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NAPHTHOQUINOLINONE DERIVATIVE WITH ANTI- PLASMODIAL ACTIVITY FROM V*ITEX DONIANA* [SWEET] STEM BARK EXTRACTS

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ABSTRACT
In an effort to identify promising phytotherapeutical substances from plants, the bark of Vitex doniana was collected, air dried and pounded. The powder was extracted with ethanol. The crude ethanol (VD1) was macerated sequentially. Four fractions obtained namely petroleum ether (VD1-01), ethyl acetate (VD1-02), chloroform (VD1-03) and methanol (VD1-04). These fractions were assayed at various concentrations (250µg/ml, 500µg/ml and 1000µg/ml) for anti-malaria activity against Plasmodium falciparum and were also screened for the presence of secondary metabolites. The ethanol extract (VD1) and methanol soluble fraction (VD1-04) exhibited the highest activity against the test organism with 68.0% and 78.0% elimination at 500µg/ml after 72 hours respectively. The VD1-04 was subjected to activity guided column chromatography that lead to isolation of pure compound VD1-04-198 named as 3-ethyl-3,4,4a,5,6,6a,10a,11,12,12a-decahydro-1H-naphtho[2,3,-g]quinolin-2-one. This was active against the malaria parasite with 89.0% elimination at 500µg/ml after 48 hours. The phytochemical analysis showed that the plant contain flavonoids, tannins, alkaloid, reducing sugar and steroids.

Keywords: anti-malaria, Vitex doniana, Naphthoquinolinone, phytotherapy, extract

INTRODUCTION

In recent years, there has been increase in human and financial commitments to malaria control, nationally and internationally, partly due to the need to meet the targets set in the Millennium Development Goals (MDGs). Present estimates suggest that around 350–500 million clinical disease episodes occur annually (Bawah and Binka, 2005). Around 60% of clinical cases and over 80% of the deaths due to malaria occur in Africa south of the Sahara (Alaba, 2005). The above has serious implication for economic growth and welfare. Malaria is responsible for an estimated average annual reduction of 1.3% in economic growth for those countries with the highest burden Nigeria inclusive (WHO, 1995, WHO, 2000, WHO, 2002 and WHO, 2005). A cause for worry at the moment is the growing resistance of parasitic diseases to cheap first line drugs and the need for the more expensive anti-malaria combination therapy (ACT). Given that malaria is endemic throughout Nigeria, and that more than half of the country's population are living below poverty line. Malaria incidence may increase significantly in Nigeria because many may not be able to afford the newly introduced drugs due to poverty (Castro and Mokate, 1988; DeLeive and Manning, 2004).

Vitex doniana SWEET (black plum) is specie of the plant belonging to family *verbenaceae*; it is called "Dinya" in Hausa and "Orunla" in Yoruba (Atawodi *et al*., 2003). *V doniana* (Verbenaceae) is a deciduous tree up-to 15 to 20m high, the tallest and most frequent Pan African *Vitex* species. It has a pale brown to gray white bark with palmate fingered leaves. Flowers are white or yellowish white with blue red center. The fruits are green, glabrous, up-to 3cm with small white dots and later turn yellowish brown then black when mature (Sofowora, 1984; Hans-Jorgen, 1990).

In Nigeria the genus of *Vitex doniana* is abundant in the Northern region, including some part of the neighboring countries. The plant is used in traditional medicine either alone or mixed with one or more parts of other plants. The bark of *V doniana* is used in Kano as narcotic or anti-epilepsy and against female sterility. The bark together with the root of *Balanite aegyptiaca* (L) DEL (Balanitaceae) and the leaves of *Acacia albida* DEL (Miniosaceae) is used as remedy for dysentery and constipation. Mixture of the bark of *V doniana* and the root of *Ziziphus mauritiana* LAM (Rhaminaceae) is used in Maiduguri for treatment of stomach troubles and abdominal pain. In Bauchi, the bark of *V doniana* and the leaves of *A albida* are used for treatment of skin infections. Also, in some parts of Bauchi, the chewed leaves are put on wounds to improve healing. The boiled mixture of the bark of *V doniana* and that of *Fiscus syncomorus* L (Moraceae) serve as antidote against snakebites. Generally, in the Northern Nigeria the bark of *V doniana* mixed with some part(s) of *Combrentum micramthum* G. DON (Combretaceae) are used for treatment of leprosy, aesthesia, anemia and hepatic disorder (Irvine, 1961 and Personnel contacts with traditional heealers). The roots and leaves are used for nausea, colic and epilepsy (Bouquet *et al*., 1971; Iwu, 1993).

Currently, claims are maintained that the bark of the *Vitex doniana* could be of value as a source for phytotherapy.

To substantiate the hypothesis, this study was aimed at evaluating the anti-malaria activity of *Vitex doniana* bark extracts. The study also aimed at identifying the types of secondary metabolites present in the portions assayed.

MATERIALS AND METHODS

Sample Collection

Bark of *Vitex doniana* were collected at Sumaila town, Sumaila Local Government Area, Kano State, on September 22, 2010. The plant was identified at the Herbarium of the Department of Biological Sciences, Bayero University, Kano, and with the help of compiled work of Aliyu (2004).

Extraction and Fractionation

The air-dried powdered sample (200g) was percolated using Ethanol (1.3L) for a period of 14 days. The mixtures were filtered and concentrated under reduced pressure using Rotavapor (R110 at 40°C). The ethanol extract was allowed to dry and its weight was recorded. This was labeled as VD1 (Ethanol extracts *V doniana*). Ethanol extract (10g) was macerated with petroleum ether (60-80 °C, 150ml), chloroform (150ml), Ethyl acetate (150ml) and methanol (150 ml) sequentially. All fractions obtained were collected in weighted beakers and were labeled as VD1-1 to VD1-4 (Sofowora, 1984).

Phytochemical Analysis

Twenty milligram (20mg) of VDI, VDI-1, VDI-2, VDI-3, VDI-4 and VDI-5 extracts was placed in five separate test tubes. Distilled water (5ml) was added to VDI. While ethanol (1ml) followed by distilled water (4ml) was added to other fractions to facilitates dissolutions of the fraction. Each sample was shaken rapidly and vigorously and distributed in to separate test tubes for the test of the presence of tannin, alkaloid, flavanoid, reducing sugar and steroid (Fatope *et al.*, 1993).

Malaria Parasite Assay

Sourcing of Malaria Parasites for the Assay

Haematology Department, Bayero University Health Services Unit, (Old Campus) Kano, provided clinical blood samples containing heavy parasitaemia of *Plasmodium falciparum*. Venous blood from patients recommended for malaria parasites test (MP test) was collected out using 5ml disposable plastic syringes and needles (BD and 20 SWG). The samples were immediately transferred into K3-EDTA disposable plastic sample bottles with tightly fitted plastic corks and mixed thoroughly and then transported to the Microbiology laboratory at Bayero University in a thermoflask containing water maintained at 4°C as demonstrated by Dacie and Lewis (1968).

Preparation of *Plasmodium falciparum* Culture Medium

Venous blood (2ml) from the main vein of white healthy rabbit's pinnae was withdrawn using a disposable 5ml syringe (BD 20S WG). This was defibrinated by allowing it to settle for at least one hour (Dacie and Lewis, 1968). The defribranated blood was centrifuged at 1500rpm using spectre merlin centrifuge for 10 minutes and the supernatant layer was collected in a sterilized tube. The sediment was further centrifuged at 1500rpm for five minutes, and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with the salt of RPMI 1640 medium (KCl 5.37mM, NaCl 10.27mM, $MgS0_4$ 4.00 mM, $NaHPO_4$ 17.73mM, $Ca(NO_3)_2$ 0.42mM, $NaHCO_3$ 2.5mM, and glucose 11.0 mM. (BDH ltd, UK) as demonstrate by Devo et al (1985). The medium was sterilized by the addition of 40µg/ml gentamicin sulphate (Trager, 1982).

In-vitro Assay of the Activity of the Extracts on *Plasmodium falciparum* Culture

A 0.1ml of tested solutions and 0.2ml of the culture media (RPM 1640) were added into test tubes containing 0.1ml of 5% parasitaemia erythrocytes and mixed thoroughly. The sensitivity of the parasites to the test fractions was determined microscopically after incubation for 24 and 48 hours at 37°C.The incubation was undertaken in glass bell jar in which a candle was lighted for one minute to ensure the supply of required quantity of CO_2 (about 5%) 0_2 gas, 2% and about 93% nitrogen gas as demonstrated by Mukhtar *et al*, (2006).

Determination of the Activity

At the end of the incubation periods 24 and 48 hours, a drop of a thoroughly mixed aliquot of the culture media was smeared on microscopic slides and stained by Giemsa's stain techniques. The mean number of erythrocytes appearing as blue discoid cells containing life rings of the parasite (that appeared red pink) was estimated and the average percentage elimination by the samples was determined. The activity of the test concentrations of the extracts was calculated as the percentage elimination of the parasites after incubation period – 24 and 48 hours, using the formula below:

$$\%A \equiv \frac{N}{Nx} \times 100$$

Where, %A = Percentage activity of the extracts
N = Total number of cleared RBC, Nx = Total number of parasitized RBC
Note: RBC = Red Blood Cells (Muktar et al., 2006.)

Purification of fraction VD1-04

Fraction VD1-04 (2.0 g) was mixed with silica gel-sand mixture and loaded on a silica gel column (100.0 g). The column was eluted with solvent mixture and the eluantes collected in fractions of 100.0 ml. Petroleum ether (1.1 l), petroleum ether: dichloromethane (2.5 l, 4:1), petroleum ether: dichloromethane (1.3 l, 3:2), petroleum ether: dichloromethane (1.0 l, 1:1), petroleum ether: dichloromethane (3.3 l, 2:3), petroleum ether: dichloromethane (10.3 l, 1:4), petroleum ether: dichloromethane (3.7 l, 1:9), dichloromethane (1.8 l), dichloromethane: ethylacetate (13.7 l, 19:1), dichloromethane: ethylacetate (2.0 l, 9:1), dichloromethane: ethylacetate (3.8 l, 4:1), dichloromethane: ethylacetate (3.1 l, 3:2), dichloromethane: ethylacetate (2.8 l, 1:1), dichloromethane: ethylacetate (1.7 l, 2:3), dichloromethane: ethylacetate (0.9 l, 1:4) and ethylacetate (1.4 l) were collected in weighed vials and each fraction was air dried.

The fractions were analyzed on TLC and those with similar R_f values were combined. A compound VD1-04-198, $C_{19}H_{24}O_2$ was obtained as a white solid (25 mg) with Melting Point range of 94 – 97 ° C with summary spectral data shown below:
IRmax (cm^{-1}, KBr) 2609, 2345, 1688, 1458, 1304, 928, 730 and 680.
^{1}Hnmr (400MHz, chloroform-d) δ 5.353 (H, d, H J= 5.781Hz), 5.338 (H, t, H J=3.266Hz), 5.298 (H, s, H), 2.345 (H, t, H, J= 7.505Hz), 2.173 (H, s, H), 2.011 (H, d, H, J=5.748Hz), 1.622 (H, m, H, J= 7.292Hz), 1.305 (H, s, H), 1.255 (H, s, H), 1.095 (H, s, H) and 0.880 (H, t, H, J= 6.810Hz).

^{13}Cnmr (100MHz, chloroform-d) δ 179.69 (s, C), 129.99 (d, C), 129.70 (d, C), 33.97 (t, C), 31.89 (t, C), 29 (), 27(), 24.66 (t, C) and 14.07 (t, C).
MS (relative intensity) (M+) at m/z 284 (10), 257 (15), 256 (85), 213 (08), 129 (12), 87 (10), 83 (12), 73 (32), 69 (23), 60 (80), 57 (45), 55 (56), 45 (15), 43 (100, base peak), 41 (95), 39 (28), 29 (54), 27 (35), 16 (20) and 14 (27).

RESULTS

The colour, texture and weight of extracts from *Vitex doniana* as well as the secondary metabolites present were shown below in table 1 and 2 respectively. The results of antimalaria activity of the extracts are shown in Table 3.

Table 1: Physical properties of *Vitex doniana* extract and fractions

Plant fraction	Weight	Texture	Colour
VD1	47.36	Solid	Dark green
VD1-01	85.22	Viscous liquid	Dark green
VD1-02	96.71	Viscous liquid	Dark green
VD1-03	76.45	Solid	Brownish
VD1-04	108.42	Solid	Brownish

Key: VD1=crude 95% ethanol extract, VD1-01= petroleum ether soluble fraction, VD1-02= chloroform soluble fraction, VD1-03= ethylacetate soluble fraction, and VD1-04= methanol soluble fraction

Table 2: Phytochemical constituents of *Vitex Doniana* (sweet) extract and fractions

Fraction	Alkaloid	Flavonoids	Tannins	Reducing sugar	Steroids
VD1	**+**	**+**	**+**	**+**	**-**
VD1-01	**-**	**+**	**-**	**-**	**-**
VD1-02	**-**	**+**	**-**	**-**	**-**
VD1-03	**+**	**-**	**+**	**+**	**-**
VD1-04	**+**	**+**	**+**	**+**	**-**

Key: VD1= crude 95% ethanol extract, VD1-01= petroleum ether soluble fraction, VD1-02= chloroform soluble fraction, VD1-03= ethylacetate soluble fraction, and VD1-04= methanol soluble fraction. + = Presence and - = A

Table 3: Antimalaria activity of *Vitex doniana* bark extract and fractions

Fractions	Concentrations (µg/ml)	Average Number of parasite per field before Incubation	Overall Average number of parasite after 48 Hours	Percentage (%) Elimination of parasite at the End of Incubation
Control		20	20	0
VD1	1000		05.0	75
	500	20	09.0	55
	250		13.0	35
VD1-01	1000		04.0	80
	500	20	06.5	68
	250		10.5	48
VD1-02	1000		03.0	85
	500	20	06.5	68
	250		11.0	45
VD1-03	1000		07.0	65
	500	20	09.5	58
	250		12.0	40
VD1-04	1000		03.0	85
	500	20	04.5	78
	250		16.0	20

Key: VD1=crude 95% ethanol extract, VD1-01= petroleum ether soluble fraction, VD1-02= chloroform soluble fraction, VD1-03= ethylacetate soluble fraction, and VD1-04= methanol soluble fraction

Table 4: Antimalaria activity of fraction VD1-04-198

Fractions	Concentrations (µg/ml)	Average Number of parasite per field before Incubation	Overall Average number of parasite after 48 Hours	Percentage (%) Elimination of parasite at the End of Incubation
Control		20	20	0
VD1-04-198	1000		02.0	92
	500	20	03.0	86
	250		07.0	65
Artesunate	1000		01.5	95
	500	20	02.5	89
	250		05.5	72

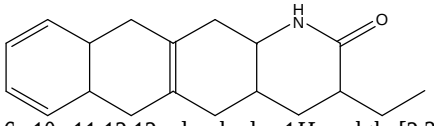
DISCUSSION

The physical characteristics of *Vitex doniana* plant extracts indicated that VD1, VD1-03, and VD1-04 were solids, while VD1-01 and VD1-02 were viscous liquid.

The phytochemical screening result showed variation in the distribution and type of secondary metabolites in the extracts. The ethanol extract (VD1) have shown the presence of alkaloid, flavonoids, reducing sugars and tannins. They were reflected in the other fractions, such that flavonoids appeared in all the fractions, while reducing sugars and tannins are reflected in VD1, VD1-03, and VD1-04. The activity of the isolated compound (VD1-04-198) was found to closer at all concentrations with the activity of the positive control (atesunate).

Anti-malaria activity was found to be dose dependent especially for fraction VD1 which demonstrated a remarkable activity at all concentrations. The most interesting anti-plasmodial activity was obtained with VD1-04, in which the microscopic examination of Giemsa's stained slides showed complete absence of the parasite at 500µg/ml after 48 hours. These observations suggested that the extract may be cytotoxic for *P falciparum*, thereby inhibiting their development. MP test result on VD1 has shown almost 85% elimination of the parasite, whereas the fractions VD1-02 and VD1-04 have about 68.0% and 78.0% elimination of the parasite respectively (after 48 hours at 500µg/ml).

From the proposed formula; $C_{19}H_{24}NO$ the degree of unsaturation = 19 – (24/2) + 1 = 8. Since the degree of unsaturation is 8 then an aromatic ring or carbonyl, double bonds (isolated or conjugated), or rings were suspected. The band at 1688 indicates a carbonyl, probably a saturated aliphatic lactam. The bands at 3000-2850 indicate C-H alkane and alkene stretches. The bands in the region 1458 to1304 could be due to C-N stretch, consistent with a lactam. The ^{13}C nmr has three signal in olefinic/ aromatic region [179.69 (s, C), 129.99 (d, C), 129.70 (d, C)] assign to one lactem carbonyl and two vinyl carbons.



3-Ethyl-3,4,4a,5,6,6a,10a,11,12,12a-decahydro-1*H*-naphtho[2,3-*g*]quinolin-2-one

CONCLUSION AND RECOMMENDATION

The present work showed that, the extracts of the bark of *Vitex doniana* (sweet) were significantly active against the *Plasmodium falciparum* with the pure compound VD1-4-198 isolated having a reasonable potential as a candidate for use against the malaria parasite.

It is recommended that further pharmacochemical work be carried out on this newly recognized compound to be reputed as antiplasmodial agent.

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