

Original Research Article

***In vitro* Antiplasmodial and Toxicity Activities of Crude Extracts and Compounds from *Euclea latideus* (Ebenaceae)**

ABSTRACT

Aims: In our search for new antiplasmodial agents, *in vitro* antiplasmodial activities of the crude extracts and isolated pure compounds were determined. In addition to the *in vitro* assays *in vivo* acute toxicity of the crude extracts was investigated to assess the safety of the plants. Furthermore structure elucidation of the pure compounds was also carried out to determine the identity of the isolated compounds.

Study design: Extraction of the root crude extracts of *Euclea latideus* was done using four solvents; hexane, dichloromethane, ethyl acetate and methanol. Isolation and purification was carried out on only the dichloromethane and ethyl acetate crude extracts.

Place and Duration of Study: The study was carried in Egerton University, Kenya; from November 2014 to March 2016.

Methodology: Three solvent; hexane, dichloromethane, ethyl acetate and methanol were used to carry out the extraction process of the crude samples. Isolation and purification of crude extracts was achieved using chromatographic techniques which included column and thin layer chromatography (TLC). The characterization of the isolated compounds was determined using NMR spectroscopic techniques.

In vitro antiplasmodial activity was performed on two strains of *Plasmodium falciparum* (3D7 CQ sensitive and DD2 CQ resistant) using a non-radioactive fluorescence based 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: Results of acute toxicity studies showed that all crude extracts of *E. latideus* had LD₅₀ > 5000 mg/kg and therefore regarded as a non-toxic plant. The four crude extracts of *E. latideus* had good activity in the range of (IC₅₀) 3D7: (9.75 ± 0.85 – 37.72 ± 0.0) µg/mL and DD2: (2.76 ± 0.0 – 38.39 ± 0.0) µg/mL. The resistance indices for *E. latideus* crude extracts ranged between 0.1- 1.43. Isolation resulted in the identification of three known compounds which include; three triterpenoids Lupeol (**EL1**), betulin (**EL2**), 3β-(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**). Among the pure compounds **EL2** had the highest

activity against both strains (IC_{50}) 3D7: $1.66 \pm 0.0 \mu\text{g/mL}$ and DD2: $15.44 \pm 4.63 \mu\text{g/mL}$ while Lupeol (**EL1**) displayed moderate activity with (IC_{50}) 3D7: $23.96 \pm 0.0 \mu\text{g/mL}$, DD2: $25.14 \pm 0.0 \mu\text{g/mL}$. The antiplasmodial activity of the crude extracts and pure compounds were significantly different ($P < 0.05$) from that of the reference standards (Chloroquine diphosphate and mefloquine hydrochloride). Both the crude extracts ($2.76\text{-}38.39 \mu\text{g/mL}$) and pure compounds ($1.66\text{-}25.14 \mu\text{g/mL}$) showed a significant decrease in activity compared to the reference standards ($0.0056\text{-}0.0440 \mu\text{g/mL}$). Significant difference ($P < 0.05$) also existed between the antiplasmodial activities of the crude extracts, which showed the same trend with that of the pure compounds.

Conclusion: The results show that the root crude extracts and pure compounds of the plant has got good antiplasmodial activity and low toxicity which can be exploited for malaria therapy. Therefore this justifies their ethnomedicinal use of the plant by the local communities in the treatment of malaria.

KEY WORDS: *Euclea latideus*, antiplasmodial, acute toxicity, betulin, in vitro

1. INTRODUCTION

Malaria is the world's most important tropical disease. It is prevalent in about 100 countries and around 2,400 million people are at risk [1]. Ninety percent of malaria cases in the world are estimated to be in Africa where the disease is endemic [2, 3]. Malaria is Africa's leading cause of infant mortality (20 per cent) and constitutes 10 per cent of the continent's overall disease burden. It accounts for 40 per cent of public health expenditure, 30-50 per cent of inpatient admissions, and up to 50 per cent of outpatient visits in areas with high malaria transmission [4]. The annual conservative estimate of 1-2.7 million deaths are due to malaria globally of which about 90% of these occur in Africa alone [5]. Children under five years of age and pregnant women are most severely affected [6]. The use of chloroquine (CQ) to prevent and treat *falciparum* malaria has led to the wide-spread appearance of CQ-resistant strains of *P. falciparum* throughout the affected regions. The resistance has at the same time increasingly extended to other available antimalarial drugs [7]. The increasing global spread of drug resistance to most of the available and affordable antimalarial drugs is a major concern and requires innovative strategies to combat the disease. Therefore there is an urgent need for new chemotherapeutic compounds, which are easy to administer and store, and which are of low cost. One possible source for such affordable

treatments lies in the use of traditional herbal remedies. Such a situation has opened the need for alternative antiparasitic therapy. The therapeutic success of two important plant-derived compounds, quinine and artemisinin, against malaria has inspired researchers to search for new antimalarial drugs from plants. Since little scientific data exist to validate antimalarial properties of these medicinal plants, it is important that their claimed antimalarial properties are investigated. This is done in order to establish their efficacy, safety and determine their potential as sources of new antimalarial drugs.

E. latideus belongs to Ebenaceae family which is found mainly in the lowlands of the tropical and, to a lesser extent, in subtropical regions of the World. The small genus *Euclea* is restricted to Africa and Southern Arabia whereas the large genus *Diospyros* is pantropical. Many of its species are usually small to medium-sized trees in the forest understory, with an often remarkably low population density. Only few species penetrate the mountains and extratropical warm temperate regions. The Ebenaceae are the source of several economically important products, the most valuable being their fruits and timber (ebony).

Among the phytochemical compounds, naphthoquinones, terpenoids (especially lupanes, ursanes, oleananes, and taraxeranes), benzopyrones, polyphenols, and tannins are all widely distributed, and very characteristic for Ebenaceae. The other compounds include steroids, naphthalene-based aromatics, hydrocarbons, lipids, amino acids, carotenoids, and sugars [8]. The naphthoquinones occur in several parts of the plants, especially in the bark, and are active against fungi, bacteria, mollusks, insects, worms, termites [9]. Their derivatives and oxidative decomposition products are responsible for the dark brown to black colored tissues of the bark, heartwood, fruits, and leaves [9]. Idioblasts containing tannins occur in various parts, including fruits [10].

Studies on the *in vitro* antiparasitic and toxicity properties of crude extracts sequentially extracted from solvents of different polarities are nonexistent in the literature. In our search for more effective drugs against *P. falciparum* and as a continuation of our investigation of plants used traditionally in Uganda to treat malaria [11], special attention was focused to *E. latideus* (Ebenaceae).

In the current study, the solvent extracts of the roots of *E. latideus* were tested for their antiparasitic activity against CQ-sensitive (3D7) and CQ-resistant (DD2) strains of *P. falciparum* in

culture using the fluorescence-based SYBR Green assay. The crude extracts were also tested for *in vivo* acute toxicity against mice using Lorke's method [12] assay.

2. MATERIAL AND METHODS

2.1 Plant material

The plant was identified and documented as an antimalarial remedy in an ethnobotanical survey conducted [11]. Medicinal plants were photographed, collected, dried and taken for identification by a taxonomist. The voucher specimens (KP 914) was deposited at the Department of Botany herbarium for future specifications.

2.2 Preparation of crude extracts

Plant materials were washed, cut into small parts and then air dried at room temperature in a shade for 21 days [13]. 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). The powdered sample was bagged in black plastic bags and stored in an air-tight container ready for extraction. Crude plant extracts were prepared by maceration of 1000 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 for 72 hours [14] in Winchester bottles of 2.5 L with intermittent agitations. 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). They 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 shown below. Samples of the solid residues obtained were transferred into sterile containers and kept in a refrigerator at 4°C for antiplasmodial and toxicity assays. Extract yield (%) = $\frac{W_1}{W_2} \times 100$, 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 length. This was followed by development with anisaldehyde spray reagent consisting of anisaldehyde, conc. H₂SO₄ and methanol in a ratio of 1:2:97 followed by heating in an oven at a temperature of 100° C.

The brown dichloromethane crude extract (7.24 g) was eluted with increasing polarity of EtOAc : hexane (0-100%), followed by 100% MeOH. Eleven broad fractions M (1-11) were collected which pooled fraction M (3-10) (**Figure 1**). This combined fraction was eluted with EtOAc : hexane (1:4, 3:7) then washed with 100% EtOAc to give 45 fractions B (1-45). These fractions pooled fraction B (15-23) that was eluted with EtOAc : hexane, 1:4 to give 61 fractions D (1-61). Repeated column chromatography with elution of EtOAc : hexane (1:3, 1:4) followed by diethyl ether-DCM (1:24) yielded black crystals of a pure compound (**EL3**) (172.3 mg, 2.38 %) that was UV active.

The light green crude extract of ethyl acetate (5.18 g) was eluted with step wise gradient of EtOAc :hexane (0% - 100%) followed by MeOH 100% to yield 14 broad fractions J (1-14). This gave pooled fraction J (4 and 5) which was eluted with increasing polarity of EtOAc : hexane (1:3, 3:7) followed by 100% EtOAc that resulted in 33 fractions EA (1-33). The pooled fraction EA (14-33) was eluted with diethyl ether : dichloromethane, 2:23 followed by 100 % EtOAc to afford two pure compounds (**EL1**) (26.0 mg, 0.50%) UV active and **EL2** (189.6 mg, 3.66%), UV inactive which were all white powders. Three pure compounds were isolated from *E. latideus*, two were from the ethyl acetate crude extract and one from the dichloromethane.

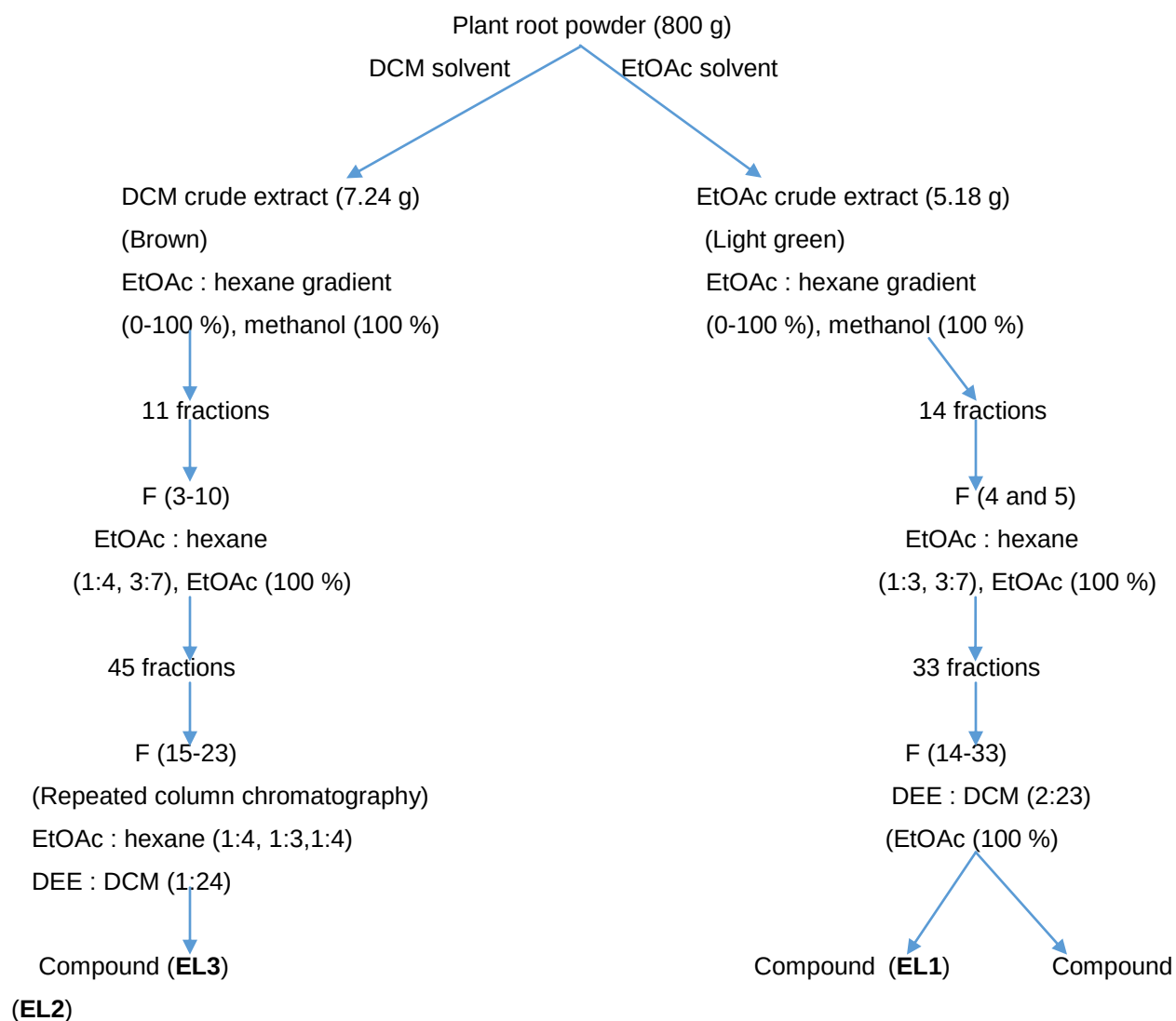


Figure 1: Flow chart showing procedure for isolation of pure compounds from *E. latideus*

2.4 Structure elucidation of pure compounds

Identification of the pure compounds was carried out by spectrophotometric methods that included 1-D and 2-D NMR. The 1-D consisted of ^1H , ^{13}C and DEPT NMR while the 2-D involved COSY, NOESY, ROESY, HSQC and HMBC. This was analyzed using a Bruker avance ^1H 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. This study gives the first report of isolation of Lupeol (**EL1**), betulin (**EL2**) and 3 β -(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**) from the roots of *E. latideus*.

2.5 Antiplasmodial bioassay activity

The extracts and pure compounds were assayed using a non-radioactive Malaria SYBR Green I assay technique [15] with modifications [16] to determine a concentration that inhibits growth of 50% of parasites in culture (IC₅₀). Two different *P. falciparum* strains, chloroquine sensitive (3D7) and chloroquine-resistant (DD2) were used. These were donated by the Division of Experimental Therapeutics, Walter Reed Army Institute of Research, Washington DC. They were grown as described [17]; with minor modifications [18]. Drugs, extracts and pure compounds were dissolved in 99.5% dimethylsulfoxide (DMSO) (Sigma–Aldrich). This was followed by dilution in complete Roswell Park Memorial Institute 1640 series of Cell Culture Media (RPMI 1640) enriched with human serum. The RPMI 1640 medium was prepared accordingly as described [19]. In summary, the basic culture medium was prepared from RPMI 1640 powder (10.4 g; Invitrogen, Inc. augmented with 2 g glucose (Sigma Inc.) and 5.95 g of HEPES (Sigma Inc.), dissolved to homogeneity in 1 L of de-ionized water and sterilized with a 0.2 μ M filter). Complete RPMI 1640 media, used for all parasite culture and drug dilutions, consisted of basic RPMI 1640 media with 10% (v/v) human ABO pooled plasma, 3.2% (v/v) sodium bicarbonate (Thermo Fisher Scientific Inc.) and 4 mg/mL hypoxanthine (Sigma Inc.). At the same time two-fold serial dilutions of drugs were prepared to generate 10 IC₅₀ concentrations of each drug for testing that consisted of chloroquine (1.953-1000 ng/mL), mefloquine (0.488-250 ng/mL) and test sample (97.7-50,000 ng/mL). These were prepared on a 96-well plate, such that the final proportion of DMSO was equal to or less than 0.0875%. Chloroquine and mefloquine were used as positive controls while 0.4% DMSO was used as the negative control. The culture adapted *P. falciparum* at 2% hematocrit and 1% Parasitemia, were then added on to the plate containing dose range of drugs and incubated in a gas mixture (5% CO₂, 5% O₂, and 90% N₂) at 37° C. The assay was terminated 72 hours later by freezing at -80°C for 24 hours. After thawing 100 μ L of SYBR Green I solution (0.2 μ L of 10,000 x SYBR Green I (Invitrogen)/mL) in lysis buffer {Tris (20 mM; pH 7.5), EDTA (5 mM), saponin (0.008%, w/v), and Triton X-100 (0.08%, v/v)} was added to each well. This was followed by gently mixing using the Beckman Coulter Biomek 2000 automated laboratory work station (Beckman Coulter, Inc., Fullerton, CA). The plates were incubated for

5-15 minutes at room temperature in the dark. Parasite growth inhibition was quantified by measuring the per-well relative fluorescence units (RFU) of SYBR Green 1 dye using the Tecan Genios Plus (Tecan US, Inc., Durham, NC). This was done with excitation and emission wavelengths of 485 and 535 nm, respectively, and with the gain set at 60. The reference anti-malarial drugs, chloroquine and mefloquine were tested along the test compounds and a minimum of three separate determinations was carried out for each sample. Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentration (IC_{50} 's) for each drug. The IC_{50} values were given as mean of two or three independent experiments and the results were presented as mean $IC_{50} \pm$ SEM (standard error mean). The resistance index (RI) for each crude extract and isolated compounds tested 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).

2.6 *In vivo* acute toxicity (LD_{50}) test:

The estimated lethal dose (LD_{50}) of the crude extracts in mice was performed using the method described [12]. A total of 230 mice weighing (13.0 – 30.0) g obtained from an inventive youth group that deals in experimental animals 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. After which the LD₅₀ was determined.

2.7 Data analysis

2.7.1 Antiplasmodial bioassay activity tests

In vitro antiplasmodial test results were given as the inhibition concentration (IC₅₀), this represents the drug concentration capable of inhibiting 50 % of the *P. falciparum*. Differential counts of relative fluorescence units (RFUs) were used in calculating 50% inhibition concentration (IC₅₀'s) 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) [18, 20]. IC₅₀ values above 100 µg/mL were considered inactive [21]. This is in line with World Health Organization guidelines [22] 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 IC₅₀ >200 µM, whereas those with an IC₅₀ of 100–200 µM are considered to have low activity; IC₅₀ of 20–100 µM, moderate activity; IC₅₀ of 1–20 µM good activity; and IC₅₀ <1 µM excellent/ potent antiplasmodial activity [23]. Similarly activities of crude extracts are classified into five classes according to their IC₅₀ values: high activity (IC₅₀ < 5 µg/mL); promising activity (5 µg/mL < IC₅₀ < 15 µg/mL); moderate activity (15 µg/mL < IC₅₀ < 50 µg/mL); weak activity (50µg/mL < IC₅₀ < 100 µg/mL), inactive IC₅₀ > 100 µg/mL [22].

2.7.2 Toxicity bioassay activity tests

The LD₅₀ values were calculated as the geometric mean of the highest non-lethal dose (with no deaths) preceding the lowest lethal dose (where deaths occurred). $LD_{50} = \sqrt{(D_0 \times D_{100})}$, Where LD₅₀ = median lethal dose, D₀ = highest dose that gave no mortality, D₁₀₀ = 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) [22].

2.7.3 Statistical analysis

For the statistical analysis, the Microsoft Excel 2013 was used to calculate means and percentages and numerical data expressed as mean $\pm$ Standard Error of Mean (SEM) for each group of experiments. 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. These were transformed in *P*-values to compare results at 95% confidence level ($\alpha = 0.05$). This was used to compare results between doses, among treatment and control dose levels. The differences between means was considered significant when $P < 0.05$ and $P > 0.05$ not significant [24].

3. RESULTS AND DISCUSSION

3.1 Test substances for bioassay activity screening

The yields for the crude extracts were: (0.36-2.36) % and (0.50-3.66) % for the pure compounds. The methanol crude extract had the highest percentage (2.36 %), while hexane crude extract had the lowest percentage yield (0.36 %). Among the pure compounds betulin (**EL2**) had the highest percentage yield (3.66 %) and Lupeol (**EL1**) with the lowest (0.50 %) (**Table 1**).

274 **Table 1. Percentage yields of crude extracts and pure compounds**

Species name	Weight of Plant	Extraction	Crude extract		Pure compounds		
	material (g)	solvent					
<i>E. latideus</i>	1000		Weight (g)	Yield % w/w	Name	Weight (mg)	Yield % w/w
		Hex	3.60	0.36			
		DCM	10.22	1.02	3 β -(5-hydroxyferuloyl)lup-20(30)-ene (EL3)	172.30	2.38
		EtOAc	8.21	0.82	Lupeol (EL1)	26.00	0.50
					Betulin (EL2)	189.60	3.66
		MeOH	23.60	2.36			

3.1 Characterization of compounds from the root extract

The phytochemical analysis of the roots of *E. latideus* led to the isolation of three compounds identified as Lupeol (**EL1**), betulin (**EL2**) and 3 β -(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**) (**Figure 2**).

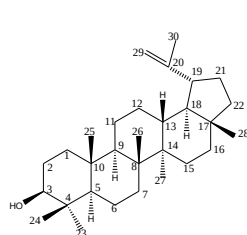
The three compounds obtained, as mentioned in the experimental section, were subjected to spectroscopic analysis for identification. The details of the spectral peaks were noted as follows:

Compound (**EL1**): Lupeol: 172.3 g, white powder. ¹H NMR (500 MHz, CDCl₃). δ_H : 0.89-1.94 (20 H, m, 2H-1, 2, 6, 7, 11, 12, 15, 16, 21, 22), 1.27-1.68 (3H, m, H-9, 13, 18), 3.22 (1H, dd, J = 4.95, 11.22 Hz, H-3), 0.70 (1H, d, J = 9.14 Hz, H-5), 2.38 (1H, ddd, J = 5.84 11.01 Hz, H-19), 0.99 (3H, s, C-23), 0.78 (3H, s, C-24), 0.85 (3H, s, C-25), 1.05 (3H, s, C-26), 0.97 (3H, s, C-27), 0.81 (3H, s, C-28), 1.71 (3H, s, C-30), 4.59 (1H, br, s, H $_{\alpha}$ -29), 4.72 (1H, br, s, H $_{\beta}$ -29). ¹³C NMR (125 MHz, CDCl₃). δ_C : 38.7 (C-1), 27.5 (C-2), 79.0 (C-3), 38.9 (C-4), 55.3 (C-5), 18.3 (C-6), 34.3 (C-7), 40.9 (C-8), 50.5 (C-9), 37.2 (C-10), 20.9 (C-11), 25.2 (C-12), 38.0 (C-13), 42.9 (C-14), 27.4 (C-15), 35.6 (C-16), 43.0 (C-17), 43.3 (C-18), 48.0 (C-19), 151.0 (C-20), 29.9 (C-21), 40.0 (C-22), 28.0 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.6 (C-27), 18.0 (C-28), 109.3 (C-29), 19.3 (C-30).

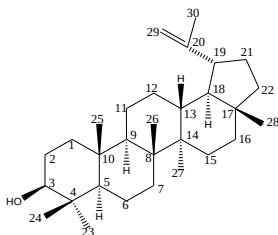
Compound (**EL2**): Betulin: 26.0 g, white powder. ¹H NMR (500 MHz, CDCl₃). δ_H : 0.90-1.98 (20 H, m, 2H-1, 2, 6, 7, 11, 12, 15, 16, 21, 22), 3.18 (1H, dd, J = 4.81, 11.43 Hz, H-3), 0.68 (1H, d, J = 10.64 Hz, H-5), 1.19-1.63 (3H, m, H-9, H-13, H-18), 2.38 (1H, ddd, J = 5.85, 10.96 Hz, H-19), 0.94 (3H, s, C-23), 0.75 (3H, s, C-24), 0.83 (3H, s, C-25), 1.02 (3H, s, C-26), 0.95 (3H, s, C-27), 1.68 (3H, s, C-30), 3.26 (1H, d, J = 10.80 Hz, H $_{\alpha}$ -28), 3.73 (1H, d, J = 10.80 Hz, H $_{\beta}$ -28), 4.52 (1H, br, s, H $_{\alpha}$ -29), 4.62 (1H, br, s, H $_{\beta}$ -29). ¹³C NMR (125 MHz, CDCl₃). δ_C : 38.7 (C-1), 27.0 (C-2), 79.2 (C-3), 38.9 (C-4), 55.5 (C-5), 18.3 (C-6), 34.2 (C-7), 41.1 (C-8), 50.6 (C-9), 37.2 (C-10), 20.8 (C-11), 25.2 (C-12), 37.3 (C-13), 42.9 (C-14), 27.4 (C-15), 29.2 (C-16), 28.0 (C-17), 48.9 (C-18), 48.0 (C-19), 150.7 (C-20), 29.7 (C-21), 34.0 (C-22), 21.1 (C-23), 15.4 (C-24), 16.1 (C-25), 16.0 (C-26), 14.8 (C-27), 60.7 (C-28), 109.9 (C-29), 19.1 (C-30).

Compound (**EL3**): 3 β -(5-hydroxyferuloyl)lup-20(30)-ene: 189.6 g, black crystalline solid. ¹H NMR (500 MHz, CDCl₃). δ_H : 1.01-1.69 (20H, m, 2H-1, 2, 6, 7, 11, 12, 15, 16, 21, 22), 1.26-4.59 (5H, m, H-3, 5, 9, 13, 18), 2.37 (1H, dd, J = 5.77, 11.0 Hz, H-19), 0.89 (3H, s, C-23), 0.92 (3H, s, C-24), 0.88 (3H, s, C-25), 1.04 (3H, s, C-26), 0.95 (3H, s, C-27), 0.79 (3H, s, C-28), 1.69 (3H, s, C-29), 3.91 (3H, s, H-7'), 4.57 (1H, d, J = 2.47 Hz, H $_{\alpha}$ -30), 4.69 (1H, d, J = 3.47 Hz, H $_{\beta}$ -30), 7.53 (1H, d, J = 15.86 Hz, H-32), 6.27 (1H,

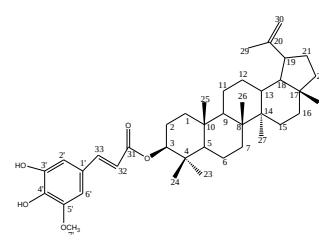
d, $J = 15.86$ Hz, H-33), 6.82 (1H, d, $J = 1.56$ Hz, H-2'), 6.65 (1H, d, $J = 1.74$ Hz, H-6'). ^{13}C NMR (125 MHz, CDCl_3). δ_{C} : 38.4 (C-1), 23.8 (C-2), 80.9 (C-3), 38.7 (C-4), 55.4 (C-5), 18.2 (C-6), 34.2 (C-7), 40.9 (C-8), 50.4 (C-9), 37.1 (C-10), 21.0 (C-11), 25.1 (C-12), 38.1 (C-13), 42.9 (C-14), 27.4 (C-15), 35.6 (C-16), 43.0 (C-17), 48.0 (C-18), 48.3 (C-19), 151.0 (C-20), 29.8 (C-21), 40.0 (C-22), 28.0 (C-23), 16.7 (C-24), 16.2 (C-25), 16.0 (C-26), 14.5 (C-27), 18.0 (C-28), 19.3 (C-29), 109.4 (C-30), 167.1 (C-31), 144.5 (C-32), 116.9 (C-33), 126.7 (C-1'), 109.3 (C-2'), 144.0 (C-3'), 134.6 (C-4'), 147.0 (C-5'), 102.9 (C-6'), 56.3 (C-7').



Lupeol (**EL1**)



Betulin (**EL2**)



3β-(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**)

Figure 2: Structures of compounds isolated from *E. latideus*

The interpretation of the spectral peaks for each of the compounds was as described below:

Compound (**EL1**): The ^1H NMR spectrum showed the presence of seven tertiary methyl protons at δ_{H} 0.78 (C-24), 0.81 (C-28), 0.85 (C-25), 0.97 (C-27), 0.99 (C-23), 1.05 (C-26), 1.71 (C-30) (each 3H, s) showing a lupane type of structure. It also showed two olefinic protons that appeared as broad singlets at δ_{H} 4.72 (1H, br, s, H-29b) and 4.59 (1H, br, s, H-29a) representing two non-equivalent germinal exomethylene protons of a terminal bond. The HSQC spectrum showed resonances of these two olefinic protons at δ_{H} 4.59 and 4.72 that correlated with methylene carbon at δ_{C} 109.3 (C-29). A doublet of doublets was seen at δ_{H} 3.22 (1H, dd, $J = 4.95, 11.22$ Hz, H-3). This was confirmed by the HSQC spectra which showed the placement of the methine proton that resonated at δ_{H} 3.22 on this carbon. This represented an axial oxymethine proton (H-3 α) attached to a secondary carbon atom that is bonded to an equatorial hydroxyl group (3 β -OH), [25]. A doublet was observed at 0.70 (1H, d, $J = 9.14$ Hz, H = 5) and a doublet of doublet of doublets at δ_{H} 2.38 (1H, ddd, $J = 5.84, 11.01$ Hz, H-19). At δ_{H} 1.71 (3H, br s, H-30) a broad 3H- proton singlet was seen indicating an isopropenyl group. The remaining protons were seen between δ_{H} 1.10 and 1.94 as complex multiplets (**Table 2**).

The ^{13}C NMR spectrum of compound (**EL1**) showed thirty carbon atoms (signals), which is typical of a lupine type of triterpenoid skeleton. The DEPT spectrum showed seven methyl groups at δ_{C} : 14.5 (C-27), 15.4 (C-24), 16.0 (C-26), 16.1 (C-25), 18.0 (C-28), 19.3 (C-30) and 28.0 (C-23). The peaks of two olefinic carbons showing an exomethylene group at 151.0 (C-20) and 109.3 (C-29) were seen indicating the presence of a double bond. With also the aid of the DEPT spectrum eleven methylene, six methine and six quaternary carbon atoms were determined. The quaternary carbon atoms at δ_{C} : 151.0 (C-20), 43.0 (C-17), 42.9 (C-14), 40.9 (C-8), 38.9 (C-4) and 37.2 were confirmed by their absence in the DEPT spectrum. The spectrum also showed a deshielded signal at δ_{C} 79.0 (C-3) indicating a carbinol carbon.

The proposed structure of compound (**EL1**) confirmed using 2-D NMR experiments that included COSY and HMBC. In the COSY spectrum cross peak correlations were seen between a methine proton at δ_{H} 2.38 (H-19) with a methylene proton peak (δ_{H} 1.34, H-21) and a methine proton signal (δ_{H} 1.38, H-18). Another cross peak correlation was seen between oxygenated methine proton signal (δ_{H} 3.22, H-3) and sp^3 methylene peak (δ_{H} 1.38, H-2). There was a correlation between sp^3 methine proton signal (δ_{H} 1.38, H-18) with (δ_{H} 2.38, H-19). Another cross peak correlation existed between the methylene proton (δ_{H} 1.40, H-22) with (δ_{H} 1.34, H-21). In the HMBC spectrum the methine proton at (δ_{H} 3.22, H-3) showed correlation with the methyl carbon signals of (δ_{C} 28.0, C-23), (δ_{C} 15.8, C-24) and methylene carbon signal δ_{C} 18.3, C-6). The sextet signal at (δ_{H} 2.38, H-19) showed cross peak correlations with two methylene carbon signals (δ_{C} 29.9, C-21) and δ_{C} 109.3 C-29), a methine carbon signal δ_{C} 48.3, C-18), a methyl carbon signal δ_{C} 19.3, C-30) and with a quaternary carbon signal δ_{C} 151.0, C-20). The pair of broad singlets of olefinic protons at (δ_{C} 4.59 and 4.72, H-29) showed cross peak correlation with a methine carbon signal at δ_{C} 48.0, C-19) and a methyl peak (δ_{C} 19.3, C-30). Therefore from the above spectroscopic data the structure of compound (**EL1**) was suggested to be that of Lupeol also known as lup-20-(29)-en-3 β -ol (**Figure 2**). This was after comparison of its ^1H and ^{13}C NMR spectral data with that from reported literature [26, 27, 28]. There is no reported literature on the isolation of Lupeol from *E. latideus*, therefore this is the first time that Lupeol is isolated from this plant species.

356 **Table 2. ^1H NMR and ^{13}C NMR spectroscopic data for Lupeol (EL1) compared against literature**
 357 **values. Literature [27] [^1H NMR 400 MHz, ^{13}C NMR 100 MHz CDCl_3 , J in Hz]**

Position of Carbon	^{13}C NMR (125 MHz)	^{13}C NMR literature	^1H NMR (500 MHz)	^1H NMR literature
1 α	38.7 (CH_2)	38.6	0.89 m	-
1 β			1.68 m	-
2 α	27.5 (CH_2)	27.7	1.68 m	-
2 β			1.58 m	-
3	79.0 (CH)	78.6	3.22 (1H, dd, $J = 4.95, 11.22$ Hz)	3.25 (1H, dd, $J = 5.6, 10.8$ Hz)
4	38.9 (C)	38.8	-	-
5	55.3 (CH)	55.8	0.70 (1H, d, $J = 9.14$ Hz)	(1H, d, $J = 9.1$ Hz)
6 α	18.3 (CH_2)	18.7	1.38 m	-
6 β			1.50m	-
7 α	34.3 (CH_2)	34.6	1.30 m	-
7 β			1.41 m	-
8	40.9 (C)	40.8	-	-
9	50.5 (CH)	50.8	1.27 m	-
10	37.2 (C)	37.7	-	-
11 α	20.9 (CH_2)	21.4	1.28 m	-
11 β			1.43 m	-
12 α	25.2 (CH_2)	25.5	1.10 m	-
12 β			1.67 m	-
13	38.0 (CH)	38.7	1.68 m	-
14	42.9 (C)	43.3	-	-
15 α	27.4 (CH_2)	27.7	1.05 m	-
15 β			1.62 m	-
16 α	35.6 (CH_2)	35.9	1.48 m	-
16 β			1.52 m	-
17	43.0 (C)	43.3	-	-
18	43.3 (CH)	48.7	1.38 m	-
19	48.0 (CH)	48.4	2.38 (1H, ddd, $J = 5.84, 11.01$ Hz)	2.35 (1H, dt, $J = 5.5, 10.9$ Hz)
20	151.0 (C)	151.5	-	-
21 α	29.9 (CH_2)	29.4	1.34 m	-
21 β			1.94 m	-
22 α	40.0 (CH_2)	39.8	1.22 m	-
22 β			1.40 m	-
23	28.0 (CH_3)	28.5	0.99 (3H, s)	0.98 (3H, s)
24	15.4 (CH_3)	15.8	0.78 (3H, s)	0.75 (3H, s)
25	16.1 (CH_3)	16.6	0.85 (3H, s)	0.85 (3H, s)
26	16.0 (CH_3)	16.3	1.05 (3H, s)	1.08 (3H, s)
27	14.6 (CH_3)	15.3	0.97 (3H, s)	0.96 (3H, s)
28	18.0 (CH_3)	18.4	0.81 (3H, s)	0.80 (3H, s)
29 α	109.3 (CH_2)	108.8	4.59 (1H, br, s)	4.58 (1H, s)

29 β			4.72 (1H, br, s)	4.68 (1H, s)
30	19.3 (CH ₃)	19.5	1.71 (3H, s)	1.75 (3H, s)

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359

360 Compound (**EL2**): The ¹H NMR spectrum of compound (**EL2**) displayed singlet signals of six
 361 tertiary methyl groups at δ_H : 1.68 (C-30), 1.02 (C-26), 0.95 (C-27), 0.94 (C-23) 0.83 (C-25) and 0.75 (C-
 362 24).

363 The observation of a signal at 1.68 indicated one methyl group attached to a double bond. In addition it
 364 showed a ¹H doublet of doublet (1H, dd, J = 4.81, 11.43 Hz) due to a hydroxymethine proton at δ_H 3.18.
 365 Its chemical shifts and coupling constant confirmed its assignment to the hydroxymethine at C-3, with the
 366 proton having an axial (α) orientation when an equatorial (β) hydroxyl is present (Mulholland and Nair,
 367 1994). Other doublets of two coupled protons had resonances at δ_H 3.79 and 3.32 (1H, d, J = 10.80 Hz),
 368 showing the presence of two an equivalent protons of a hydroxymethylene group (-CH₂OH, C-28). This
 369 was confirmed by proton resonances in the HSQC spectrum of the methylene carbon that had a signal at
 370 δ_C 60.7. The protons signals at δ_H 4.62 and 4.52 (1H, br, s, C-29) indicated the presence of two
 371 nonequivalent germinal exomethylene protons of terminal methylene group. This corresponded well with
 372 ¹³C NMR signal at 109.9 in the HSQC spectrum. There were other major signals of doublet of doublet of
 373 doublets at δ_H 2.38 (1H, ddd, J = 5.84, 10.96 Hz, C-19) and a doublet signal at δ_H 0.68 (1H, d, J = 10.64
 374 Hz, C-5). The remaining protons were assigned as multiplets (**Table 3**).

375 The ¹³C NMR spectrum showed thirty carbon signals that were clarified by DEPT experiments
 376 that indicated that compound (**EL2**) was a triterpenoid. It showed the presence of six methyl groups at δ_C
 377 19.09 (C-30), 16.1 (C-25), 16.0 (C-26), 15.4 (C-24), 14.8 (C-27) and 21.1 (C-23). It also revealed the
 378 presence of twelve methylene, six methine and six quaternary carbon atoms that were confirmed by their
 379 absence in the DEPT spectrum at δ_C : 150.7 (C-20), 42.7 (C-14), 41.0 (C-8), 38.9 (C-4), 37.2 (C-10), 28.0
 380 (C-17) in the molecule. The quaternary carbon δ_C 150.7 (C-20) and one methylene carbon δ_C 109.9 (C-
 381 29) confirmed the presence of a terminal double bond.

382 Complete assignment of protons of the proposed structure was established using 2-D NMR
 383 experiments that included COSY, HMBC and ROESY spectra. In the COSY cross peak correlations
 384 existed between olefinic protons δ_H 4.52 (H $_{\alpha}$ -29) and δ_H 4.62 (H $_{\beta}$ -29) with the methyl proton signal δ_H 1.68

(H-30). Cross peak correlation were established between the two hydroxymethylene groups δ_H 3.79 (H_{α} -28) and δ_H 3.32 (H_{β} -28). There were other cross peak correlation between the 23ethane proton δ_H 1.68 (H-5) with a methylene proton δ_H 1.53 (H_{α} -6). In the HMBC spectrum there was correlation between protons and carbons. The olefinic protons δ_H 4.52 (H_{α} -29) and 4.62 (H_{β} -29) correlated with the 23ethane carbon δ_C 48.0 (C-19) and methyl carbon δ_C 19.1 (C-30). The hydroxymethine proton δ_H 3.18 (H-3) showed cross peak correlation with carbons δ_C 15.4 (C-24), 27.0 (C-2), 38.7 (C-1) and 38.9 (C-4). Correlations existed of 2 and 3 bonds between the methylene protons δ_H 1.38 (H_{β} -11) and δ_H 1.17 (H_{α} -11) with carbons at δ_C : 55.5 (C-5), 42.9 (C-14), 41.0 (C-8), 34.2 (C-7). The 24ethane proton that resonated at δ_H 2.38 (H-19) showed cross peak correlations with carbons at δ_C : 19.1 (C-30), 29.7 (C-21), 37.3 (C-13), 48.9 (C-18) and 109.9 (C-29). Correlations were observed in the ROESY spectrum some of them included that between the olefinic protons δ_H 4.52 (H_{α} -29) and 4.62 (H_{β} -29) with the methine protons at δ_H 1.61 (H-18), 1.61 (H-18) and 2.38 (H-19). Space cross peak correlations were seen between the methine proton δ_H 2.38 (H-19) with methylene protons δ_H 3.79 (H_{α} -28), 4.62 (H_{β} -29), 1.85 (H_{β} -22), 1.61 (H_{α} -12) and a methine proton 1.61 (H-13). Space coupling correlations were seen between the methyl protons δ_H 0.95 (3H-27) with the methylene protons δ_H : 4.62 (H_{β} -29), 1.61 (H_{α} -12), 1.54 (H_{β} -15) and the methine protons of δ_H 1.61 (H-13), 1.61 (H-18). Proton cross peak correlations existed between the carbinol proton δ_H 3.18 (H-3) with methylene protons δ_H : 1.53 (H_{β} -6), 1.32 (H_{α} -6), 1.53 (H_{α} -2), 0.90 (H_{α} -1), 1.62 (H_{β} -1), methine proton 0.68 (H-5) and methyl protons 0.83 (3H-25). After comparing the ^1H NMR and ^{13}C NMR spectral data with that from literature the structure of compound was proposed to be a pentacyclic triterpene, betulin (**Figure 2**) also known as lup-20(29)-ene- β ,28-diol [29, 30]. Although the isolation and biological activities of betulin is well published, no literature is available concerning its isolation from *E. latideus*. Therefore this study reports the isolation of betulin for the first time from *E. latideus*.

Table 3. ^1H and ^{13}C NMR spectroscopic data for betulin (EL2) in $CDCl_3$ compared against literature values. Literature [30] [^1H NMR 300 MHz, ^{13}C NMR 75 MHz, $CDCl_3$, J in Hz]

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR literature	^1H NMR (500 MHz)	^1H NMR literature
1 α	38.7 (CH ₂)	38.7	0.9 m	-

1 β			1.62 m	1.63 m
2 α	27.0 (CH ₂)	27.1	1.53 m	-
2 β			1.57 m	1.59 m
3	79.2 (CH ₂)	78.9	3.18 (1H, dd, J = 4.81, 11.43 Hz)	3.17 (1H, dd, J = 4.90, 10.80 Hz)
4	38.9 (C)	38.9	-	-
5	55.5 (CH)	55.3	0.68 (1H, d, J = 10.64 Hz)	0.65 (1H, d, J = 9.4 Hz)
6 α	18.3 (CH ₂)	18.3	1.32 m	1.40 m
6 β			1.53 m	-
7 α	34.2 (CH ₂)	34.3	1.32 m	-
7 β			1.00 m	1.05 m
8	41.1 (C)	41.0	-	-
9	50.6 (CH)	50.4	1.19 m	1.23 m
10	37.2 (C)	37.2	-	-
11 α	20.8 (CH ₂)	20.1	1.17 m	1.15 m
11 β			1.38 m	-
12 α	25.2 (CH ₂)	25.2	1.64 m	1.51 m
12 β			1.71 m	-
13	37.3 (CH)	37.3	1.61 m	1.51m
14	42.9 (C)	42.7	-	-
15 α	27.4 (CH ₂)	27.4	0.95 m	-
15 β			1.54 m	1.56 m
16 α	29.2 (CH ₂)	29.2	1.19 m	-
16 β			1.93 m	1.93 m
17	28.0 (C)	28.0	-	-
18	48.9 (CH)	48.8	1.61 m	1.51 m
19	48.0 (CH)	47.8	2.38 (1H, ddd, J = 5.85, 10.96 Hz)	2.37 (1H, ddd, J = 4.0, 8.0 Hz)
20	150.7 (C)	150.0	-	-
21 α	29.7 (CH ₂)	29.8	1.32 m	1.35 m
21 β			1.98 m	-
22 α	34.0 (CH ₂)	34.0	1.33 m	-
22 β			1.85 m	1.83 m
23	21.1 (CH ₃)	27.9	0.94 (3H, s)	0.95 (3H, s)
24	15.4 (CH ₃)	15.3	0.75 (3H, s)	0.74 (3H, s)
25	16.1 (CH ₃)	16.1	0.83 (3H, s)	0.80 (3H, s)
26	16.0 (CH ₃)	16.0	1.02 (3H, s)	1.00 (3H, s)
27	14.8 (CH ₃)	14.8	0.95 (3H, s)	0.96 (3H, s)
28 α	60.7 (CH ₂)	60.6	3.26 (1H, d, J = 10.80 Hz)	3.31 (1H, d, J = 12.0 Hz)
28 β			3.73 (1H, d, J = 10.80 Hz)	3.78 (1H, d, J = 12.0 Hz)
29 α	109.9 (CH ₂)	110.0	4.52 (1H, br, s)	4.56 (1H, s)
29 β			4.62 (1H, br, s)	4.66 (1H, s)
30	19.1 (CH ₃)	19.1	1.68 (3H, s)	1.66 (3H, s)

Compound (**EL3**): The ^1H NMR spectrum showed eight methyl groups all being singlets.

They consisted of six tertiary methyl groups located on saturated carbons. These included: δ_{H} : 0.95 (3H, s, C-27), 1.04 (3H, s, C-26), 0.88 (3H, s, C-25), 0.92 (3H, s, C-24), 0.79 (3H, s, C-28), 0.89 (3H, s, C-23). There is one isopropylidene methyl group ($\text{MeC}=\text{CH}_2$) present at δ_{H} 1.69 (3H, s, C-29) and a methyl methoxyl ($-\text{OCH}_3$) singlet at 3.91 (3H, s, C-7'). The HSQC spectrum confirmed its location on this carbon at δ_{H} 56.3. Proton signals were observed at δ_{H} 4.69 and 4.57 due to two coupled nonequivalent germinal olefinic protons. Both of these protons were doublets 4.69 (1H, d, $J = 2.47$ Hz, C-30) and 4.57 (1H, d, $J = 2.47$ Hz, C-30). A proton signal was observed at δ_{H} 4.59 (1H, m, C-3) due to a deshielded methine proton on a carbon that has an oxygen atom attached to an ester. The HSQC spectrum established its placement at δ_{C} 80.9. Two doublets of 5-hydroxy ferulic acid signals due to coupled trans-substituted protons on the $\text{ArCH}=\text{CH}-$ were observed at δ_{H} 7.53 (1H, d, $J = 15.86$ Hz, C-32 $_{\alpha}$) and 6.27 (1H, d, $J = 15.86$ Hz, C-33 $_{\beta}$). Two doublet signals due to two aromatic methine protons appeared at δ_{H} 6.82 (1H, d, $J = 1.56$ Hz, C-2') and 6.65 (1H, $J = 1.74$ Hz, C-6'). These two protons were placed on their respective carbons by the HSQC spectrum at 109.3 (C-2') and 102.9 (C-6'). A doublet of doublets was observed at δ_{H} 2.37 (1H, dd, $J = 5.77$ Hz, 11.0 Hz, C-19). The above ^1H NMR signals established the presence of a ursane type of triterpene similar to α - amyrin esterified in position C-3 and having 5- hydroxyferulic acid.

The ^{13}C NMR and DEPT experiments established the composition of the structure to have forty carbon atoms. It showed distinct carbon signals for lupene moiety. The outstanding difference was that the C-3 signal was deshielded to δ_{C} 80.9 compared to a free alcohol at δ_{C} 78.8. This confirmed the linkage between the lupene and ferulosyl moieties at C-3. Eight methyl signals were observed at δ_{C} : 14.5 (C-27), 16.0 (C-26), 16.2 (C-25), 16.7 (C-24), 18.0 (C-28), 19.3 (C-29), 28.0 (C-23) and 56.3 (C-7') which confirmed the attachment of the methyl group to a methoxy group. The ^{13}C NMR further established six double bonds, three belonging to the aromatic ring located between δ_{C} : 126.7 (C-1'), 102.9 (C-6'), 144.0 (C-3') and 109.3 (C-2'), 134.6 (C-4') and 147.0 (C-5'). Two double bonds are located between δ_{C} : 144.5 (C-32 $_{\alpha}$) and 116.9 (C-33 $_{\beta}$), 151.0 (C-20) and 109.4 (C-30), while one is due to carbonyl group at 167.1 (C-31). The methoxy carbon atom was seen to absorb at δ_{C} 56.3 (C-7'). The ^{13}C NMR and DEPT experiments further determined the presence of eleven methylene and ten methine groups. The

DEPT spectrum confirmed the presence of eleven quaternary carbon atoms by their not appearing in its spectrum, these include δ_C : 167.1 (C-31), 151.0 (C-20), 147.0 (C-5'), 144.0 (C-3'), 134.6 (C-4'), 126.7 (C-1'), 38.7 (C-4), 43.0 (C-17) 42.9 (C-14), 40.9 (C-8) and 37.1 (C-10) (**Table 4**).

The ^1H and ^{13}C NMR, HSQC assignments were confirmed with COSY, HMBC and NOESY experiments. In the COSY spectrum the methine proton δ_H 7.53 (H-32) showed correlation with another methine proton δ_H 6.27 (H-33). The olefinic protons at 4.57 $_{\alpha}$ and 4.69 $_{\beta}$ (C-30) showed cross peak correlation with the methyl protons δ_H 1.69 (H-29). The methine proton δ_H 2.37 (H-19) showed correlation with another methine proton δ_H 1.37 (H-18) and the methylene proton δ_H 1.91 $_{\beta}$ (H-19). The methylene protons δ_H 1.40 $_{\alpha}$ and 2.05 $_{\beta}$, showed cross peak correlation with a methine proton δ_H 1.26 and the methylene protons δ_H 1.69 $_{\beta}$ and 1.07 $_{\alpha}$ (H-12). In the HMBC spectrum the olefinic methine proton δ_H 7.53 (H-32 $_{\alpha}$) correlated with carbons δ_C : 167.1 (C-31), 126.7 (C-1'), 116.9 (C-33), 109.3 (C-2') and 102.9 (C-6'). The olefinic methylene protons (δ_H 4.57 $_{\alpha}$, 4.69 $_{\beta}$, C-30) were observed to correlate with carbons δ_C 48.3 (C-19), 19.3 (C-29).

The hydroxymethine proton δ_H 3.91 (H-3) showed cross peak correlation with carbons δ_C 167.1 (C-31) and 16.7 (C-24). The correlation between the hydroxymethine proton and with carbon δ_C 167.1 (C-31) proved the linkage between the lupene and ferulosyl moieties. Furthermore the methine proton δ_H 2.37 (H-19) showed cross peak correlation with carbons δ_C 151.0 (C-20), 109.4 (C-30), 48.0 (C-18), 38.1 (C-13), 29.8 (C-21) and 19.3 (C-29). The methyl protons δ_H 0.79 (H-28) correlated with carbons 48.0 (C-18), 48.3 (C-19), 35.6 (C-16) and 43.0 (C-17). In the NOESY spectrum correlations were seen between olefinic proton δ_H 7.53 (H-32 $_{\alpha}$) with protons δ_H 6.82 (H-2'), 6.65 (H-6') and 6.27 (H-33 $_{\beta}$). The olefinic proton δ_H 6.27 (H-33 $_{\beta}$) was observed to correlate with protons δ_H 7.53 (H-32 $_{\alpha}$), 6.82 (H-2') and 6.65 (H-6'). The olefinic protons δ_H 4.57 $_{\alpha}$ and 4.69 $_{\beta}$ (C-30) showed cross peaks with protons δ_H 2.37 (H-19), 1.37 (H-18) and 1.69 (C-29). The methyl protons δ_H 1.69 (H-29) correlated with olefinic protons δ_H (4.57 $_{\alpha}$, 4.69 $_{\beta}$, C-30) and the methine proton 2.37 (H-19).

Comparison of the above spectral data with that reported in literature, the structure of the compound was proposed to be 3 β -(5-hydroxyferuloyl) lupen-20(30)-ene [31, 32] (**Figure 2**). The isolation of this compound from *E. latideus* is being reported for the first time.

465 **Table 4.** ^1H and ^{13}C NMR spectroscopic data for 3β -(-5-hydroxyferuloyl) lupen-20(30)-ene (EL3) in
 466 CDCl_3 compared against literature values. Literature [32] [^1H NMR 400 MHz, ^{13}C NMR 100
 467 MHz, CDCl_3 , J in Hz.

Position of Carbon	^{13}C NMR (125 MHz)	^{13}C NMR literature	^1H NMR (500 MHz)	^1H NMR literature
1 α	38.4 (CH_2)	38.4	1.02 m	-
1 β			1.69 m	-
2 α	23.8 (CH_2)	23.8	1.69 m	-
2 β			1.91 m	-
3	80.9 (CH)	80.9	4.59 m	4.50-4.60 m
4	38.7 (C)	39.0	-	-
5	55.4	55.4	1.4 m	-
6 α	18.2 (CH_2)	18.2	1.4 m	-
6 β			1.5 m	-
7 α	34.2 (CH_2)	34.2	1.02 m	-
7 β			1.38 m	-
8	40.9 (C)	40.8	-	-
9	50.4 (CH)	50.3	1.26 m	-
10	37.1 (C)	37.1	-	-
11 α	21.0 (CH_2)	20.9	1.21 m	-
11 β			1.40 m	-
12 α	25.1 (CH_2)	25.1	1.07 m	-
12 β			1.69 m	-
13	38.1 (CH)	38.0	1.69 m	-
14	42.9 (C)	42.8	-	-
15 α	27.4 (CH_2)	27.4	1.01 m	-
15 β			1.69 m	-
16 α	35.6 (CH_2)	35.6	1.37 m	-
16 β			1.46 m	-
17	43.0 (C)	43.0	-	-
18	48.0 (CH)	48.0	1.37 m	-
19	48.3 (CH)	48.3	2.37 (1H, dd, $J = 5.77, 11.0$ Hz)	2.30 m
20	151.0 (C)	150.9	-	-
21 α	29.8 (CH_2)	29.8	1.26 m	-
21 β			1.91 m	-
22 α	40.0 (CH_2)	40.0	1.20 m	-
22 β			1.40 m	-
23	28.0 (CH_3)	28.0	0.89 (3H, s)	0.87 (3H, s)
24	16.7 (CH_3)	16.7	0.92 (3H, s)	0.89 (3H, s)
25	16.2 (CH_3)	16.2	0.88 (3H, s)	0.86 (3H, s)
26	16.0 (CH_3)	16.0	1.04 (3H, s)	1.02 (3H, s)

27	14.5 (CH ₃)	14.5	0.95 (3H, s)	0.93 (3H, s)
28	18.0 (CH ₃)	18.0	0.79 (3H, s)	0.76 (3H, s)
29	19.3 (CH ₃)	19.3	1.69 (3H, s)	1.66 (3H, s)
30 α	109.4 (CH ₂)	109.3	4.57 (1H, d, J = 2.47 Hz)	4.58 (1H, br d, J = 2.4 Hz)
30 β			4.69 (1H, d, J = 3.47 Hz)	4.70 (1H, br d, J = 2.4 Hz)
31	167.1 (C)	167.2	-	
32	144.5 (CH)	144.4	7.53 (1H, d, J = 15.86 Hz)	7.54 (1H, d, J = 15.80 Hz)
33	116.9 (CH)	116.8	6.27 (1H, d, J = 15.86 Hz)	6.29 (1H, d, J = 15.80 Hz)
1'	126.7 (C)	126.6	-	
2'	109.3 (CH)	109.2	6.82 (1H, d, J = 1.56 Hz)	6.79 (1H, br, d, J = 1.80 Hz)
3'	144.0 (C)	143.9	-	-
4'	134.6 (C)	134.5	-	-
5'	147.0 (C)	146.9	-	-
6'	102.9 (CH)	102.9	6.65 (1H, d, J = 1.74 Hz)	6.63 (1H, br, d, J = 1.80 Hz)
7'	56.3 (CH ₃)	56.2	3.91 (3H, s)	3.88 (3H, s)

3.2 Antiplasmodial activity of crude extracts and pure compounds

The extracts and the pure compounds were active against the 3D7 (chloroquine sensitive) and DD2 (chloroquine resistant) strains of *P. falciparum* (Table 5). The root crude extracts of *E. latideus* exhibited activities of: (IC₅₀) 3D7: 37.72 $\pm$ 0.0 μ g/mL (hexane extract); (28.07 $\pm$ 1.16 μ g/mL) (dichloromethane extract) and (IC₅₀) DD2: 38.39 $\pm$ 0.0 μ g/mL (hexane) all displayed moderate activity. Promising activity was shown by crude extracts (IC₅₀) 3D7: (12.86 $\pm$ 1.08 μ g/mL) (ethyl acetate extract) and DD2: (13.97 $\pm$ 1.49 μ g/mL) (methanol extract). High activity from this plant was displayed by the crude extracts: (IC₅₀) DD2: 2.76 μ g/mL (dichloromethane extract); (4.37 $\pm$ 0.57 μ g/mL) (ethyl acetate extract) and 3D7: (9.75 $\pm$ 0.85 μ g/mL) (methanol extract) (Table 5.). The antiplasmodial activities displayed by the crude extracts were not due to the toxicity of the plants but the real activity of the compounds because all the crude extracts of *E. latideus* were practically non-toxic. (Table 6). Antiplasmodial activities of crude extracts (Hex, DCM, EtOAc, MeOH) (2.76-38.39) μ g/mL and pure compounds (1.66-25.14) μ g/mL showed significant difference with the reference standards (0.0056-0.0440) μ g/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 antiparasmodial activity for the two strains *Plasmodium* compounds compared to the two positive controls (CQ and MQ) (Table 5).

E. latideus root crude extracts of hexane, dichloromethane, ethyl acetate and methanol had good activity (IC_{50}) 3D7: ($9.75 \pm 0.85 - 37.72 \pm 0.0$) $\mu\text{g/mL}$ and DD2: ($2.76 \pm 0.0 - 38.39 \pm 0.0$) $\mu\text{g/mL}$. The activities of the four extracts were classified as highly active and having moderate activity. Some antiparasmodial activity studies have been investigated on some *Euclea* species. In a study of South African medicinal plants against Chloroquine-sensitive strain (D10) of *P. falciparum* [33] reported antiparasmodial activity of 134 species. The roots, leaves and twigs of *Euclea natalensis* and *Euclea undulata* were among the plants tested. All the water crude extracts were inactive with $IC_{50} > 100 \mu\text{g/mL}$ while the actives of the DCM/MeOH extracts had IC_{50} between 4.6-11.0 $\mu\text{g/mL}$. Another investigation was carried out on *Euclea divinorum* [34]. The crude extracts were screened against chloroquine (CQ) susceptible and resistant strains of *P. falciparum* (D6 and W2, respectively). The CH_2Cl_2 and EtOAc crude extracts obtained from the leaves showed high *in vitro* antiparasmodial activity in the range of $IC_{50} = 6.12 \pm 0.45 - 17.29 \pm 1.44 \mu\text{g/mL}$ and $8.42 \pm 1.06 - 12.09 \pm 0.67 \mu\text{g/mL}$ against CQ-susceptible strain, respectively. In Nigeria [35] carried out antiparasmodial activity using solvents of different polarities (petroleum ether, DCM, MeOH and aqueous). The extracts were from the leaves and gave high activity of IC_{50} (9.1, 7.6, 12.1 and 15.9) $\mu\text{g/mL}$ against *P. falciparum* KI parasites respectively. A study [36] on the crude $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) extract of the aerial parts of *Tephrosia subtriflora* showed similar results of moderate antiparasmodial activity against KSM 009 strain of *P. falciparum* with IC_{50} value of $11.39 \pm 1.06 \mu\text{g/mL}$. Also [37] in their search for new antimalarial compounds studied the $\text{CH}_2\text{Cl}_2/\text{CH}_3\text{OH}$ (1:1) extract of the roots of *Tephrosia aequilata* which caused 100% mortality of the chloroquine-sensitive (3D7) strain of *P. falciparum* at a 10 mg/mL concentration. The activities of the crude extracts studied above ranged from highly active, moderately active to being inactive. These activities are similar to those exhibited by the crude extracts of *E. latideus*.

Extracts of *E. latideus* gave good resistance indices of 0.10 and 0.34 for dichloromethane and ethyl acetate crude extracts respectively. The hexane and methanol crude extracts displayed quite different indices of 1.02 and 1.43 respectively against .The resistance indices of all the crude extracts of

the plant were better than the reference standards which had 2.88 and 4.73 for mefloquine and chloroquine respectively (**Table 5**). The resistance indices exhibited by the crude extracts of the plant suggest that some of these extracts have promising activity against CQ resistant DD2 strain of *P. falciparum*. These results indicate the possible explanation for the traditional use of this medicinal plant against malaria.

The investigation showed for the first time that compounds present in extracts of *E. latideus*, have high antiplasmodial activity. *E. latideus* displayed indices between 1-1.43. Similar investigations were carried out [38] on extracts of ethyl acetate and methanol of *Phyllanthus emblica*, *Syzygium aromaticum*, and *Abrus precatorius*. The extracts were found to be as active against CQ-resistant Dd2 and INDO as against CQ-sensitive 3D7 giving resistance indices of 1-1.5. However, the ethyl acetate extracts from *P. emblica* and *S. aromaticum* gave resistance indices of 2.06 (Dd2) and 0.76 (INDO), respectively. Extracts of *A. squamosa* showed good response against CQ-resistant strains than against 3D7 giving resistance indices in a range of 0.5 - 0.82. Extracts of *G. superba* exhibited different resistance indices with values >1 for Dd2 and <1 for INDO strains. Another study [39] on antiplasmodial activity of ethyl acetate crude extracts of plant species; *Zingiber officinale*, *Ficus religiosa*, *Murraya koenigii*, *Ficus benghalensis*, *Aspergus racemosus*, *Artemisia pallens*, *Ficus racemosa*, *Phyllanthus emblica* and *Hemidesmus indicus* found out the resistance indices of the plants ranging from 0.78 to 1.28, which was in the same range as the crude extracts for *E. latideus*.

In the current study three compounds were isolated and their structures elucidated. However only two compounds were assessed for their *in vitro* antiplasmodial activities. These included Lupeol (**EL1**) and betulin (**EL2**). The activity of 3 β -(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**) could not be determined because it was not soluble in DMSO. Isolation and purification of the ethyl acetate crude extracts of *E. latideus* afforded triterpenoids Lupeol and betulin that were tested for antiplasmodial activity. Lupeol had activity of IC₅₀ 3D7; 23.96 $\pm$ 0.0 μ g/mL and DD2: 25.14 $\pm$ 0.0 μ g/mL. This activity was low as compared to that of its ethyl acetate crude extract which was highly active on both strains of parasite (IC₅₀ 3D7: 4.69 $\pm$ 0.0 μ g/mL, DD2: 3.5 $\pm$ 0.0 μ g/mL). The crude also had a resistance index of 0.75 which low as compared to that of the pure compound that had 1.09. This suggests that there was enhancement of activity by other chemical constituents present in the extract which may have acted synergistically. The

crude extract was also more active against the CQ resistance strain than the pure compound as shown by their indices. Similar findings in which the crude extract had high activity compared to the crude extract from which it was isolated was also recorded [40]. In their study the isolated four diterpenes had (IC_{50}) D6: 14.3-24.4 $\mu\text{g/mL}$, W2: 17.5-18.4 $\mu\text{g/mL}$ which were less active compared to the chloroform crude extract that displayed (IC_{50}) D6: 7.89 $\mu\text{g/mL}$, W2: 8.74 $\mu\text{g/mL}$. Antiplasmodial activity screening of Lupeol is documented. Some of the studies were investigated [41], on 3D7 and Dd2 parasitic strains. Their research gave IC_{50} values of 80.30 and 54.22 μM on the two strains of parasites respectively, on Lupeol isolated from *Ampelozizyphus amazonicus* from Nigeria. Another study on Lupeol isolated from the ethyl acetate leaf extract of *Cassia siamea* [42] gave high activity of IC_{50} 5.0 $\mu\text{g/mL}$ on PfKI strain of parasites. Also [43] carried out antiplasmodial screening of Lupeol isolated from the DCM/MeOH crude extract of the bark of *Albizia zygia* on PfKI strain that gave IC_{50} of $> 0.078 \mu\text{g/mL}$. Another report was given [44] on the *in vitro* inhibitory activity of Lupeol against CQ-sensitive 3D7 strain of *P. falciparum*. Their study gave an IC_{50} value of 11.8 mg/mL which was shown to cause a human erythrocyte shape towards stomatocytes. There was difference in the activity of Lupeol isolated from *E. latideus* and that from other plant species. However all plant species used recorded activity that ranged from highly active to moderate activity.

The second compound betulin isolated from *E. latideus* displayed antiplasmodial activity of (IC_{50}) 3D7: $1.66 \pm 0.0 \mu\text{g/mL}$, DD2: $15.44 \pm 4.63 \mu\text{g/mL}$. It exhibited high activity compared to the ethyl acetate crude extract from which it was isolated. It also had a resistance index of 9.30 showing that it was less reactive to the CQ DD2 resistance strain compared to the ethyl acetate crude extract which had a value of 0.34. These results are similar to those in a study carried out by [45] in which the activity of the pure compounds was higher than the crude extracts. They evaluated the ethyl acetate and aqueous extracts of *Neoboutonia macrocalyx* against CQ sensitive (D6) and CQ resistant (W2) strains of *P. falciparum*. The isolated pure compounds montanin and neoboutomacroin had IC_{50} 2.3-3.9 $\mu\text{g/mL}$ and 3.6-4.9 $\mu\text{g/mL}$ against CQ D6 and CQ W2 strains respectively. The ethyl acetate had 12.7-5.7 $\mu\text{g/mL}$ while the aqueous extract was inactive. Antiplasmodial activity screening investigated on betulin isolated from different plant species has been reported in literature. An *in vitro* antiplasmodial activity was conducted [46] against the *P. falciparum* CQ-susceptible strain (D10). The study was investigated on betulin isolated from the

dichloromethane leaf crude extract of *Scheffleria umbellifera* which gave good activity of IC₅₀ of 3.2 µg/mL. According [47] betulin isolated from several plant families such as Rhamnaceae (*Ziziphus vulgaris*) and Labiatae (*Zataria multiflora*) showed moderate activity (IC₅₀ < 12 µg/mL and < 27 µM, respectively). Another investigation was conducted [41] on betulin isolated from *Ampelozizyphus amazonicus*. Their findings gave IC₅₀ values of 17.08 and 14.22 µM against 3D7 CQ sensitive and Dd2 CQ resistant parasites respectively. An evaluation was also conducted [48, 49] and they found out that the betulin isolated from *Uapaca nitida* was inactive with IC₅₀ of 500 µg/mL. However [50] reported betulin isolated from *Croton argrophylloides* to inhibit *P. falciparum* strains with an IC₅₀ of 12 µg/mL. The activities of betulin isolated from *E. latideus* was in agreement with the values documented in literature above.

Table 5. *In vitro* antiplasmodial activities of crude extracts and isolated compounds against 3D7 and DD2 strains of *P. falciparum*

Name	Test samples	Antiplasmodial activity IC ₅₀ (µg/mL)	
		IC ₅₀ ± SEM	
		3D7 strain (CQ Sensitive)	DD2 strain (CQ Resistant)
<i>E. latideus</i>	Hex	37.72 ± 0.0 ^a	38.39 ± 0.0 (1.02) ^b
	DCM	28.07 ± 1.16 ^a	2.76 ± 0.0 (0.098) ^b
	EtOAc	12.86 ± 1.08 ^a	4.37 ± 0.57 (0.34) ^b
	MeOH	9.75 ± 0.85 ^a	13.97 ± 1.49 (1.43) ^b
Pure compounds	Lupeol (EL1)	23.96 ± 0.0 ^a	25.14 ± 0.0 (1.09) ^b
	Betulin (EL2)	1.66 ± 0.0 ^a	15.44 ± 4.63 (9.30) ^b
	3β-(5-hydroxyferuloyl)lup-20(30)-ene (EL3)	NT	NT
Reference	Chloroquine diphosphate (CQ)	0.0093 ± 0.0057 ^a	0.0440 ± 0.0 (4.73) ^b
Standards	Mefloquine hydrochloride (MQ)	0.0056 ± 0.0011 ^a	0.0161 ± 0.0 (2.88) ^b

Values are expressed as mean ± S.E.M. (n = 3).

Values with the same superscript in the same column are significantly different (*P* < 0.05)

^aValues enclosed in parenthesis represent resistance index ratio of IC₅₀ CQ-resistant strain (DD2 /IC₅₀ CQ sensitive strain 3D7).

NT – Not tested

3.3 Acute toxicity of crude extracts

The present work on *in vivo* acute toxicity was approved by the Research Ethical Committee for using animals at Makerere University, Department of Pharmacology (No.1250). According to the LD₅₀ values of acute toxicity determined for the crude extracts of *E. latideus* were > 5000 mg/kg and considered to be practically non-toxic. These extracts included that of hexane, dichloromethane, ethyl acetate and methanol (**Table 6**). During the first phase of administration for *E. latideus* extracts at doses 500, 1000 and 2000 mg/kg, for the hexane extract animals looked normal after 6 hours of dosing. When the dose was increased to 3000 and 4000 mg/kg the animals rested for about 15 minutes and at 5000 mg/kg their movements in the cage slowed down in first 30 minutes after dosing. The dichloromethane extract had the same observations at 500 and 1000 mg/kg as that for the hexane extract, however at 2000, 3000, 4000 mg/kg there was a small reduction in the movement of the animals. Finally at dose of 5000 mg/kg the animals were observed not to move freely as before. For the ethyl acetate crude extract at all doses 500-5000 mg/kg there was no observable effect in the behavior of the animals. Also no signs of toxicity was seen for the methanol crude extract at doses 500 - 200 mg/kg, however at 3000 and 4000 mg/kg the movement of the animals slowed down in the first hour after doing. The movement of the animals reduced for about an hour and then they rested in the corners of the cage.

Determination of acute toxicity the first step in the toxicological analyses of herbal drugs. In the present study Lorke's procedure [12] 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. In the current study the acute toxicity of most of the crude extracts of the *E. latideus* had LD₅₀ > 5000 mg/kg and considered non-toxic. In a study [51] on toxicological studies of the aqueous leaf extracts of *Combretum micranthum* on rats was investigated. The acute toxicity was assessed in two phases using Lorke's method (1983). In the first phase rats received doses of 10-1000 mg/kg while the second phase doses of 1250 - 5000 mg/kg were administered. The administration of the extract up to 5000 mg/kg caused 0% death. The result of acute toxicity studies in rats indicated that the extract was less toxic and can be used as medicine. In another study [52] also determined the toxicity of methanol-dichloromethane extract and fractions in an investigation on the antibacterial, antifungal and wound healing potentials of *Nauclea diderrichii* root bark. The estimated lethal dose (LD₅₀) of the plant extract was also performed in accordance with the method described [12].

The extract gave an estimated LD₅₀ > 5000 mg/kg as no death was observed in the groups after 48 hours treatment. Also no visible sign of acute toxicity was observed. Therefore, the crude extract could be reasonably safe to animals and humans. Acute toxicity was determined in another study still [53] on antimicrobial effects of a lipophilic fraction and kaurenoic acid isolated from the root bark extracts of *Annona senegalensis*. The same method of acute toxicity determination was used to carry out the procedure. Death occurred at the maximum dose (5000mg/kg). Their medium lethal dose (LD₅₀) of the isolated compound was calculated to be 3800 mg/kg in mice indicating the good level of safety.

Table 6. *In vivo* acute toxicity activities of crude extracts against mice

Scientific name	Extraction solvent	Mortality						
		Phase 1 dose (mg/Kg)			Phase 2 dose (mg/Kg)			LD ₅₀ (mg/Kg)
		500	1000	2000	3000	4000	5000	
<i>E. latideus</i>	Hex	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a	0/3 ^a	>5000
	DCM	0/3	0/3	0/3	0/3	0/3	0/3	>5000
	EtOAc	0/3	0/3	0/3	0/3	0/3	0/3	>5000
	MeOH	0/3	0/3	0/3	0/3	0/3	0/3	>5000

^a Number of animals dead/number of animals used

4. CONCLUSION

The analyses revealed for the first time that compounds present in the crude extracts of *Euclea latideus* have good antiplasmodial activity. In addition the resistance indices of the crude extracts were low showing that they were equally active against the chloroquine resistant DD2 parasite strain. This shows that the plant has promising antiplasmodial activity against CQ resistant DD2 strain of *P. falciparum*. Therefore the plant can be taken in its crude form to offer prophylactic cure. The plant crude extracts were found to be practically non-toxic, this shows that the good antiplasmodial activity of the crude extracts is not due to their

toxicity and they have a large safety margin. The apparent lack of toxicity of the crude extracts of the plant possibly explains why it taken without any side effects. Therefore the medicinal plant can be considered to be safe for consumption in its crude form. This accounts for collectively the historical and enduring use of the plant against malaria by the local communities.

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.

COMPETING INTERESTS

The authors declare that they have no competing interests.

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