Journal of Advances in Medical and Pharmaceutical Sciences

21(1): 1-14, 2019; Article no.JAMPS.44550
ISSN: 2394-1111

Assessment of Antiplasmodial Activity and Toxicity of Crude Extracts and Isolated Compounds from *Oncoba spinosa* (Flacourtiaceae)

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Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/JAMPS/2019/v21i130124
Editor(s):
(1) Dr. Cyprian Ogbonna Onyeji, Professor, Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Obafemi Awolowo University, Ile-Ife, Nigeria.
Reviewers:
(1) Kesara Na-Bangchang, Thammasat University, Thailand.
(2) Tapiwa Manyarara, College of Health Sciences, University of Zimbabwe, Zimbabwe.
Complete Peer review History: http://www.sdiarticle3.com/review-history/44550

Original Research Article

Received 28 September 2018
Accepted 05 December 2018
Published 27 July 2019

ABSTRACT

Aims: The medicinal plant *Oncoba spinosa* is used by the local communities in Butebo County in Eastern Uganda for treatment of malaria and other diseases. *In vitro* antiplasmodial activities of the crude extracts and isolated compounds were screened against chloroquine sensitive 3D7 and resistant Dd2 strains. *In vivo* acute toxicity of the extracts and structure elucidation were also determined in the study.
Experimental: Crude extracts of: *n*-hexane, dichloromethane, ethyl acetate and methanol were prepared. Isolation and purification of these extracts were done using chromatographic techniques which consisted of column and thin layer chromatography. The structures were elucidated on the

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basis of spectroscopic evidence. *In vitro* antiplasmodial activity was performed on chloroquine sensitive 3D7 and resistant Dd2 strains of *Plasmodium falciparum* using SYBR Green 1 assay technique. Lorke's method of acute toxicity was used to determine the *in vivo* acute toxicity of the crude extracts in mice.

Results: The root ethyl acetate crude extract had highest antiplasmodial activity of IC_{50}:4.69 ± 0.01 µg/mL and 3.52 ± 0.02 µg/mL against 3D7 and Dd2 strains respectively while the remaining three were inactive against both strains of *Plasmodium.* Isolation resulted in the identification of three known compounds which included: β-sitosterol, benzoic acid and chaulmoogric acid. Among the tested compounds β-sitosterol showed the highest activity of IC_{50} 3D7: 5.51 µM. Dichloromethane and hexane extracts were non-toxic with LD_{50} > 5000 mg/kg while the EtOAc and MeOH extracts were slightly toxic with LD_{50} of 547.72 mg/kg. Statistically significance existed between the antiplasmodial activity of the crude extracts and compounds when compared with the controls at (p < 0.05). Extracts and compounds exerted a significant (P < 0.05) decrease in antiplasmodial activity compared to the positive controls.

Conclusion: The findings confirm the ethnobotanical use of *O. spinosa* by the local communities in Butebo County for the treatment of malaria. The results also suggest that the crude extract of this plant is safe and possesses antimalarial activity which can be used as a basis for *in vivo* and clinical studies to be done. Therefore the plant can offer a potential drug lead for developing a safe, effective and affordable antimalarial.

Keywords: Oncoba spinosa; β-sitosterol; in vitro; antiplasmodial; acute toxicity; malaria.

1. INTRODUCTION

Malaria remains a devastating scourge on the lives and livelihoods to the millions of global citizens living in the tropics. Despite the significant advances made in lessening the burden of malaria in recent years, the disease still remains a major public health problem affecting many people in tropical and subtropical regions [1]. This is especially the case in sub-Saharan Africa where 90% of the estimated annual global malaria deaths occur [2]. Most of the conventional drugs are no longer effective due to the emergence of drug resistant strains.

In Uganda, malaria is the most common disease and accounts for 25-40% of out-patient attendance at health facilities, and 20% of inpatient admissions. It also kills at least 9 to 14% of all in-patients. Children aged five years and below, as well as pregnant women, are the most affected. In Uganda, more than 200 children die daily from this disease [3] and people largely rely on traditional medicine [4].

Oncoba spinosa belongs to the family Flacourtiaceae (Salicaceae). It is a small tree of about 13 m high which grows under conditions of higher rainfall, deciduous, secondary and fringing forest from Senegal to West Cameroon. It is widely distributed in tropical Africa and Arabia [5]. The leaves of the plant are traditionally reputed for its medicinal potential particularly in South-West of Nigeria for the treatment of diabetes and cancer, while the seed oil is drunk as a fever remedy. In Ivory Coast, the plant has a good reputation as an aphrodisiac and in Tanzania the leaf sap is drunk as a remedy for malaria cure [6].

O. spinosa is used in the treatment of malaria and fever by the local communities in Butebo County, Pallisa District in Eastern Uganda. Investigations on the antiplasmodial and toxicity properties of crude extracts of *O. spinosa* have not been determined. In our search for more effective drugs against *P. falciparum* and as a continuation of our investigation of medicinal plants used traditionally in Uganda to treat malaria [7], special attention was focused to *O. spinosa*.

In search for new antimalarial principles, *O. spinosa* normally used to treat malaria and other ailments among the local communities in Butebo County Pallisa District, Eastern Uganda was investigated. The crude extracts and pure compounds were screened against chloroquine (CQ susceptible and resistant strains of *P. falciparum* (3D7 and Dd2, respectively) using the fluorescence-based SYBR Green assay. Isolation and purification of bioactive principles present in the crude extracts was done using chromatographic techniques (column and thin layer chromatography). Structure determination was done using NMR spectroscopic techniques. The *in vivo* acute toxicity assay was also carried out in mice using Lorke's method [8].

2. MATERIALS AND METHODS

2.1 Plant Material

Oncoba spinosa (Fig. 1) was identified and documented as an antimalarial remedy in an ethnobotanical survey that was conducted in Butebo County to document antimalarial medicinal plants. [7]. The plant was photographed, collected, dried and taken for identification by a taxonomist at Makerere University, Department of Botany. The voucher specimen (KP 904) was deposited at the Department of Botany herbarium for future specifications.

2.2 Extraction

Plant materials (roots) were washed, cut into small parts and then air dried at room temperature in a shade for 21 days [9]. The dried plant material was pounded using a clean mortar and pestle and then blended into fine powder with electric blender (Thomas-Wiley Mill Model 4). Crude plant extracts were prepared by maceration of 800 g of air dried powdered plant material. This was done in sequential cold extractions with 1200-2000 mL of *n*-hexane, dichloromethane (DCM), ethyl acetate (EtOAc) and methanol (MeOH) at room temperature (25°C) for 72 hours in Winchester bottles (2.5 L) with intermittent agitations [10]. The process was repeated twice and after the third extraction, the same crude plant powder was air dried and further treated three times with the next solvent. In all the three stages, the extracts were filtered through cotton wool, then Whatman filter paper (Whatman® No. 1). Finally after filtration the crude extracts were concentrated, under reduced pressure in a water bath at 40-45°C, by using a rotatory evaporator machine (BUCHI-R 205). The plant extracts were then transferred to weighed containers and put in the oven to dry completely at 40°C to produce solid materials. Their mass yields were calculated based on dry weight and expressed as percentage yield of the crude extract (Table 1) using the equation: Extract yield (%) = $\frac{W1}{W2}$ × 100, where, W1 = net weight of crude extracts (grams), W2 = total weight of medicinal plant powder (grams).

2.3 Isolation and Fractionation of Bioactive Compounds

The isolation of the pure compounds was achieved by a combination of column chromatography and thin layer chromatography (TLC). Column chromatography was run on silica gel 60 (70-230 mesh) and analytical TLC was carried out on pre-coated silica gel 60 F254 (Merck). The detection of the spots was accomplished by the TLC plates first being visualised with a UV lamp at 254 and 365 nm wave lengths. This was followed by development with anisaldehyde spray reagent consisting of anisaldehyde, conc. H_2SO_4 and methanol in a ratio of 1:2:97 followed by heating in an oven at a temperature of 100°C.

The DCM crude extract (2.6 g) yellow in colour was eluted with hexane: ethyl acetate mixtures of increasing polarity (0-100% EtOAc) in the ratios of 100:0 to 0:100 then washed with 100% MeOH. A total of 12 broad fractions (S1-S12) were collected and combined on the basis of their TLC profiles. Fraction (S2 and S3) were eluted with a

Fig. 1. Photo of *O. spinosa*

gradient of EtOAc-hexane (2:3, 1:1) respectively then washed with 100% EtOAc, to give 29 fractions labeled A1-A29. TLC analysis pooled combined fractions of A2-A10, A11 and A12. Combined fraction A2-A10), was eluted with 100% hexane then EtOAc: hexane (1:19, 1:4) respectively to give a white powder that was a pure compound (OS2) (293.4 mg, 1.41%). Fractions (S6 and S7) were eluted with solvent system of increasing polarity of EtOAc-hexane (2:3, 1:1) respectively followed by 100% EtOAc. This gave 28 fractions labeled G1-G28). TLC profile analysis gave pooled combined fraction, G21 and G22 which was eluted with EtOAc-hexane, 1:1 followed by 100% EtOAc to give a pure compound (OS1) (29.8 mg, 1.15%), of white crystals.

The brown EtOAc crude (6.84 g), was eluted with hexane-EtOAc mixture of increasing polarity of (0-100%), finally washed with 100% MeOH. This resulted in 13 broad fractions identified as OE1-OE13. The fractions were combined according to their TLC profiles to give fraction OE3-OE12 that was eluted with EtOAc-hexane (3:17, 1:4) respectively then followed by 100% EtOAc. This yielded 38 fractions labelled AE1-AE38. This pooled fraction AE1-AE24 was eluted with diethyl ether-DCM (13:87, 4:21) respectively followed by 100% EtOAc to give compound (OS3) (40.9 mg, 0.60%) with white crystals. Four pure compounds were isolated from this plant, three from the dichloromethane and one from the ethyl acetate crude extracts.

2.4 Structure Elucidation of Pure Compounds

Identification of the pure compounds was carried out by spectroscopic methods that included 1-D and 2-D NMR. The 1-D consisted of ^{1}H, ^{13}C and DEPT NMR while the 2-D involved COSY, NOESY, ROESY, HSQC and HMBC. This was analysed using a Bruker avance ^{1}H NMR (500 MHz) and ^{13}C NMR (125 MHz) to get the spectral data using TMS as the residual solvent signal reference. The spectral data obtained was compared with that reported from literature in order to elucidate the structures of the isolated compounds.

2.5 Antiplasmodial Bioassay Activity

The extracts and pure compounds were assayed using non-radioactive malaria SYBR Green I assay technique [11] with modifications [12] to determine a concentration that inhibits growth of 50% of parasites in culture (IC_{50}). Two different *P. falciparum* strains, chloroquine sensitive (3D7) and chloroquine-resistant (Dd2) were used. These isolates were grown as described with minor modifications [13,14]. The RPMI 1640 medium was prepared accordingly as described [15]. Chloroquine and mefloquine were used as standard controls while 0.4% DMSO was used as the negative control. The IC_{50} values were given as mean of two or three independent experiments and the results were presented as mean IC_{50} ± SD (standard deviation). The resistance index (RI) for each crude extract and isolated compounds was also determined to assess the activity of the *Plasmodium* on the CQ resistant strain. It was calculated as the ratio between IC_{50} of the resistant value of the strain to the sensitive value of the strain. RI = IC_{50} of resistant strain (Dd2) ⁄ IC_{50} of sensitive strain (3D7). The RI value determines whether the test samples have activity against the resistant strain of *P. falciparum.*

2.6 *In vivo* Acute Toxicity (LD_{50}) Test

The present work was approved by the Ethical Committee for using animals at Makerere University, Department of Pharmacology (number 1250). The estimated lethal dose (LD_{50}) of the crude extracts in mice was performed using the method described [8]. A total of 86 mice weighing (13.0-30.0) g obtained from Department of Pharmacology Makerere University were used to carry out the *in vivo* acute toxicity experiments. The mice were kept in cages in a ventilated room and fed with a pelletised grower mash. They were also provided with clean drinking water. The weight of each mouse was measured and the dose calculated for all the dose levels. The tests were done in two phases. In the first phase, nine (9) mice were divided into 3 groups of 3 mice per group. After overnight fast (24 hours) the animals in the first phase received doses of 500, 1000, and 2000 mg/kg weight body. The remaining surviving animals were sacrificed under chloroform anaesthesia. When no death was observed in the first phase, then higher doses were administered in the second phase. In the second phase, also 9 mice, 3 per group were treated with doses of 3000, 4000 and 5000 mg/kg body weight. One mouse was used as a control and received an equivalent volume of distilled water. When death occurred in the first phase then four groups of four animals each was used. These group of animals received doses of 600, 700, 800, and 900 mg/kg body weight. The stock

solution was prepared by dissolving 0.2 g of the crude extract in 2 mL of distilled water to give a concentration of 100 mg/mL. The crude extracts were then administered using a cannula attached to a graduated syringe. The animals were given food and water four hours post drug administration. Toxicity signs such, writhing, decreased motor activity, decreased body/limb tone, decreased respiration, loss of appetite, feeling sleep, depression, gasping for air, palpitation and mortality (death) that occurred within 24 hours was recorded. This was followed by determination of the lethal dose (LD_{50}).

2.7 Data Analysis

2.7.1 Antiplasmodial bioassay activity tests

Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentration (IC_{50}) for each drug by an equation generating a sigmoidal concentration-response curve (variable slope), with log transformed drug concentrations on the X-axis and relative fluorescent units (RFUs) on the Y-axis (Graphpad Prism for Windows, version 4.0; Graphpad Software, Inc., San Diego, CA) [13, 16]. IC_{50} values above 100 µg/mL were considered inactive [17]. This is in line with World Health Organization guidelines [18] and basic criteria for antiparasitic drug discovery. In describing *in vitro* antiplasmodial activities of natural products, pure compounds are considered to be inactive when they have IC_{50} > 200 µM, whereas those with an IC_{50} of 100-200 µM are considered to have low activity; IC_{50} of 20-100 µM, moderate activity; IC_{50} of 1-20 µM good activity; and IC_{50} < 1 µM excellent/potent antiplasmodial activity [19]. Similarly activities of crude extracts are classified into five classes according to their IC_{50} values: high activity (IC_{50} < 5 µg/mL); promising activity (5 µg/mL < IC_{50} < 15 µg/mL); moderate activity (15 µg/mL < IC_{50} < 50 µg/mL); weak activity (50 µg/mL < IC_{50} < 100 µg/mL), inactive IC_{50} > 100 µg/mL [18].

2.7.2 Toxicity bioassay activity tests

The LD_{50} values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) preceding the lowest lethal dose (where deaths occurred). $LD_{50} = \sqrt{(D_o \times D_{100})}$, Where LD_{50} = median lethal dose, D_o = highest dose that gave no mortality, D_{100} = lowest dose that produced mortality. The general toxicity activity was considered: ≤ 1 mg/kg (extremely toxic), 1-50 mg/kg (highly toxic), 50-500 mg/kg (moderately toxic), 500-5000 mg/kg (slightly toxic), 5000-15000 mg/kg (practically non-toxic) and ≥ 15000 mg/kg (relatively harmless) [18].

2.7.3 Statistical analysis

Data on *Parasitemia*, was analysed using windows SPSS version 16. Statistical significance was determined with the Biostat 1.0 software package using one way ANOVA and student's *t*-test. The experimental results were expressed as mean ± standard deviation (SD) for each group of experiments. These were transformed in *P*-values to compare results at 95% confidence level (α = 0.05). This was used to compare results between doses, among treatment and control dose levels. The differences between means was considered significant when $P < 0.05$ [20].

3. RESULTS AND DISCUSSION

3.1 Test Samples for Bioassay Activity Screening

The yields of the four crude extracts ranged between (0.51-3.01) percent while for the pure compounds the yields were (0.10-11.28) percent. These were calculated based on the weight of medicinal plant powder (for crude extracts) and crude extracts (pure compounds). Among the extracts the MeOH extract gave the highest yield (3.01%) and the dichloromethane had the lowest with 0.51%. Chaulmoogric acid (OS2) (11.28%) was the most abundant while OS3 had the lowest yield of 0.60% among the isolated pure compounds (Table 1).

3.2 Elucidation of Compounds from the Root Extract

The phytochemical analysis of the roots of *O. spinosa* led to the isolation of three compounds identified as a steroid, β-sitosterol (OS1), Chaulmoogric acid (OS2) and benzoic acid (OS3) (Fig. 2). The three compounds obtained, as mentioned in the experimental section, were subjected to spectroscopic analysis for identification. The details of the spectral peaks were noted as follows:

Compound (OS1): β-sitosterol, 29.8 mg, white crystals. ^{1}H NMR (500 MHz, $CDCl_3$). δ_H: 0.91-2.29 (22 H, m, 2H-1, 2, 4, 7, 11, 12, 15, 16, 22, 23, 28), 0.92-1.66 (7 H, m, H-8, 9, 14, 17, 20, 24, 25), 0.68 (3H, s, H-18), 0.81 (3H, s, d, *J* = 6.78

Hz, H-26), 0.82 (3H, t, *J* = 6.9 Hz, H-29), 0.84 (3H, s, H-19), 0.92 (3H, d, *J* = 6.55 Hz, H-21), 1.01 (3H, s, H-27), 3.52 (1H, tdd, *J* = 4.42, 11.11 Hz, H-3), 5.35 (1H, br, d, *J* = 5.2 Hz, H-6). ^{13}C NMR (125 MHz, $CDCl_3$). δ_C: 37.5 (C-1), 31.9 (C-2), 72.0 (C-3), 42.5 (C-4), 141.0 (C-5), 121.9 (C-6), 34.2 (C-7), 32.1 (C-8), 50.4 (C-9), 36.7 (C-10), 21.3 (C-11), 40.0 (C-12), 42.5 (C-13), 57.0 (C-14), 24.5 (C-15), 28.5 (C-16), 56.3 (C-17), 12.1 (C-18), 19.6 (C-19), 36.4 (C-20), 19.0 (C-21), 35.9 (C-22), 26.3 (C-23), 46.1 (C-24), 29.4 (C-25), 20.0 (C-26), 19.3 (C-27), 23.3 (C-28), 12.2 (C-29).

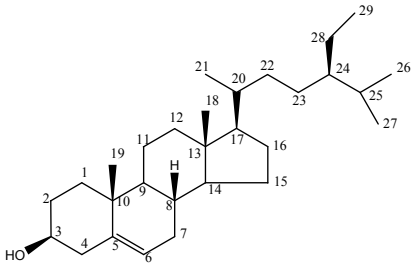
Compound (OS2): Chaulmoogric acid, 186.9 mg, white crystals. ^{1}H NMR (500 MHz, $CDCl_3$). δ_H: 5.68 (1H, tdd, *J* = 2.05, 4.02 Hz, H-2′), 5.68 (1H, tdd, *J* = 2.05, 3.94 Hz, H-3′), 2.61 (1H, br, s, H-1′), 2.35 (2H, t, *J* = 7.49 Hz, H-2), 2.24 (2H, m, H-4′), 2.02 (2H, m, H-5′), 1.63 (2H, qn, *J* = 7.49 Hz, H-3), 1.26 (26H, br s, H-4-H-13), 10.30 (1H-OH). ^{13}C NMR (125 MHz, $CDCl_3$). δ_C: 178.8 (C-1), 33.9 (C-2), 24.7 (C-3), 29.2 (C-4), 29.4 (C-5), 29.6 (C-6), 29.7 (C-7), 29.6 (C-8), 29.9 (C-9), 29.7 (C-10), 28.0 (C-11), 29.1 (C-12), 36.2 (C-13), 45.6 (C-1′), 135.5 (C-2′), 130.0 (C-3′), 32.0 (C-4′), 29.9 (C-5′)

Compound (OS3): Benzoic acid, 40.9 mg, white crystalline solid. ^{1}H NMR (500 MHz, $CDCl_3$). δ_H: 8.08 (1H, dd, *J* = 7.89, 1.70 Hz, H-6, H-2), 7.46 (1H, dd, *J* = 7.15, 1.13 Hz, H-5, H-3), 7.47 (1H, t, 7.15 Hz, H-4). ^{13}C NMR (125 MHz, $CDCl_3$). δ_C: 130.6 (C-1), 130.3 (C-2, 6), 129.2 (C-3, 5), 134.0 (C-4), 169.6 (C-7).

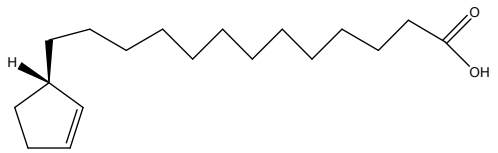
Compound (OS1): This compound showed a phenomenon that is seen by the frame work of steroids [21].

Table 1. Percentage yields of crude extracts and pure compounds

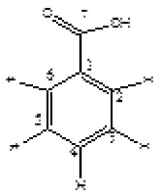
Species name	Weight of plant material (g)	Extraction solvent	Crude extract		Pure compounds		
			Weight (g)	Yield % w/w	Name	Weight (g)	Yield% w/w
O. spinosa	800	Hex	9.50	1.19			
		DCM	4.11	0.51	β-sitosterol (**OS1**)	29.80	1.15
					Chaulmoogric acid (**OS2**)	293.40	11.28
		EtOAc	10.46	1. 33	Benzoic acid (**OS3**)	40.90	0.60
		MeOH	24.11	3.01			



--sitosterol (OS1)



Chaulmoogric acid (OS2)



Benzoic acid (OS3)

Fig. 2. Structures of isolated compounds

Table 2. ^{1}H and ^{13}C NMR spectroscopic data for β-sitosterol (OS1) in $CDCl_3$ compared against literature values. Literature [23] [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz]

Position of caebon	^{13}C NMR (125 MHz)	^{13}C NMR (100 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) Literature
1α	37.5 (CH_2)	37.6	1.82 m	-
1β			1.85 m	-
2α	31.9 (CH_2)	31.5	1.95 m	-
2β			1.99 m	-
3	72.0 (CH)	71.6	3.52 (1H, tdd, *J* = 4.42, 11.11 Hz)	3.52 m
4α	42.5 (CH_2)	42.8	2.24 m	-
4β			2.29 m	-
5	141.0 (C)	140.5	-	-
6	121.9 (CH)	121.5	5.35 (1H, br, d, *J* = 5.20 Hz)	5.37 m
7α	34.2 (CH_2)	33.9	1.00 m	-
7β			1.51 m	-
8	32.1 (CH)	31.8	1.51 m	-
9	50.4 (CH)	50.4	0.92 m	-
10	36.7 (C)	36.7	-	-
11α	21.3 (CH_2)	21.1	1.46 m	-
11β			1.50 m	-
12α	40.0 (CH_2)	39.9	1.99 m	-
12β			2.02 m	-
13	42.5 (C)	42.8	-	-
14	57.0 (CH)	56.5	1.00 m	-
15α	24.5 (CH_2)	24.5	1.06 m	-
15β			1.58 m	-
16α	28.5 (CH_2)	28.5	1.25 m	-
16β			1.84 m	-
17	56.3 (CH)	57.3	1.11 m	-
18	12.1 (CH_3)	12.0	0.68 (3H, s)	0.75 (3H, s)
19	19.6 (CH_3)	19.6	0.84 (3H, s)	1.09 (3H, s)
20	36.4 (CH)	35.9	1.36 m	-
21	19.0 (CH_3)	18.7	0.92 (3H, d, *J* = 6.55 Hz)	0.98 (3H, d, *J* = 6.50 Hz)
22α	35.9 (CH_2)	34.2	0.91 m	-
22β			1.35 m	-
23α	26.3 (CH_2)	26.3	1.15 m	-
23β			1.83 m	-
24	46.1 (CH)	46.4	0.93 m	-
25	29.4 (CH)	29.2	1.66 m	-

Position of caebon	^{13}C NMR (125 MHz)	^{13}C NMR (100 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (400 MHz) Literature
26	20.0 (CH_3)	19.8	0.81 (3H, d, *J* = 6.78 Hz)	0.85 (3H, d, *J* = 6.70 Hz)
27	19.3 (CH_3)	19.2	1.01 (3H, s,)	0.81 (3H, d, *J* = 6,7 Hz)
28α	23.3 (CH_2)	23.5	1.22 m	-
28β			1.25 m	-
29	12.2 (CH_3)	11.8	0.82 (3H, t, *J* = 6.90 Hz)	0.92 (3H, t, *J* = 7.4 Hz)

Table 3: ^{1}H and ^{13}C NMR spectroscopic data for Chaulmoogric acid (OS2) in $CDCl_3$ compared against literature values. Literature [27] [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (100 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR literature (400 MHz)
1	178.8 (C)	172.4	-	-
2α	33.9 (CH_2)	34.1	2.35 (2H, t, *J* = 7.49 Hz)	2.30 (2H, t, *J* = 7.2 Hz
2β			2.36	-
3α	24.7 (CH_2)	24.9	1.63 (2H, qn, *J* = 7.49 Hz)	1.60 (2H, s)
3β			1.7 m	-
4	29.2 (CH_2)	29.1	1.26 (br, s)	1.26 (br, s)
5	29.4 (CH_2)	29.4	1.26 (br, s)	1.26 (br, s)
6	29.6 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
7	29.7 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
8	29.6 (CH_2)	29.6	1.26 (br, s)	1.26 (br, s)
9	29.9 (CH_2)	29.8	1.26 (br, s)	1.26 (br, s)
10	29.7 (CH_2)	29.8	1.26 (br, s)	1.26 (br, s)
11	28.0 (CH_2)	28.0	1.26 (br, s)	1.26 (br, s)
12α	29.1 (CH_2)	29.2	1.26 (br, s)	1.26 (br, s)
12β			1.37 m	-
13α	36.2 (CH_2)	36.2	1.26 (br, s)	1.26 (br, s)
13β			1.35 m	1.35
1'	45.6 (CH)	45.5	2.61 (1H, br, s)	2.60 (1H. br, s)
2'	135.5 (CH)	135.1	5.68 (1H, tdd, *J* = 2.05, 4.02 Hz	5.66 m
3'	130.0 (CH)	129.7	5.68 (1H, tdd, *J* = 2.05, 3.94 Hz	5.66 m
4'α	32.0 (CH_2)	32.0	2.24 m	2.21 m
4'β			2.36 m	-
5'α	29.9 (CH_2)	29.9	1.26 (br, s)	1.26 (br, s)
5'β			2.02 m	2.01 m

Table 4. ^{1}H and ^{13}C NMR spectroscopic data for benzoic acid (OS3) in $CDCl_3$ Compared against literature values. Literature [28] [^{1}H NMR 600 MHz, ^{13}C NMR 150 MHz, MeOD, *J* in Hz

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR (150 MHz) literature	^{1}H NMR (500 MHz)	^{1}H NMR (600 MHz) literature
1	130.6 (C)	129.4	-	-
2	130.3 (CH)	130.3	8.08 (1H, dd, *J* = 7.89, 1.70, H-6)	8.12 (1H, dd, *J* = 7.68, 1.68, H-6)
3	129.2 (CH)	128.4	7.46 (1H, dd, *J* = 7.15, 1.13, H-5)	7.45 (1H, dd, *J* = 7.20, 1.08, H-5)
4	134.0 (CH)	133.8	7.57 (1H, t, *J* = 7.15)	7.62 (1H, t)
5	129.2 (CH)	128.4	7.46	7.45
6	130.3 (CH)	130.3	8.08	8.12
7	169.6 (C)	172.8	-	-

Table 5. *In vitro* antiplasmodial activities of crude extracts and isolated compounds against 3D7 and Dd2 strain of *P. falciparum*

Name	Test samples	Antiplasmodial activity IC_{50} (µg/mL)	
		3D7 strain (CQ sensitive)	**Dd2 strain (CQ Resistant)**
O. spinosa	Hex	> 50[a]	> 50[b] (> 1)
	DCM	> 50	> 50 (> 1)
	EtOAc	4.69 ± 0.01[a]	3.5 ± 0.0 (0.75)
	MeOH	> 50	> 50 (> 1)
Pure compounds	β-sitosterol (**OS1**)	2.28 ± 0.01 [5.51]*[a]	> 50 [120.77]*[b] (> 21.93)
	Chaulmoogric acid (**OS2**)	> 50 [> 178.57]*	18.76 ± 3.23 [67.00]*[b] (< 0.38)
Reference standards	Chloroquine diphosphate (CQ)	0.0093 ± 0.0099[a]	0.0440 ± 0.0102b (4.73)
	Mefloquine hydrochloride (MQ)	0.0056 ± 0.0011[a]	0.0161 ± 0.0132b (2.88)

Hex-hexane, DCM-Dichloromethane, EtOAc-Ethyl acetate, MeOH-Methanol
Values are expressed as mean ± SD (n = 3)
Values with the same superscript in the same column are significantly different (P < 0.05)
*Values in *IC_{50} are expressed in µM (Micromolar)*
Values enclosed in parenthesis represent resistance index ratio (RI) of CQ resistant strain Dd2 to IC_{50} CQ sensitive strain 3D7

The ^{13}C NMR signals seen are also in agreement with that reported in literature [22,23] (Table 2). Therefore based on the 1-D and 2-D NMR experiments and comparison with data reported from literature [24,23], the structure of compound (OS1) was proposed to be that of β-sitosterol also known as stigmast-5-en-3β-ol. This investigation reports for the first time the isolation of β-sitosterol from *O. spinosa*.

Compound (OS2): The spectroscopic analysis results of ^{1}H and ^{13}C NMR assignments were in correct agreement with that reported in literature [25, 26, 27] (Table 3). The complete assignment of protons was achieved by the HSQC, HMBC COSY and NOESY spectroscopic data. Basing on reported spectral data the structure of compound (OS2) was proposed to be of chaulmoogric acid also known 13-(2-cyclopentenyl) tridecanoic acid. This is the first report on the isolation of this compound from *O. spinosa.*

Compound (OS3): ^{1}H and ^{13}C NMR spectral data was in agreement with that reported in literature [26,28] (Table 4). The structure of compound (OS3) was proposed to that of a phenolic compound known as benzoic acid.

3.3 Antiplasmodial Activity of Crude Extracts and Pure Compounds

In vitro antiplasmodial activity the root extracts of hexane, dichloromethane and methanol with IC_{50} > 50 µg/mL against both 3D7 and Dd2 strains were considered inactive. The root EtOAc crude extract had high activity of 4.69 ± 0.01 µg/mL and 3.52 ± 0.02 µg/mL against 3D7 and Dd2 strains respectively. This extract had the highest antiplasmodial activity out of the four crude extracts tested for this plant against both strains of *Plasmodium*. The root ethyl acetate extract of was found to be active against both CQ sensitive 3D7 and CQ resistant Dd2 strains with resistance index of 0.75 than the hexane, dichloromethane and methanol extract which showed resistance indices of > 1. The resistance indices of all the crude extracts were better than the reference standards which had 2.88 and 4.73 for mefloquine and chloroquine respectively (Table 5).

The pure compound with (IC_{50}) 3D7: 5.51 µM (β-sitosterol (OS1) showed good activity while chaulmoogric (OS2) acid displayed moderate activity of IC_{50} Dd2: 67.00 µM**.** The two compounds were found to be inactive on both strains of parasite with (IC_{50}) 3D7: > 178.57 µM (chaulmoogric acid) and Dd2: > 120.77 µM (β-sitosterol (OS1)). The antiplasmodial activities of the isolated compounds correlate well with the activities of the crude extracts from which they were isolated. Therefore they were responsible for the various activities demonstrated by the crude extracts. One compound was not very reactive against the CQ resistant strain as shown by its resistance index: β-sitosterol (OS1) (> 21.93). Chaulmoogric acid showed the highest cross resistance against both strains with resistance index of < 0.38. Antiplasmodial activities of crude extracts of the plant (Hex, DCM, EtOAc, MeOH) (IC_{50}: 3.52- > 50) µg/mL and pure compounds (IC_{50}: 5.51- > 178.57) µM showed significant difference with the reference standards (IC_{50}: 0.0056-0.0440) µg/mL (CQ and MQ) for both the two strains of parasite CQ sensitive 3D7 and CQ resistant Dd2 at (P < 0.05). There was also significant difference observed between the antiplasmodial activities of the crude extracts of different extraction solvents (Hex, DCM, EtOAc, and MeOH). All the crude extracts and pure compounds exerted a significant (P < 0.05) decrease in antiplasmodial activity for the two strains of *Plasmodium* compared to the two standard controls (CQ and MQ) (Table 5).

Similar studies on antiplasmodial activities of crude extracts have been investigated on the Flacourtiaceae family which to *O. spinosa* belongs. Evaluation of antiplasmodial activity on the aerial parts of *Scolopia zeyheri* (Flacourtiaceae) was studied [29]. The hexane, DCM and MeOH extracts were tested against CQ resistant FcBI and CQ sensitive F32 strains of parasite. Their findings gave (IC_{50}) FcBI: 24.5 ± 2.12 µg/mL (hexane extract), 29.3 ± 6.7 µg/mL (DCM extract), > 50 µg/mL (MeOH). The CQ resistant strain exhibited (IC_{50}) F32: > 50 µg/mL (hexane and DCM extracts), 7.5 ± 2.1 µg/mL (MeOH extract). Another *in vitro* antiplasmodial investigation was conducted in South Africa on root DCM, MeOH/MeOH and water crude extracts of *Flacourtia indica* (Flacourtiaceae) against CQ sensitive D10 *P. falciparum* strain using the parasite lactate dehydrogenase (pLDH) assay [30]. Their findings gave IC_{50}: 86.5 µg/mL (DCM), 78 µg/mL (DCM/MeOH), 78 µg/mL (water) which showed that the extracts were inactive. A study from the same Flacourtiaceae family was assessed on *Trimeria grandifolia* DCM/MeOH leaf extracts against CQ sensitive 3D7 strain [31]. Their results also gave IC_{50} >50 µg/mL, which was regarded inactive. These

results are also in agreement with those reported in which MeOH crude extracts of the seeds of *O. spinosa* had IC_{50} >100 µg/mL and also regarded inactive [32]. These results are in the same range to those got for *O. spinosa* in the current study where the EtOAc crude extracts of *O. spinosa* had IC_{50} values of 3.52 ± 0.02 µg/mL and 4.69 ± 0.01 µg/mL for 3D7 and Dd2 strains respectively, while other crude extracts were found inactive (IC_{50}: > 50 µg/mL for both strains of parasite (Table 5). The resistance index for EtOAc crude extract was 0.75 while that for hexane, DCM and MeOH were >1. Therefore, these extracts and the pure compound may lack cross-resistance with CQ resistant strain. This phenomenon can be attributed to differences in the mode of actions of the different bioactive compounds in the extracts and that of the pure compound [33].

In the present study three compounds were isolated and their structures elucidated, however only two compounds were assessed for their *in vitro* antiplasmodial activities. These included β-sitosterol (OS1), and chaulmoogric acid (OS2). Benzoic acid (OS3) had poor yields which only enabled the determination of spectroscopic data. The *in vitro* antiplasmodial activity of compounds was assessed on the two strains of *Plasmodium* (CQ sensitive 3D7 and CQ resistant Dd2). In the current investigation β-sitosterol isolated from this plant had (IC_{50}) 3D7: 5.51 µM, Dd2: > 120.77 µM. Both the DCM crude extract from which it was isolated and the β-sitosterol had resistance indices in the same range > 1 and > 21.93 respectively. This indicates that both the crude and isolated compound were not very active against the resistant Dd2 strain. *In vitro* antiplasmodial screening on β-sitosterol isolated from MeOH crude extract of leaves of *Teclea trichocarpa* was assessed against CQ resistant *P. falciparum* KI strain [34]. Their investigation gave IC_{50} of 8.20 µg/mL (19.81 µM), which correlates with the IC_{50} value got from this study. However, the DCM extract from which it was isolated was found to be inactive against both strains of parasite with IC_{50} > 50 µg/mL. This shows that there was antagonic interaction of the compounds in its crude form, which explains the increased activity of pure compound alone. Increased activity of compounds than their crude extracts was also cited [35]. In their investigation on *S. pinnata* DCM/MeOH (1:1) whole plant crude extracts against CQ NF54 sensitive strain gave IC_{50} of 2.19 µg/mL. This IC_{50} value was less than those of the isolated compounds schkuhrin I and schkuhrin II with (IC_{50}) NF54: 2.05 and 1.67 µg/mL respectively. A bioassay-guided fractionation of the trunk bark extract of *Laetia procera* (Flacourtiaceae) that led to the isolation of six clerodane diterpenoids: casearlucin A, casamembrol A and four laetiaprocerines were investigated [36]. The diterpenoids exhibited antiplasmodial activity with IC_{50} values of 0.57-6.04 µM on F32 strain and 0.54-27.5 µM on FCb1 strain. In another study on hexane and DCM extracts of the bark of *Casearia grewiifolia* (Flacourtiaceae) four new clerodane diterpenes (caseargrewiins) and two known clerodane diterpenes were isolated [37]. All compounds exhibited antimalarial activity against *P. falciparum* K1 with IC_{50} values of 3.6-7.9 µM, but they were also cytotoxicity. This shows that the Flacourtiaceae family has some species that exhibit high antiplasmodial activity.

Another pure compound that was tested for antiplasmodial activity from this study was chaulmoogric acid. This compound exhibited activity of IC_{50} 3D7: > 178.57 µM and Dd2: 67.00 µM which was different from the DCM crude extract from which it was isolated. The DCM crude extract was completely inactive against both strains while this compound showed increased activity in its pure form on Dd2 strain. This result is similar to a study in which the isolated compound showed increased activity than the crude extract from which it was isolated [31]. The isolated compounds methyl canadine, nitidine and chelerythine (IC_{50}) 3D7: 2.01, 0.17 and 1.35 µg/mL respectively from the ethanol root bark of *Zanthoxylum chalybeum* had increased activity than its crude extract (IC_{50} 42.5 and 41.5) µg/mL of MeOH and DCM respectively.

3.4 Acute Toxicity of Crude Extracts

According to the LD_{50} values of acute toxicity calculated, the crude extracts where classified into two groups. The first group included extracts in which the LD_{50} > 5000 mg/kg and considered to be practically nontoxic. These extracts consisted of hexane and DCM extracts with LD_{50} > 5000 mg/kg. The second group was categorised as slightly toxic with LD_{50} of 547.72 mg/kg, this consisted of EtOAc and MeOH extracts.

There no observable change in behavior of the mice for hexane and DCM crude extracts for at all doses 500-5000 mg/kg. The EtOAc extract recorded no observable change in behavior at a

dose of 500 mg/kg. At doses of 1000 and 2000 mg/Kg, there was retarded movement, restless in breathing and animals became less active and all the tested animals died. The same trends of results were recorded for the MeOH crude extract. When doses were changed to 600, 700, 800 and 900 mg/kg the EtOAc and MeOH extracts recorded mortality death.

Determination of acute toxicity is the first step in the toxicological analyses of herbal drugs. In the present study Lorke's procedure was used because it offers the advantage that when doses are correctly chosen adequate information is obtained using only few animals, irrespective of the material tested and the route of administration [7]. In the current study the acute toxicity of most of the crude extracts of the three plants had LD_{50} > 5000 mg/kg and these were considered nontoxic. The LD_{50} was 547.72 mg/kg in only two crude extracts which were regarded as slightly toxic. The methanol crude extract of the seeds of this plant was investigated [32]. Their results showed that the seed crude extract was non-toxic to larvae of *brine shrimps* at IC_{50} of 250 µg/mL. However, the antiplasmodial activity of the MeOH seed extract was found to be inactive (IC_{50} >100 µg/mL), these results correlate well with the findings from the current study.

4. CONCLUSION

The phytochemical study of the crude extracts of *O. spinosa* roots afforded known compounds including β-sitosterol (OS1), chaulmoogric acid (OS2) and benzoic acid (OS3). The EtOAc crude extract and compounds (OS1, OS2) possess significant antiplasmodial activity with the crude extracts having no toxicity. This justifies the use of the plant in treating malaria and therefore it can be used as a phytomedicine at low cost that is easily affordable by the local community a waiting clinical studies.

CONSENT

It is not applicable

ETHICAL APPROVAL

The present work was performed according to the approved guidelines of animal experiments of the Research Ethical Committee at the Department of Pharmacology Makerere University, Kampala, Uganda (NUMBER 1250).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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