Trop J Nat Prod Res, July 2019; 3(7):221-224 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

Antimalarial Activity of Leaf Extract, Fractions and Isolation of Sterol from *Alstonia boonei*

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ARTICLE INFO

Article history:
Received 23 February 2019
Revised 19 July 2019
Accepted 21 August 2019
Published online 25 August 2019

ABSTRACT

Bioactive compounds in plants have found a wide application in medicines due to their pharmacological or toxicological effects in man and animals. *In vitro* anti-plasmodial activity of the leaves of *Alstonia boonei* was studied to support its traditional use as a remedy for malaria. The ethyl acetate extract was subjected to *in vitro* anti-plasmodial assays. The ethyl acetate extracts and fractions were found to be active against *Plasmodium falciparum* with percentage elimination range of 16.67 to 47.22%. The fractions were monitored using chromatographic techniques and this led to the isolation of *β*-Sitosterol, a plant derived sterol. The structure of the isolated compound was elucidated using spectroscopic methods including FTIR and NMR. *B*-Sitosterol has been reported to have antimalarial properties giving credence to the traditional uses of this plant for the treatment of malaria.

Keywords: Anti-plasmodial, *Alstonia boonie*, Leaves extracts, *β*-Sitosterol.

Introduction

Medicines from medicinal plants form the basis of primary healthcare for majority of the people living in rural and urban areas. World Health Organization has stated that more than 80% of people living in developing countries still depend on herbal medicines to treat common diseases including malaria.[1] This has been a general trend which is still being practiced till date.

Alstonia boonei is a large evergreen tree belonging to the family Apocynaceae and one of the widely used medicinal plants in different parts of Africa. It is distributed throughout the tropics and the rain forest of west and Central Africa and has been found effective against several pathogens. Pharmacological studies on the various parts of the plant have indicated that it possesses anti-inflammatory properties.[2,3] Immuno-stimulant property, diuretic, spasmolytic and hypotensive properties, antipsychotic and anxiolytic effect,[4] antimalarial, antipyretic, analgesic, anthelmintic and reversible antifertility effect. [5,6] The stem bark of *A. boonei* is used in traditional medicine to treat fever, painful micturition, insomnia, malaria and chronic diarrhea, rheumatic pains, as anti-venom for snake bites and in the treatment of arrow poisoning. It is also used in treating painful micturition and rheumatic conditions.[5] *A. boonei* has a history of use in traditional medicine in Nigeria and Central Cameroon for the treatment of malaria, insomnia, chronic diarrhea, and rheumatic pains, it also serves as anti-venom for snake bites and in the treatment of arrow poisoning.[3,7]

In Africa and elsewhere, plant extracts are still widely used in the treatment of malaria and other ailments, and up to 80% of the African population use traditional medicines for primary health care.[8]

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Citation: Adeyemi MM, Habila JD, Enemakwu TA, Okeniyi SO, Salihu L. Antimalarial Activity of Leaf Extract, Fractions and Isolation of Sterol from *Alstonia boonei*. Trop J Nat Prod Res. 2019; 3(7):221-224. doi.org/10.26538/tjnpr/v3i7.1

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Malaria is a vector born disease, caused by protozoan parasites of the genus *Plasmodium* transmitted from the blood of an infected person and passed to a healthy human by female anopheles mosquito bites and is still a prevalent issue in Nigeria. Malaria is a worldwide public health concern, the World Health Organization (WHO) malaria world report,[1] gave an estimate of 207 million cases and 627,000 deaths globally in 2012 alone. It has been reported that over 70 countries are malaria endemic and globally, an estimated 3.4 billion people are at risk of malaria infection.[1]

In this study, the antimalarial activity of pooled fractions from the ethyl acetate extract of *A. boonei* leaves was carried out with the intent of providing scientific proof of the claim by traditional healers of its uses in traditional medicine especially as a herbal treatment for malaria.

Materials and Methods

General Experimental Procedures

The melting point of the isolated compound was recorded on an Ernst Leitz Wetziar micro-hot stage melting point apparatus (uncorrected). Thin layer Chromatography (TLC) profiling was carried out on Silica gel 60 F_{254} (Merck), spots on TLC plates were visualized under UV light and by spraying with 10% sulphoric acid (H_2SO_4) followed by heating at 100^{o}C for at least 5 min. Column Chromatography was performed using Silica gel (Merck, 60 – 120 mesh size). ^{1}H and ^{13}C-NMR) were recorded in chloroform on a Bruker Avance 400 MHz spectrophotometer, chemical shifts were reported in ppm relative to TMS.

Plant collection and authentication

The fresh leaves of *Alstonia boonei* were collected from Eke-Avrugo Community in Igalamela-Odolu LGA of Kogi State. Identification and authentication was done by Prof. M.O. Nwosu of the Department of Plant Science and Biotechnology, University of Nigeria, Nsukka with a voucher number (UNH No. 12a).

The leaves were washed and air-dried for two weeks after which they were ground into powder form, sieved and stored in air-tight containers for further use. Extraction and fractionation was done according to standard protocol.[9] The powdered plant material (200 g) was percolated with ethyl acetate (1000 cm^3) for one week. The ethyl acetate soluble fraction was evaporated to dryness at room temperature and a solid brown residue weighing 10.70 g was obtained.

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Extraction and isolation
The ethyl acetate extract (10.70 g) was subjected to silica gel column chromatography (300 g; 85 cm × 6.5 cm). The column was eluted with n-hexane:ethyl acetate solvent mixture in the following percentage order; 100:0, 97.5:2.5, 95:5, and 90:10. The eluates were collected in portions of about 50 mL each. A total of 125 fractions were collected. Each fraction was concentrated and analyzed on TLC plates; similar fractions were pooled together on the basis of their TLC profiles. Fraction $AB_{A\text{-}1\text{-}5}$ was further subjected to column purification from which a white crystalline solid was obtained which gave a single spot on TLC plate and subjected to spectroscopic analysis.

Anti-Malaria Assay
Malaria parasites
Confirmed *Plasmodium falciparum* infected blood sample of patients were collected from the Hematology Department, Bayero University Teaching Hospital, Kano. The samples were immediately transferred into K3-EDTA disposable plastic sample bottles corked and transported to the Microbiology laboratory at Bayero University in a thermo flask containing water maintained at 4°C. The preparation of the malaria parasite, separation of the erythrocytes from the serum of the blood samples, preparation of *Plasmodium falciparum* culture medium were carried out using standard methods.[10-14]

Ethical Consideration
Ethical approval for the use of human tissue was obtained from the Health Research Ethics Committee of the federal ministry of Health and Human Services, Kaduna state with approval reference number MOH/ADM/744/VOL.1/740.

Preparation of Plasmodium falciparum Culture Medium
Venous blood (2 mL) from the main vein of healthy albino rabbits was withdrawn using a disposable 5 mL syringe (BD 205 WG). This was defibrinated by allowing it to settle for one hour. The defibrinated blood was centrifuged at 1500 rpm using spectre merlin centrifuge for 10 min and the supernatant was collected in a sterilized tube. The sediment was further centrifuged at 1500 rpm for 5 min, and the supernatant layer was added to the first test tube. The sediments were discarded and the serum collected was supplemented with RPMI 1600 medium [KCl 5.37 mM, NaCl 10.27 mM, $MgSO_4$ 0.4mM, $NaHPO_4$ 17.73 mM, $Ca(NO_3)_2$ 0.42 mM, $NaHCO_3$ 2.5 mM, and glucose 11.0 mM (BDH Ltd, UK)]. The medium was sterilized with 40 µg/mL gentamicin sulphate.[11]

Separation of the Erythrocytes from the Serum of the Blood Samples
About 50% dextrose solution (0.5 mL) was added to each of the blood samples (5 mL) defibrinated, and then centrifuged at 2500 rpm for 15 min in a spectra merlin centrifuge. Supernatant were separated from the sediments. The latter was diluted with normal saline,[10] and centrifuged at 2500 rpm for 10 min. The resulting supernatants were discarded. Samples with higher parasitaemia (above 0.5%) were diluted with fresh malaria parasite negative erythrocytes.[13]

In-Vitro Assay of the Activity of the Fractions on the Plasmodium falciparum Culture
A 0.1 mL of test fractions (1 and 2 mg/mL in 10% DMSO) and 0.2 mL of the culture medium were added into a tube containing 0.1 mL of 0.5% parasitized erythrocytes and mixed thoroughly. The sensitivity of the parasites to the test samples was determined microscopically after incubation for 48 h at 37°C. The incubation was undertaken in glass bell jar containing a lighted candle to ensure the supply of required quantity of 5% CO_2, 2% O_2 gas, and 93% nitrogen gas.[14] Artemether/lumefantrine in 0.5 – 5.0 mg/cm^3 was used as the positive control.

Determination of the Activity
At the end of the incubation period, a drop of a thoroughly mixed aliquot of the culture medium was smeared on microscopic slides and stained by Giemsa's staining 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 samples was calculated as the percentage elimination of the parasites after incubation period of 48 h, using the formula below;[14]

$$\% = \frac{N}{Nx} \times 100$$

Where, % = Percentage activity of the test sample
N = Total number of parasites eliminated by the sample,
Nx = Total number of parasitized in blood cells.

Statistical analysis

Data were analyzed by using statistical software package (SPSS for Windows, Version 18, IBM Corporation, NY, USA) using Turkey's-HSD multiple range post-hoc test. Values were considered significantly different at $P< 0.05$. All data are presented as the mean ± SD.

Results and Discussion

The results of the column chromatography (Table 1) of ethyl acetate extract as well as anti-malarial parasite assay of the pooled column fractions (Table 2), led to the selection of fraction $AB_{A\text{-}1\text{-}5}$ for further purification. The average percentage column fractions recovery was calculated as 87.33%. From the column collection, $AB_{A\text{-}1\text{-}5\text{-}3}$ was obtained as crystalline white substance (Yield, 0.0346 g (18.47%); Melting point; 138-140°C) with single spot on TLC plate.
Table 4 shows the ^{13}C-NMR and ^{1}H-NMR data of compound $AB_{A\text{-}1\text{-}5\text{-}3}$ and comparison with literature data. The FTIR results showed the following characteristics absorption bands at 3306.1 cm^{-1} which is typical of O-H stretch vibration. Absorption at 2944.7 cm^{-1}and 2832.9 cm^{-1} is due to aliphatic C-H stretch vibrations. Other absorption frequencies include 1449.6 cm^{-1} as a result of the C=C stretch. From the ^{1}H-NMR data, the signal for H-3 proton appeared at δ 3.54 as a triplet of a doublet with a *J* value of 4.5 and 1.1 Hz. Olefinic proton (H-6) showed a multiplet signal at δ 5.34, six methyl proton signals at δ 1.28, δ 1.23, δ 1.12, δ 1.09, δ 1.04 and 1.02. The ^{13}C-NMR showed recognizable signals at 140.71 and 121.73 ppm, which were assigned to C-5 and C-6 double bonds, respectively. The value at 19.32 ppm corresponds to angular carbon (C-19). The Spectra obtained from the DEPT experiments showed twenty-nine carbon signal including six methyls, eleven methylenes, nine methine and from this three quaternary carbons were identified.[15-17] From the spectral data and with comparison with those described in the literatures the compound was identified as *β*-Sitosterol[18] (Figure 1).
Table 3 shows the results of the antimalarial activity of the positive control (Artemether/lumefantrine). At 5.0 mg/mL, the percentage parasite elimination (% activity) was 93.18% and at 0.5 mg/mL, the percentage activity was 75.00%.
Recent studies have shown that *β*-sitosterol have been tested in both *in vivo* and *in vitro* assays as antimalarial agents and this compound has been isolated from the root of plant materials.[20] Other researchers have also reported the *in vivo* antimalarial activity of *β*-sistoterol in mice infected with W2 strain of *P. falciparum.*[21]

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Table 1: Weight of macro column chromatography analysis and malaria parasite assay

Pooled Fractions	fractions	Weight (g)	% Activity at 2.0 mg/mL
1 to 5	AB_{A-1-1}	2.024	16.67
6 to 8	AB_{A-1-2}	1.8521	22.22
9 to 12	AB_{A-1-3}	1.91	41.67
13 and 14	AB_{A-1-4}	0.952	27.78
15 to 19	AB_{A-1-5}	2.6071	47.22

Table 2: Weight of micro column chromatography analysis and malaria parasite assay of AB_{A-1-5}

Pooled Fractions	fractions	Weight (g)	% Activity at 1.0 mg/mL
1 to 6	$AB_{A-1-5-1}$	0.0301	12.42
7 to 10	$AB_{A-1-5-2}$	0.0222	10.08
11 to 21	$AB_{A-1-5-3}$	0.0346	18.47

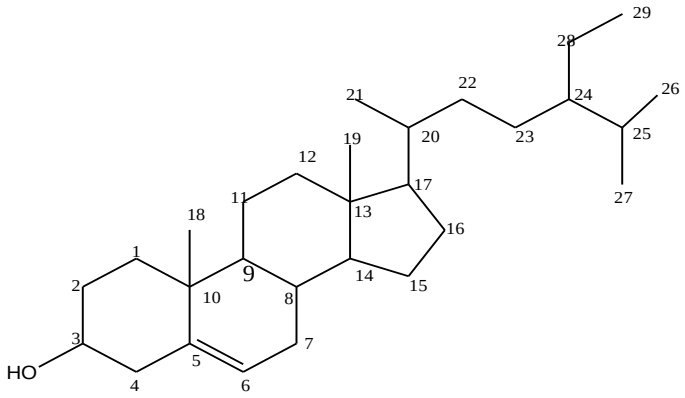

Figure 1: Molecular Structure of compound $AB_{A-1-5-3}$ (β-sitosterol)

Table 3: Results of antimalarial activity of positive control (Artemether/lumefantrine).

Concentration (mg/mL)	Average No. of parasite before Incubation	Average Number of parasite after 48 h incubation	Percentage elimination after Incubation (%)
0.5	44	11	75
1.0	44	6	86.36
2.0	44	4	90.90
5.0	44	3	93.18

Table 4: ^{13}C and ^{1}H-NMR (400 MHz, $CDCl_3$) data of compound $AB_{A-1-5-3}$ in comparison with literature data

	Experimental		Literature	
Position	^{13}C	^{1}H	^{13}C	^{1}H
C-1	37.21		37.22	
C-2	31.61		31.62	
C-3	71.81	3.539 (tdd	71.77	3.53 (tdd, 1H, *J* = 4.5, 4.2, 3.8 HZ)
C-4	42.25		42.26	
C-5	140.71	5.383	140.72	5.36 (t, 1H, *J* = 6.4HZ)
C-6	121.73		121.70	
C-7	31.89		31.88	
C-8	31.87		31.86	
C-9	50.08		50.08	
C-10	36.48		36.47	
C-11	21.05		21.05	
C-12	39.73		39.74	
C-13	42.29		42.29	
C-14	56.72		56.72	
C-15	24.28		24.28	
C-16	28.23		28.22	
C-17	56.00		56.00	
C-18	11.84		11.84	
C-19	19.38	0.985	19.38	0.93 (d, 3H, *J* = 6.5 HZ)
C-20	36.12		35.77	
C-21	18.75		18.69	
C-22	36.12		36.16	
C-23	23.02		23.80	
C-24	39.73	0.86	39.48	0.84 (t, 3H, *J* = 7.2HZ)
C-25	28.23		27.99	
C-26	22.65	0.84	22.65	0.83 (d, 3H, *J* = 6.4HZ
C-27	22.68	0.82	22.61	0.81 (d, 3H, *J* = 6.4)
C-28	24.2	0.66	24.2	0.68 (s, 3H)
C-29	11.8	1.02	11.81	1.01 (s, 3H)

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Conclusion

The identified compound *β*-sistoterol is one of the several phytosterols with structure similar to that of cholesterol. Phytochemical screening of the plant extract had indicated the presence of steroids and sterols.[22] This study has proven that *A. boonei* leaves used for this study is active against *Plasmodium falciparum* parasite.

Conflict of interest

The authors declare no conflict of interest.

Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

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