Original research paper

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

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 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 ± 0.01µg/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. MATERIAL AND METHODS

2.1 Plant material

Oncoba spinosa (Figure 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.

Figure 1: **Photo of *O. spinosa***

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 ºC) 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 ºC, 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 visualized 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 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 labeled 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 analyzed 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 $IC_{50} \pm$ 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 pelletized 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 anesthesia. 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 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 (50µg/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 analyzed 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**).

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			

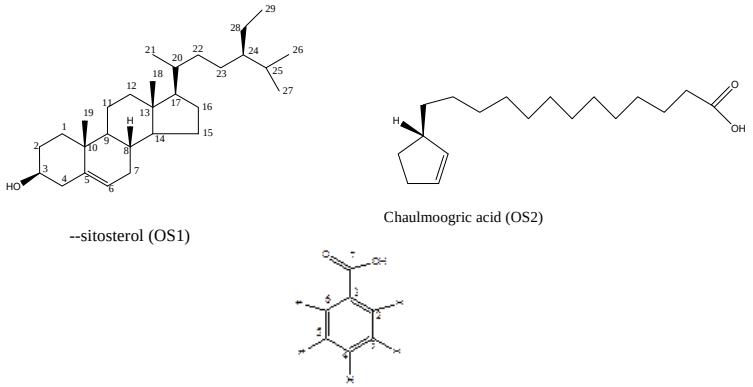
3.2 Elucidation of compounds from the root extract

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**) (**Figure 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).



--sitosterol (OS1)

Chaulmoogric acid (OS2)

Benzoic acid (OS3)

Figure 2: **Structures of isolated compounds**

222 Compound (**OS1**): This compound showed a phenomenon that is seen by the frame work of steroids
223 [21].
224 The ^{13}C NMR signals seen are also in agreement with that reported in literature [22, 23] **(Table 2)**.
225 Therefore based on the 1-D and 2-D NMR experiments and comparison with data reported from
226 literature [24, 23], the structure of compound (**OS1**) was proposed to be that of β-sitosterol also
227 known as stigmast-5-en-3β-ol. This investigation reports for the first time the isolation of β-sitosterol
228 from *O. spinosa*.
229
230 **Table 2: ^{1}H and ^{13}C NMR spectroscopic data for β-sitosterol (OS1) in $CDCl_3$**
231 **compared against literature values. Literature [23] [^{1}H NMR 400 MHz, ^{13}C NMR**
232 **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	-
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)

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

237 spectral data the structure of compound (**OS2**) was proposed to be of chaulmoogric acid also known
238 13-(2-cyclopentenyl) tridecanoic acid. This is the first report on the isolation of this compound from *O.*
239 *spinosa.*
240
241 **Table 3**: **^{1}H and ^{13}C NMR spectroscopic data for Chaulmoogric acid (OS2)**
242 **in $CDCl_3$ compared against literature values. Literature [27] [^{1}H NMR**
243 **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,	5.66 m

			4.02 Hz	
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

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.

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	-	-

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**).

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 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 ±

296 2.1 µg/mL (MeOH extract). Another *in vitro* antiplasmodial investigation was conducted in South Africa
297 on root DCM, MeOH/MeOH and water crude extracts of *Flacourtia indica* (Flacourtiaceae) against CQ
298 sensitive D10 *P. falciparum* strain using the parasite lactate dehydrogenase (pLDH) assay [30]. Their
299 findings gave IC_{50}: 86.5 µg/mL (DCM), 78 µg/mL (DCM/MeOH), 78 µg/mL (water) which showed that
300 the extracts were inactive. A study from the same Flacourtiaceae family was assessed on *Trimeria*
301 *grandifolia* DCM/MeOH leaf extracts against CQ sensitive 3D7 strain [31]. Their results also gave IC_{50}
302 >50 µg/mL, which was regarded inactive. These results are also in agreement with those reported in
303 which MeOH crude extracts of the seeds of *O. spinosa* had IC_{50} >100 µg/mL and also regarded
304 inactive [32]. These results are in the same range to those got for *O. spinosa* in the current study
305 where the EtOAc crude extracts of *O. spinosa* had IC_{50} values of 3.52 ± 0.02 µg/mL and 4.69 ± 0.01
306 µg/mL for 3D7 and Dd2 strains respectively, while other crude extracts were found inactive (IC_{50}: > 50
307 µg/mL for both strains of parasite (**Table 5**). The resistance index for EtOAc crude extract was 0.75
308 while that for hexane, DCM and MeOH were >1. Therefore, these extracts and the pure compound
309 may lack cross-resistance with CQ resistant strain. This phenomenon can be attributed to differences
310 in the mode of actions of the different bioactive compounds in the extracts and that of the pure
311 compound [33].
312
313 In the present study three compounds were isolated and their structures elucidated, however only two
314 compounds were assessed for their *in vitro* antiplasmodial activities. These included β-sitosterol
315 (**OS1**), and chaulmoogric acid (**OS2**). Benzoic acid (**OS3**) had poor yields which only enabled the
316 determination of spectroscopic data. The *in vitro* antiplasmodial activity of compounds was assessed
317 on the two strains of *Plasmodium* (CQ sensitive 3D7 and CQ resistant Dd2). In the current
318 investigation β-sitosterol isolated from this plant had (IC_{50}) 3D7: 5.51 µM, Dd2: > 120.77 µM. Both the
319 DCM crude extract from which it was isolated and the β-sitosterol had resistance indices in the same
320 range > 1 and > 21.93 respectively. This indicates that both the crude and isolated compound were
321 not very active against the resistant Dd2 strain. *In vitro* antiplasmodial screening on β-sitosterol
322 isolated from MeOH crude extract of leaves of *Teclea trichocarpa* was assessed against CQ resistant
323 *P. falciparum* KI strain [34]. Their investigation gave IC_{50} of 8.20 µg/mL (19.81 µM), which correlates
324 with the IC_{50} value got from this study. However, the DCM extract from which it was isolated was
325 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 categorized 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.

REFERENCES

1. Alshawsh MA, Mothana RA, Al-shamahy HA, Alsllami SF, Lindequist U. Assessment of antimalarial activity against *Plasmodium falciparum* and phytochemical screening of some Yemeni medicinal plants. *Evidence-Based Complementary and Alternative Medicine*. 2009; 6(4):453-456.
2. World Health Organization (2013). Factsheet on the World Malaria Report http://www.who.int/malaria/media/world-malaria report-2013/en/(accessed 12.07.2014).
3. Tabuti JR. Herbal medicines used in the treatment of malaria in Budiope County, Uganda. *Journal of Ethnopharmacology*. 2008; 116(1):33-42.
4. Ssegawa P, Kasenene JM. Plants for malaria treatment in southern Uganda: traditional use, preference and ecological viability. *Journal of Ethnobiology*. 2007; 27(1):110-131.
5. Hutchinson J and Dalziel J. M. (1954). Flacourtiaceae. In: Keay R.W.J, editor. Flora of West Tropical Africa. (vol. 1, part 1). 2nd ed. London: Crown Agents for Overseas Governments and Administration: pp. 185-191.
6. Balogun O, Oladosu I, Akinnusi A, Zhiqiang L. Fatty acids composition, α-glucosidase inhibitory potential and cytotoxicity activity of *Oncoba spinosa* Forssk. *Elixir Appllied Chem*istry. 2013; 59:15637-15641.
7. Kodi P, Mwangi ME, Kiplagat CP, Kariuki TS. Ethnobotanical survey of antimalarial medicinal plants used in Butebo County, Eastern Uganda. *European Journal of Medicinal Plants*. 2017; 21(4):1-22.
8. Lorke D. A New Approach to Practical Acute Toxicity Testing. *Archives Toxicology*. 1983; 54(4):275-287.

412 9. Sofowora A. Medicinal plants and traditional medicine in Africa: John Wiley and sons LTD;
413 1982.
414 10. Newton SM, Lau C, Gurcha SS, Besra GS, Wright CW. The evaluation of forty-three plant
415 species for *in vitro* antimycobacterial activities; isolation of active constituents from *Psoralea*
416 *corylifolia and Sanguinaria canadensis. Journal of Ethnopharmacology*. 2002; 79(1):57-67.
417 11. Smilkstein M, Sriwilaijaroen N, Kelly JX, Wilairat P, Riscoe M. Simple and inexpensive
418 fluorescence-based technique for high-throughput antimalarial drug screening. *Antimicrobial*
419 *Agents and Chemotherapy*. 2004; 48(5):1803-1806.
420 12. Yenesew A, Akala HM, Twinomuhwezi H, Chepkirui C, Irungu BN, Eyase FL, et al. The
421 antiplasmodial and radical scavenging activities of flavonoids of *Erythrina burttii*. *Acta Tropica*.
422 2012; 123(2):123-127.
423 13. Trager W, Jensen JB. Human malaria parasites in continuous culture. *Science*. 1976;
424 193(4254):673-675.
425 14. Johnson JD, Dennull RA, Gerena L, Lopez-Sanchez M, Roncal NE, Waters NC. Assessment
426 and continued validation of the malaria SYBR Green I-based fluorescence assay for use in
427 malaria drug screening. *Antimicrobial Agents and Chemotherapy*. 2007; 51(6):1926-1933.
428 15. Akala HM, Eyase FL, Cheruiyot AC, Omondi AA, Ogutu BR, Waters NC, et al. Antimalarial
429 drug sensitivity profile of western Kenya *Plasmodium falciparum* field isolates determined by a
430 SYBR Green I *in vitro* assay and molecular analysis. *The American Journal of Tropical*
431 *Medicine and Hygiene.* 2011; 85(1):34-41.
432 16. Bacon DJ, Latour C, Lucas C, Colina O, Ringwald P, Picot S. Comparison of a SYBR green I-
433 based assay with a histidine-rich protein II enzyme-linked immunosorbent assay for *in vitro*
434 antimalarial drug efficacy testing and application to clinical isolates. *Antimicrobial Agents and*
435 *Chemotherapy*. 2007; 51(4):1172-1178.
436 17. O'Neil MJ, Bray DH, Boardman P, Phillpson JD, Warhurt DC. Plants as Source of
437 Antimalarial Drugs Part 1: *In vitro* Test Method for the Evaluation of Crude Extracts from
438 Plants. *Planta medica*. 1995; 51:394-398.
439 18. WHO. Working to Overcome the Global Impact of Neglected Tropical Diseases: First WHO
440 Report on Neglected Tropical diseases: Update; 2011, [Pub Med].

441 19. Batista R, De Jesus Silva Júnior A, De Oliveira AB. Plant-derived antimalarial agents: new
442 leads and efficient phytomedicines. Part II. Non-alkaloidal natural products. *Molecules*. 2009;
443 14(8):3037-3072.
444 20. Kirkwood, Betty R, Sterne JA. Essential Medical Statistics. 2nd Edition. Black WellScience Ltd.
445 Massachusetts, U.S.A. 2003; ISBN 0865428719.
446 21. Yun-Song W, Jing-Hua Y, Shi-De L, Hong-Bin Z, and Liang L. New cytotoxic steroids from
447 *Stachyurus imalaicus* var. himalaicus. *Molecules*. 2007; 12(3):536-42.
448 22. Pateh U, Haruna A, Garba M, Iliya I, Sule I, Abubakar M, et al. Isolation of stigmasterol, β-
449 sitosterol and 2-hydroxyhexadecanoic acid methyl ester from the rhizomes of *Stylochiton*
450 *lancifolius* Pyer and *Kotchy* (Araceae). *Nigerian Journal of Pharmaceutical Sciences*. 2009;
451 8(1):19-25.
452 23. Rashed K, Said A, Abdo A, Selim S. Antimicrobial activity and chemical composition of
453 *Pistacia chinensis* Bunge leaves. *International Food Research Journal*. 2016; 23(1):316-321.
454 24. Odiba J, Musa A, Hassan H, Yahay S, Okolo E. Antimicrobial activity of isolated stigmast-5-
455 en-3-β-ol (β-sitosterol) from honeybee *propolis* from North-Western, Nigeria. *International*
456 *Journal of Pharmaceutical Science Research*. 2014; 5(12):908-918.
457 25. Blaise P, Farines M, Soulier J. Identification of cyclopentenyl fatty acids by ^{1}H and ^{13}C nuclear
458 magnetic resonance. *Journal of the American Oil Chemists' Society*. 1997; 74(6):727-730.
459 26. Xu Z, Lu Y, Chai X, Ren H, Tu P. Chemical constituents from *Xylosma controversum*. *Journal*
460 *of Chinese Pharmaceutical Sciences*. 2007; 16(3):218-234.
461 27. Sivaraman G, Paulraj MG, Balakrishna K, Stephen Irudayaraj S, Ignacimuthu S, Al-Dhabi NA.
462 Biological effects of active fraction isolated from *Hydnocarpus pentandra* (Bunch.-Ham.) Oken
463 seeds against *Helicoverpa armigera* (Hub.) (Lepidoptera: Noctuidae). *Archives of*
464 *Phytopathology and Plant Protection*. 2017; 50(5-6):262-274.
465 28. Abdullah NH, Salim F, Ahmad R. Chemical constituents of Malaysian *U. cordata* var.
466 ferruginea and their *in vitro* α-glucosidase inhibitory activities. *Molecules*. 2016; 21(5):525-
467 541.
468 29. Sylvain B, Le Lamer A, Maurel-Chevalley S, Mutiso P, Souard F, Moulis C, et al. Evaluation of
469 the antiplasmodial activity of extracts of plants used in traditional medicine in Kenya.
470 *International Journal of Medicinal Plants Research.* 2013; 2:219-224.

30. Clarkson C, Maharaj VJ, Crouch NR, Grace OM, Pillay P, Matsabisa MG, et al. *In vitro* antiplasmodial activity of medicinal plants native to or naturalized in South Africa. *Journal of Ethnopharmacology*. 2004; 92(2):177-191.

31. Muganga R, Angenot L, Tits M, Frederich M. Antiplasmodial and cytotoxic activities of Rwandan medicinal plants used in the treatment of malaria. *Journal of Ethnopharmacology*. 2010; 128(1):52-57.

32. Addae-Kyereme J, Croft SL, Kendrick H, Wright CW. Antiplasmodial activities of some Ghanaian plants traditionally used for fever/malaria treatment and of some alkaloids isolated from *Pleiocarpa mutica*; *in vivo* antimalarial activity of pleiocarpine. *Journal of Ethnopharmacology*. 2001 ;76(1):99-103.

33. Muthaura C, Rukunga G, Chhabra S, Mungai G, Njagi E. Traditional antimalarial phytotherapy remedies used by the Kwale community of the Kenyan Coast. *Journal of Ethnopharmacology*. 2007; 114(3):377-386.

34. Mwangi E, Keriko J, Machocho A, Wanyonyi A, Malebo H, Chhabra S, et al. Antiprotozoal activity and cytotoxicity of metabolites from leaves of *Teclea trichocarpa*. *Journal of Medicinal Plants Research*. 2010; 4(9):726-731.

35. Mokoka TA, Xolani PK, Zimmermann S, Hata Y, Adams M, Kaiser M, et al. Antiprotozoal screening of 60 South African plants, and the identification of the antitrypanosomal germacranolides schkuhrin I and II. *Planta medica*. 2013; 79(14):1380-1384.

36. Jullian V, Bonduelle C, Valentin A, Acebey L, Duigou A-G, Prévost M-F, et al. New clerodane diterpenoids from *Laetia procera* (Poepp.) Eichler (Flacourtiaceae), with antiplasmodial and antileishmanial activities. *Bioorganic & medicinal chemistry letters*. 2005; 15(22):5065-5070.

37. Kanokmedhakul S, Kanokmedhakul K, Kanarsa T, Buayairaksa M. New Bioactive Clerodane Diterpenoids from the Bark of *Casearia g rewiifolia*. *Journal of Natural Products*. 2005; 68(2):183-188. 2