Fractions G, left in the vial overnight formed solid deposit which was decanted and the residues recrystallised using methanol. The recrystallized product afforded compound **1**. The supernatant was concentrated, subjected to vacuum column chromatography and further eluted with hex—EtOAc (8:2) to give compound **2**. Fraction H was subjected to LC-MS and GC-MS afforded compounds **3** and **4**.

The isolated compounds were successively subjected to ¹H NMR, infra red (IR), UV, MS and ¹³C NMR analysis for structure determination.

Acute Toxicity Studies: The medial lethal dose of the pet ether extract of *A.vogelii* (leaves) that would kill 50% of the animals in a population (LD₅₀) was determined intraperitoneally. Albino mice were divided into five groups of six (6) animals each weighing between (18-20g). The mice were subjected to 24h fasting (with only water) before administering the extract. The extract was suspended in vehicle (Tween 80 in distilled water, 3% v/v) was administered in dose of 1,000, 2000, 4,000, 6,000 and 8,000mg/kg i.p. The sixth group served as control received only 3% Tween 80. All animals were kept at room temperature in cross ventilated rooms without illumination at night. The mice were then observed for toxicity and fatalities over 48 hours.

Antiplasmodial Studies

Animals: About 4 weeks old Swiss albino mice (14-20g) were obtained from the Laboratory Animal Center of the College of Medicine, University of Lagos. The mice were housed in standard conditions and were maintained on a standard pelleted feed and water *ad libitum*.

Parasite Inoculation and Treatment

Oral route of administration: The blood schizontocidal action of the extract against *P. berghei* was performed using a 4-day curative standard test (Adesegun, et al., 2001; David, et

al., 2004). The mice were randomly divided into Ten groups (1- 10) of six mice each. They were all infected intraperitoneally except group one with 1×10^7 *P. berghei* –infected blood cells in a volume of 0.1 ml diluted in phosphate buffer saline (pH 7.2) (Ishih, et al., 2004). Group 1(negative control) received just distilled water and pellets. Group 2 (positive control) was inoculated with the parasite media only with no treatment. Groups 3-7 were treated with 100, 500, 750, 1000 and 1250mg/Kg of the petroleum ether leaf extract of *A. vogelii* respectively. Groups 8 and 9 were treated with reference drugs (chloroquine 10mg/Kg and artesunate combination 1.6mg/kg) (Ene, et al., 2009). Group 10 received 0.4ml of 3% w/w tween 80 (dissolving solvent where applicable to extract). The treatment was administered day-0 to day-3 using the oral cannula.

Intraperitoneal route:The mice also were infected by intraperitoneal (i.p) injection with 1×10^7 *P. berghei* –infected blood cells in a volume of 0.1 ml diluted in phosphate buffer saline (pH 7.2). Seventy two hours later, were randomly divided into groups consisting of Group A-D treated with 50, 100, 250mg/Kg of extract and decussatin (10mg/kg). Group 1, 2, E were negative, positive and reference (chloroquine, 5mg/Kg ip.) controls respectively. Treatments were performed daily for 4 consecutive days.

The animals were observed at day 4- day 7 intervals and thin blood smears were prepared from each mouse and stained with Giemsa stain and parasitaemia examined microscopically (Saidu, et al., 2000). Parasite density was determined by counting the number of plasmodium parasites against 200 white blood cells and expressing the resultant number of parasites/ μ l blood assuming a white blood cell count of 8000 per μ l of blood according to the relative value method (Earle and Perez, 1983).

Parasite density was calculated using the formula:

$$\text{Parasite count} \times 8000 / \text{WBC Counts} = \text{Parasites} / \mu\text{l}$$

Ferrous chelating ability of Extract: A slightly modified method developed by Dinis et al., 1994 was adopted. The PE extract and Decussatin (5.0ml each; 1mg/ml) including EDTA Solution (reference standard) were spiked with 0.1ml of 2 mM FeCl_2 and 0.2ml of 5mM ferrozine solution. After reaction for 10mins, the absorbance (at 562nm) of the resulting solutions was recorded. The higher the ferrous ion chelating ability of the test sample, the lower was the resulting absorbance.

The percentage of ferrous ion chelating ability was expressed by:

$$\% = [(A_0 - A_s) / A_s] \times 100$$

- A_0 = the absorbance of the control; A_s = the absorbance of the extract

Statistical analysis:The statistical analysis of all the analyses was carried out using GraphPad Prism 5 Demo. The values are represented as mean \pm SD. One way ANOVA and 2-tailed Student's t-test were also used (Microsoft Excel, 2007), with $P < 0.05$ being considered significant.

RESULTS

The results from antiplasmodial activity of petroleum ether leaf extract of *A. vogelii* showed that treatment of the *P. berghei* infected mice with the extract intraperitoneal (i.p) produced reduction in parasite density while the control groups showed an increase in parasitaemia after administration. A statistically significant difference was observed between parasite density of the infected mice treated with extract and untreated infected mice (Table 1). The untreated group showed drastic increase in parasite density from day-0 to day-7 (Table 1). There was a statistically significant difference in deduction in parasite density from day-4 compared to day-7 in groups

treated with 250mg/Kg extract and chloroquine respectively in comparison with other test groups. The reduction in parasite density of extract is significantly less than that of chloroquine. Although, parasitemia was not completely cleared in all test groups but it was reduced. However, there was no reduction in parasite density when the extract was administered orally at concentrations 100-1250mg/ml daily. Decussatin, one of the isolates showed no significant antiplasmodial activity at the maximum concentration (10mg/kg) used in the study (Table 1). The result of behavioural and toxicity studies on the extract showed that it was safe for further biological studies as no lethality was observed at 2000mg/Kg i.p. in mice (Table 2). The behavioral signs of toxicity observed in mice given above 2000mg/Kg body weight include paw licking, salivation, stretching and reduced activity. The results of iron chelating effect of the extract showed that At the tested concentration of 1 mg/ml, pet ether extract, hexane and EtOAc fractions, decussatin and positive control EDTA demonstrated good iron chelating ability, 89.53 ± 1.00 , 77.90 ± 0.52 , 97.12 ± 0.50 , 77.80 ± 0.50 and 118.50 ± 2.78 % respectively. Phytochemical investigations of the extract led to isolation of the following compounds:

Compound 1: 1 hydroxy - 3, 7, 8 trimethoxyxanthone (Decussatin); Yellow - whitish crystals, $C_{16}H_{14}O_6$; M.pt; $150^{\circ}C-154^{\circ}C$ M^+ : m/z 302, run time:11.6 mins; 287,259,227,201,171,143,122,100,79,51. **IR ν_{max} cm^{-1} :** 3100, 2919, 2849, 1743, 1658, 1596, 1571, 1480,946 for major peaks. **1H NMR** (600 MHz , $CDCl_3$): δ 13.24 (1H,s ArOH), 7.31 (1H,d, J 9.18Hz), 7.15 (1H,d, J 9.18Hz) ,6.31 (1H, d, J 2.20Hz), 6.20 (1H,d, J 2.20Hz), 3.98 (3H, s, OMe), 3.91 (3H,s,OMe), 3.86 (3H,s,OMe); **^{13}C NMR** (600 MHz, $CDCl_3$): δ ; 181.2(C-9) ,166.4(C-3) , 163.9(C- 1) , 157.1(C-4a), 151.0 (C-5a), 149.3 (C- 7), 148.9 (C-8), 120.5(C-6), 115.8 (C-8a), 112.8 (C-5), 104.1 (C-9a), 96.9 (C-2), 92.1 (C-4), 61.8 (OCH₃ – 8), 57.2(OCH₃ – 7), 55.8 (OCH₃ – 3). ^{13}C and 1H -NMR are in agreement with literature (Peres and Nagem, 1997).

Compound 2: Stigmasta-5, 22- dien-3 β -ol (Stigmasterol); colorless pleasant odour gel; $C_{29}H_{48}O$; M.pt $161^{\circ}C - 170^{\circ}C$ M^+ : m/z 413, **IR ν_{max} cm^{-1} :** 2954.08, 2900.00, 2852.55, 1460.10, 1377.16, 909.03, 721.63, 438.18 for major peaks. **1H NMR:** (400 MHz, $CDCl_3$); δ 3.50 (H-3), δ 5.85 (m, H- 6), δ 4.60 (m, H-22, H-23), multiple signals at δ 1.50 - 2.00. **^{13}C NMR:** (400 MHz, $CDCl_3$); δ ; 1 C 42.2 (C-1), 56.4 (C -2), 28.9 (C-3), 24.4 (C- 4), 57.0 (C- 5), 31.9 (C-6), 50.1 (C-7), 21.1(C-8), 39.8 (C- 9), 31.5 (C-10),121.7 (C-11), 140.7 (C-12), 36.3 (C- 13),37.4 (C- 14), 31.7 (C-15),76.7 (C-16),42.4 (C-17),12.2 (C-18), 40.5 (C-19) ,21.1 (C- 20) ,19.4 (C-21), 138.3(C-23),129.3 (C-24),51.2 (C-25) , 31.6 (C-26) , 21.6 (C-27), 25.4 (C-28).12.2 (C-29). ^{13}C and 1H -NMR are in agreement with literature (Li, et al., 2006).

Compound 3:1, 8 - dihydroxy - 3, 7- dimethoxyxanthone (Swertiaperennin) ; Pale yellow crystals; $C_{15}H_{12}O_6$; M.pt; $150^{\circ} - 153^{\circ}C$. M^+ : m/z 288.; **IR ν_{max} cm^{-1} :** 2900.00, 2849.34, 1704.62, 1667.67, 1641.30, 1603.59, 1575.86, 1505.88, 1464.27, 1436.83, 1378.45 for major peaks. **1H NMR:** (600 MHz, $CDCl_3$); δ 12.00(1H,s, ArOH), 11.40(1H,s), 7.20 (1H,d,J=7.24Hz), 6.78 (1H,d,J=9.0Hz), 6.33 (1H,d,J=2.20Hz), 6.27(1H,d,J=2.24Hz), 3.87(3H, s,OMe), 3.83 (3H,s,OMe). **^{13}C NMR :** (600 MHz, $CDCl_3$); δ ; 185.1 (C-9) , 167.5(C-3), 163.0 (C-1), 158.1(C-4a), 150.18(C-5a),149.6 (C-7), 142.9(C -8), 120.4 (C-6), 107.8 (C-8a) , 105.6 (C-5) , 102.4 (C-9a) , 97.2 (C-2), 93.0(C-4), 57.1(OCH₃ – 7), 55.9(OCH₃ – 3). ^{13}C and 1HNMR are in agreement with literature (Valento, et al., 2002)

Compound 4: Hexadecanoic acid (Palmitic acid); $C_{16}H_{32}O_2$ M^+ : m/z 270 (fragments at; 227,185, 143, 121, 97, 74, 43). Run time: 3.43 min. **IR ν_{max} cm^{-1} :** 3076.72, 1704.62, 1667.67, 978.67, 958.72 for major peaks. **1H NMR:** (600 MHz, $CDCl_3$); δ

2.3 – 2.5 (m, 31H), ¹³C NMR: (600 MHz, CDCl₃); δ 33.9, 31.9, 29.7, 29.7, 29.76, 29.6, 29.6, 29.4, 29.4, 29.3, 29.1, 28.8, 24.7, 24.6, 22.7, 14.1.

DISCUSSION

The remarkable activity of quinine and other related drugs and the success of artemisinin have stimulated the search for new plant derived antimalarials. However, the reported cases drug resistance to these antimalarial drugs including artemisinin-derivatives and its combination therapies (Wichmann, et al., 2004; Jambou, et al., 2005) made the search and development new anti-malarial agents inevitable. Natural plant products are major sources of biologically active compounds and have potential for the development of novel anti-malarial drugs (Newman, et al., 2003).

In this study, the anti-plasmodial activity of *Anthocleista vogelii* Planch was investigated using *in vivo* animal (mice) model. Mice were used to predict the antimalarial activities of the extract. Synthetic antimalarial drugs like chloroquine, mefloquine and artemisinin have been investigated using mice model (David, et al., 2004). The parasite *P. berghei* was used to predict treatment outcomes and since it is sensitive to chloroquine and artemisinin, they were used as reference drugs. About 4 weeks old mice were used in this study to avoid the effect of anaemia in the old mice and effect of physiological changes associated with aging on treatment outcome (Pierrot, et al., 2003). The 4-day test using the *P. berghei* infected mice model is widely used as a test for the *in vivo* antiplasmodial activity of potential antimalarial agents, as it provides a preclinical indication of potential bioactivity of the test sample (Munoz, et al., 1999).

Petroleum ether extract of *A. vogelii* displayed good antiplasmodial activity against the *P. berghei* parasite when administered intraperitoneally. The significant reduction in parasite density by the extract on day 4 and day 7 at 100-250mg/Kg body weight was dose dependent and is in agreement with previous report and consistent with the traditional use of the plant as a herbal remedy against the disease in south west and east Nigeria (Igoli, et al., 2005; Adebayo and Krettli, 2011). The extract however was not active when administered orally this is against the report of *in vivo* antimalarial activity of ethanolic leaf and stem bark extracts of *Anthocleista djalonensis* another species when administered through the same route (Anita, et al., 2009). This effect may be due to poor absorption and possible metabolism in gastrointestinal tract of the constituents of the extract.

The extract showed no lethality in mice up to 2,000mg/Kg body weight of petroleum ether leaf extract. This is 20 times greater than the minimum effective dose of 100mg/kg. Reports have demonstrated that if the lethal dose of a substance is three times more than the minimum effective dose, the substance is considered a good candidate for further investigations (Jutamaad, et al., 1998). The extract could therefore be described as safe and could explain its safe use by indigenous people Nigeria in management of malaria.

Phytochemical evaluation of *A. vogelii* leaf extract led to isolation of decussatin (1-hydroxy-3, 7, 8-trimethoxyxanthone) (**1**) and swertiaperennin (1, 8-dihydroxy-3,7-dimethoxyxanthone) (**3**) previously reported from the stem bark of the plant (Tene, et al., 2008). Stigmasterol (stigmasta-5, 22-dien-3-beta-ol) (**2**) and hexadecanoic acid (**4**) though not new but were being reported for the first time in the leaf of the species. However, 7 α -hydroxysitosterol and sitosterol 3-O- β -D-glucopyranoside were isolated (Tene, et al., 2008) from the stem bark of *Anthocleista vogelii*. Also, fourteen alkanes were identified from the plant, ranging from tetracosane (C₂₄H₅₀) to

heptriacontane (C₃₇H₇₆) (Sonibare, et al., 2007). Only decussatin was tested *in vivo* for antiplasmodial activity due to its relatively high yield. It however, demonstrated poor reduction in parasite density compare to untreated rats 25,980±1.50µ/l (Table 1). Thus the observed activity may due to its synergistic effect with other compounds.

The observations from iron chelating ability of the tested substances showed that petroleum ether extract and decussatin are good and promising chelators though not better than EDTA. Recent studies revealed low molecular weight iron chelators played significant role in treatment of parasitic diseases like malaria (Van Zyl, 1992; Cabantchik, et al., 1996). These compounds remove the excess, toxic iron from the patient's blood. Excess unbound iron catalyzes reactions that generate highly reactive free radicals, such as OH[•], O₂^{•-}, which in sufficient concentrations cause cellular damage and can illicit high temperature in the body system (Kushner, et al., 2001).

CONCLUSION

We have demonstrated the antiplasmodial effects of petroleum ether extract of leaf of *A. vogelii*. Phytochemical studies led to isolation of decussatin, swertiaperennin, hexadecanoic acid and stigmaterol. Decussatin showed weak reduction in the parasite density at tested concentration. However, the extract and decussatin demonstrated good iron chelating ability at the tested concentration. The extract was found to be safe till 2000mg/kg.

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Figure 1

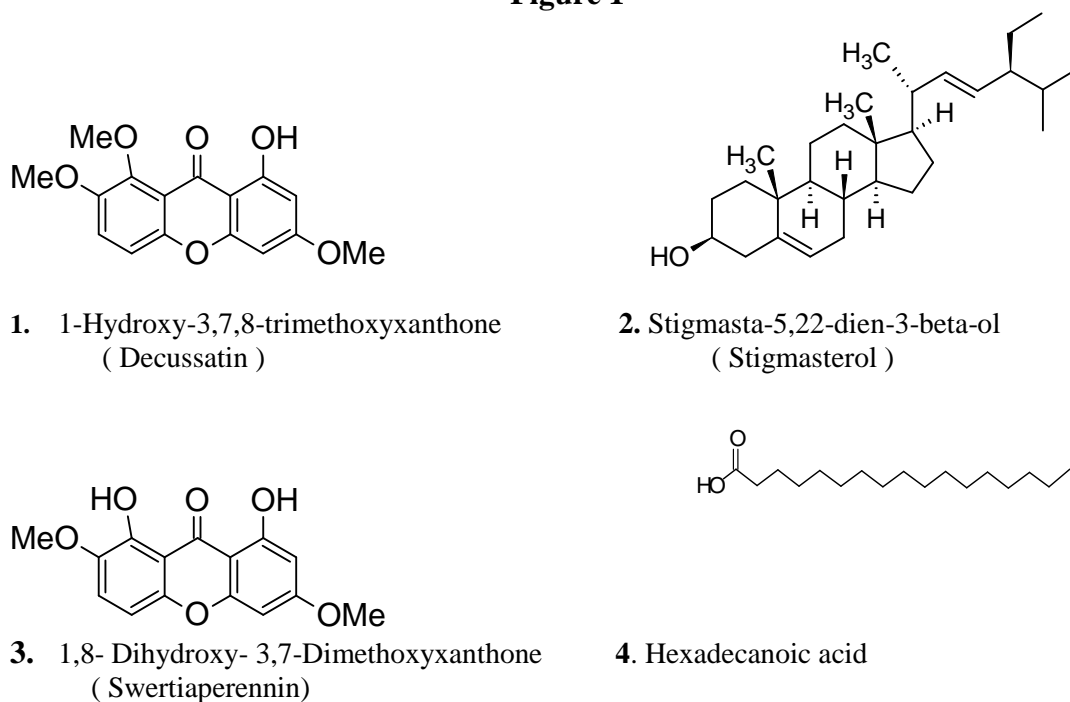


Table -1: Parasite Density (PD) of antiplasmodial studies after intraperitoneal administration (IP).

Animal Groups	Dosage (mg/kg)	Av. Weight (g)	PD before Treatment (μ /l)	PD (at day 4) (μ /l)	PD (at day 7) (μ /l)
1	Negative Control	19.35	-	-	-
2	Positive Control	15.90	21,900 \pm 0.02	45,370 \pm 0.05	47,370 \pm 1.35
A	250	16.03	12,000 \pm 0.05	9,540 \pm 0.85	2,350 \pm 0.02*
B	100	14.81	19,520 \pm 0.10	10,800 \pm 0.20	10,000 \pm 1.20*
C	50	14.20	19,231 \pm 1.52	18,550 \pm 0.05	18,900 \pm 1.40*
D	Decussatin (10mg/kg)	15.00	20,150 \pm 1.00	24,000 \pm 0.02	25,980 \pm 1.50
E	Chloroquine (5mg/kg)	15.00	19,876 \pm 0.02	5,060 \pm 1.62	1,000 \pm 0.50 *

- Results are mean \pm S.E (n = 3)
- $P < 0.05$, , when compared at day 7 for groups with significant difference in PD
- PD: Parasite density. negative control: no parasite, no drug. positive control: parasite, no drug.

Table- 2: Toxicity effects of graded doses (mg/kg body weight) of PE leaf extract of *A. vogelii* after IP administration in mice.

Dosage (mg/Kg)	Log Dose	Mortality rate	Mortality (%)	Probit value
8000	4.1801	4/5	80	5.841
6000	4.1801	4/5	80	5.841
4000	4.1000	3/5	60	5.253
2000	4.2410	0/5	0	0.000
1000	4.3000	0/5	0	0.000