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

Kodi Philip[1*], Mwangi M. Elizabeth[1], Peter K. Cheplogoi[1], Moses K. Langat[2], and M. Akala Hoseah[3]

[1]Department of Chemistry, Egerton University, P.O.Box 536 - 20115, Njoro, Nakuru, Kenya.
[2]Natural Products Research Group, Department of Chemistry, Faculty of Engineering and Physical Sciences, University of Surrey, Guilford, Surrey, Gu2 7XH, United Kingdom.
[3]Department of Emerging Infectious Diseases—Global Emerging Infections Surveillance and Response System (DEID-GEIS) Program, United States Army Medical Research Unit, Kenya. (USAMRU-K), Kenya Medical Research Institute (KEMRI)—Walter Reed Project, Kisumu, Kenya.

Authors' contributions

This work was carried out in collaboration between all the authors. Author KP designed the study, wrote the protocol, carried out the experimental work, managed the literature searches and wrote the first draft of the manuscript. Spectroscopic analysis was managed by author MKL while antiplasmodial activity assays were determined by author MAH. Characterization was carried out by authors KP, MKL, MME and PKC. All authors read and approved the final draft of the manuscript.

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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 were

**Corresponding author: E-mail: kodiphilip@yahoo.com;*

carried out on only the dichloromethane and ethyl acetate crude extracts.
Methodology: Four 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 were 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* (chloroquine [CQ]-sensitive 3D7 and CQ-resistant Dd2 strains) 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 LD_{50} > 5000 mg/kg and therefore regarded as a non-toxic plant. The four crude extracts of *E. latideus* had good activity
With range of (IC_{50}) 3D7: (9.75-38.21) µg/mL and Dd2: (2.78-38.93) µg/mL. The resistance indices for *E. latideus* crude extracts ranged between 0.10- 1.43, suggesting that some of the extracts had equal promise against the CQ resistant strain of *P. falciparum*. 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 on both strains (IC_{50}) 3D7: 1.64 ± 0.02 µg/mL and Dd2: 7.69 ± 1.21 µg/mL while Lupeol (EL1) displayed moderate activity with (IC_{50}) 3D7: 23.91 ± 0.05 µg/mL, Dd2: 25.14 ± 0.01 µg/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 IC_{50} (2.78-38.93) µg/mL and pure compounds IC_{50} (1.64-25.14) µg/mL showed a significant decrease in activity compared to the reference standards (0.0056-0.0440) µg/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 have 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 of Butebo Sub-County, in Pallisa District in Eastern Uganda in the treatment of malaria.

Keywords: Euclea latideus; antiplasmodial; acute toxicity; betulin; in vitro; Butebo.

1. INTRODUCTION

Malaria is the world's most important tropical disease. Globally, about 3.2 billion people live in areas prone to malaria transmission, and 1.2 billion are at high risk [1]. In 2016, malaria contributed to an estimated 216 million infections and about 445, 000 deaths where 91% of the deaths were in the African region and among those about 78% were children under the age of 5 years [1]. Children under five years of age and pregnant women are most severely affected [2]. 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 [3]. The increasing global spread of drug resistance to most of the convenient and affordable antimalarial drugs is a significant 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 medicines. Such a situation has opened the need for alternative antiplasmodial 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 *E. latideus*, it is important that its claimed antimalarial property is investigated. This was done in order to establish its efficacy, safety and determine its potential as a source of new antimalarial drug.

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 found in 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, naphtalene-based aromatics, hydrocarbons, lipids, amino acids, carotenoids, and sugars [4]. The naphthoquinones occur in several parts of the plants, especially in the bark, and are active against fungi, bacteria, mollusks, insects, worms, termites [5]. Their derivatives and oxidative decomposition products are responsible for the dark brown to black coloured tissues of the bark, heartwood, fruits, and leaves [5]. Idioblasts containing tannins occur in various parts, including fruits [6].

E. latideus is used in the treatment of malaria and fever by the local communities in Butebo Sub-County, Pallisa District in Eastern Uganda. Studies on the *in vitro* antiplasmodial and toxicity properties of crude extracts of *E. latideus* has not been investigated. 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 [7], special attention was focused to *E. latideus* (Ebenaceae).

In the current study, the solvent extracts of the roots of *E. latideus* and isolated pure compounds were tested for their antiplasmodial 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 assay [8].

2. MATERIALS AND METHODS

2.1 Plant Material

E. latideus was identified and documented as an antimalarial remedy in an ethnobotanical survey that was conducted in Butebo Sub-County [7]. The plant was photographed, collected, dried and taken for identification by a taxonomist at Makerere University, Department of Botany. 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 [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 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 in Winchester bottles of 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 40-45ºC, 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. 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 length. 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 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) (Fig. 1). This combined fraction (3-10) was eluted with EtOAc : hexane (1:4, 3:7) followed by 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 of fractions D(1-61) 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. This study gives the first report of isolation of Lupeol (EL1), betulin (EL2) and 3β-(5-hydroxyferuloyl)lup-20(30)-ene (EL3) from the roots of *E. latideus*.

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 a 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. The following reagent was obtained through Biodefense and Emerging Infections Research Resources Repository (BEI Resources), National Institute of Allergy and Infectious Diseases (NIAID), National Institute of Health (NIH): *Plasmodium falciparum*, Strain Dd2, MRA-150, contributed by David Walliker and 3D7, MRA-102, contributed by Daniel J. Carucci. These isolates were grown as described with minor modifications [13,14]. Drugs, extracts and pure compounds were dissolved in 99.5% dimethyl sulfoxide (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 [15]. 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 µM 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 dilutions 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 0.025-0.05%. Chloroquine and mefloquine were used as standard 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% CO_2, 5% O_2, and 90% N_2) at 37°C. The assay was terminated 72 hours later by freezing at -80°C for 24 hours. After thawing 100 µL of SYBR Green I solution which contained (0.2 µL of 10,000 x SYBR Green I (Invitrogen)/mL) in lysis buffer {Tris (20 mM; pH

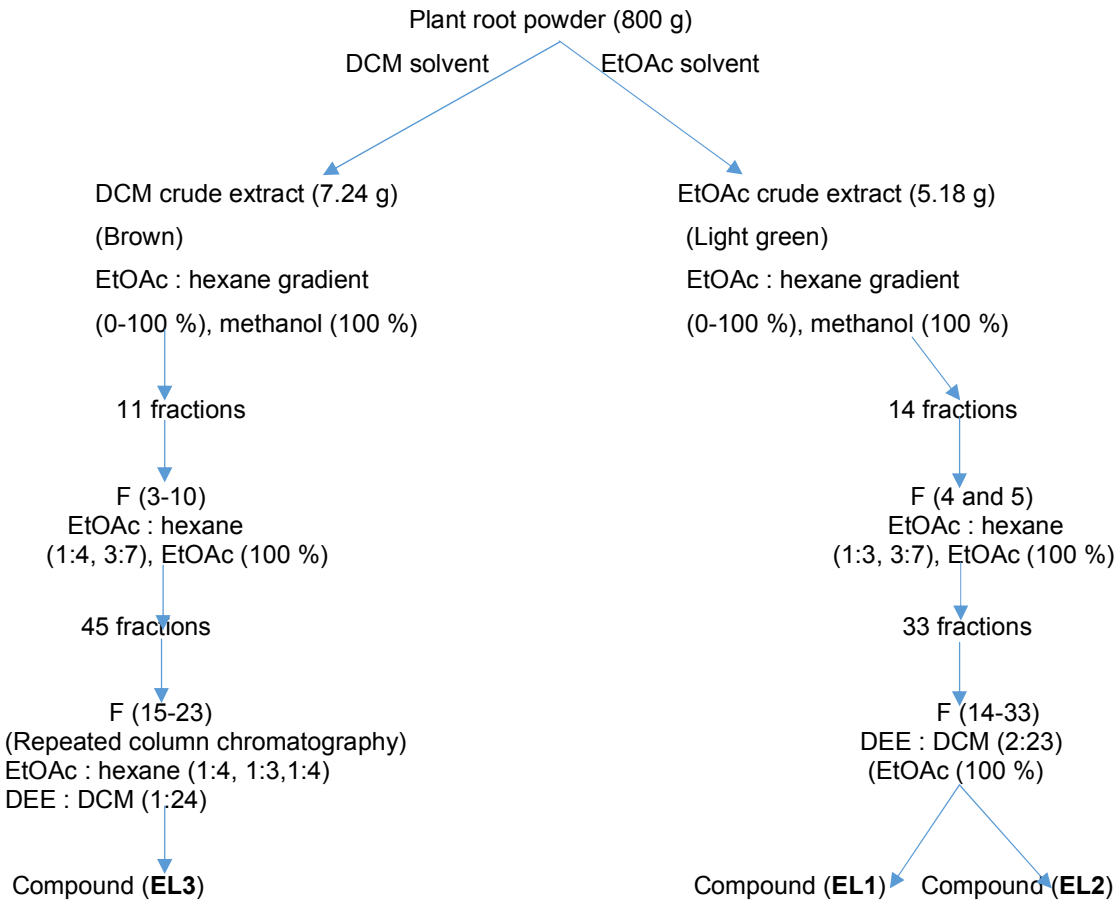


Fig. 1. Flow chart showing procedure for isolation of pure compounds from *E. latideus*

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}) 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} ± 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 estimated lethal dose (LD_{50}) of the crude extracts in mice was performed using the method described [8]. A total of 73 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. 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) [14, 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

For the statistical analysis, the Microsoft Excel 2013 was used to calculate means and percentages and numerical data expressed as mean ± standard deviation (SD) 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 (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

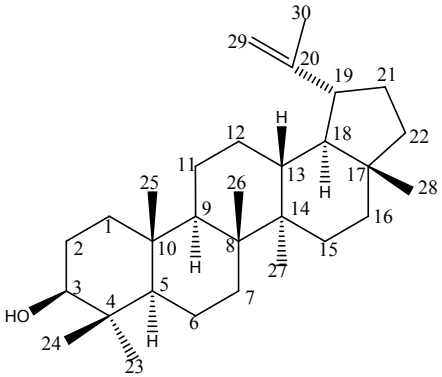
3.1 Test Samples for Bioassay Activity Screening

The yields for the crude extracts were: (0.36-2.36)% and (0.50-3.66)% for the pure compounds which were calculated based on the weight of medicinal plant powder and crude extracts, respectively.

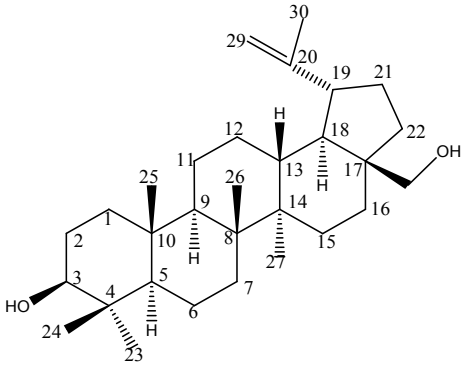
The methanol crude extract had the highest percentage (2.36%), while hexane crude extract had the lowest percentage yield (0.36%). Among the pure compounds betulin (EL2) had the highest percentage yield (3.66%) while Lupeol (EL1) with the lowest (0.50%) (Table 1).

Table 1. Percentage yields of crude extracts and pure compounds

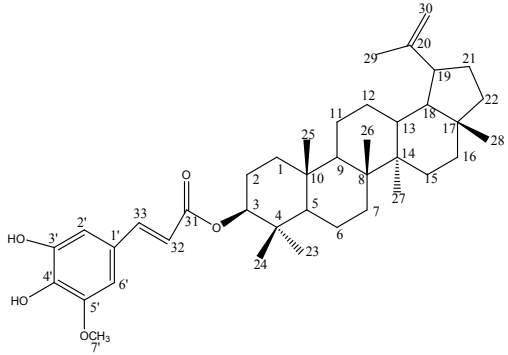
Species name	Weight of plant material (g)	Extraction solvent	Crude extract		Pure compounds		
E. latideus	1000		Weight (g)	Yield % w/w	Name	Weight (mg)	Yield I% w/w
		Hex	3.60	0.36			
		DCM	10.22	1.02	3β-(5-hydroxyferulo-yl)lup-20(30)-ene (**EL3**)	172.30	2.38
		EtOAc	8.21	0.82	Lupeol (**EL1**)	26.00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



Lupeol (**EL1**)



Betulin (**EL2**)



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

Fig. 2. Structures of compounds isolated from *E. latideus*

3.2 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 3β-(5-hydroxyferuloyl)lup-20(30)-ene (EL3) (Fig. 2).

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

Compound (EL1): Lupeol: 172.3 g, white powder. ^{1}H NMR (500 MHz, $CDCl_3$). 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, H-23), 0.78 (3H, s, H-24), 0.85 (3H, s, H-25), 1.05 (3H,s, H-26), 0.97 (3H,s, H-27), 0.81 (3H, s, H-28), 1.71 (3H, s, H-30), 4.59 (1H, br, s, H_α-29), 4.72 (1H, br, s, H_β-29). ^{13}C NMR (125 MHz, $CDCl_3$). 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. ^{1}H NMR (500 MHz, $CDCl_3$). 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, H-23), 0.75 (3H, s, H-24), 0.83 (3H, s, H-25), 1.02 (3H, s, H-26), 0.95 (3H, s, H-27), 1.68 (3H, s, H-30), 3.26 (1H, d, *J* = 10.80 Hz, H_α-28), 3.73 (1H, d, *J* = 10.80 Hz, H_β-28), 4.52 (1H, br, s, H_α-29), 4.62 (1H, br, s, H_β-29).

^{13}C NMR (125 MHz, $CDCl_3$). 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): 3β-(5-hydroxyferuloyl)lup-20(30)-ene: 189.6 g, black crystalline solid. ^{1}H NMR (500 MHz, $CDCl_3$). 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, H-23), 0.92 (3H, s, H-24), 0.88 (3H, s, H-25), 1.04 (3H, s, H-26), 0.95 (3H, s, H-27), 0.79 (3H, s, H-28), 1.69 (3H, s, H-29), 3.91 (3H, s, H-7′), 4.57 (1H, d, *J* = 2.47 Hz, H_α-30), 4.69 (1H, d, *J* = 3.47 Hz, H_β-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′).

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

Compound (EL1): The ^{1}H NMR spectrum (Appendix K1) showed the presence of seven tertiary methyl protons at δ_H 0.78 (H-24), 0.81 (H-28), 0.85 (H-25), 0.97 (H-27), 0.99 (H-23), 1.05 (H-26), 1.71 (H-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 geminal exomethylene protons of a terminal bond. The HSQC spectrum (Appendix K3) 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-3α) attached to a secondary carbon atom that is bonded to an equatorial hydroxyl group (3β-OH), [21]. 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 (Appendix K2) 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 carbon atoms were assigned. 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 (Appendix K4 & 5). 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_C19.3, 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-3β-ol (Fig. 2). This was after comparison of its ^1H and ^{13}C NMR spectral data with that from reported literature [22, 23, 24]. 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.

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

The observation of a signal at 1.68 indicated one methyl group attached to a double bond. In addition it showed a ^1H doublet of doublet (1H, dd, *J* = 4.81, 11.43 Hz, H-3) due to a hydroxymethine proton at δ_H 3.18. Its chemical shifts and coupling constant confirmed its assignment to the hydroxymethine at C-3, with the proton having an axial (α) orientation when an equatorial (β) hydroxyl is present [23]. Other doublets of two coupled protons had resonances at δ_H 3.79 and 3.32 (1H, d, *J* = 10.80 Hz), showing the presence of two an equivalent protons of a hydroxymethylene group (-CH_2OH, C-28). This was confirmed by proton resonances in the HSQC spectrum (Appendix K6) of the methylene carbon that had a signal at δ_C 60.7. The protons signals at δ_H 4.62 and 4.52 (1H, br, s, H-29) indicated the presence of two nonequivalent geminal exomethylene protons of terminal methylene group. This corresponded well with ^{13}C NMR signal at 109.9 in the HSQC spectrum. There were other major signals of doublet of doublet of doublets at δ_H 2.38 (1H, ddd, *J* = 5.84, 10.96 Hz, H-19) and a doublet signal at δ_H 0.68 (1H, d, *J* = 10.64 Hz, H-5). The remaining protons were assigned as multiplets (Table 3).

The ^{13}C NMR spectrum showed thirty carbon signals that were clarified by DEPT experiments that indicated that compound (EL2) was a triterpenoid. It showed the presence of six methyl groups at δ_C 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 presence of twelve methylene, six methine and six quaternary carbon atoms that were confirmed by their 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 (C-17) in the molecule. The quaternary carbon δ_C 150.7 (C-20) and one methylene carbon δ_C 109.9 (C-29) confirmed the presence of a terminal double bond.

Complete assignment of protons of the proposed structure was established using 2-D NMR experiments that included COSY, HMBC and ROESY spectra (Appendix K7 & 8). In the COSY cross peak correlations existed between olefinic protons δ_H 4.52 (H_α-29) and δ_H 4.62 (H_β-29) with the methyl proton signal δ_H 1.68 (H-30). Cross

Table 2. ^{1}H NMR and ^{13}C NMR spectroscopic data for Lupeol (EL1) compared against literature values. Literature [23] [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz $CDCl_3$, *J* in Hz]

Position of carbon	^{13}C NMR (125 MHz)	^{13}C NMR literature	^{1}H NMR (500 MHz)	^{1}H NMR literature
1α	38.7 (CH_2)	38.6	0.89 m	-
1β			1.68 m	-
2α	27.5 (CH_2)	27.7	1.68 m	-
2β			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)
6α	18.3 (CH_2)	18.7	1.38 m	-
6β			1.50m	-
7α	34.3 (CH_2)	34.6	1.30 m	-
7β			1.41 m	-
8	40.9 (C)	40.8	-	-
9	50.5 (CH)	50.8	1.27 m	-
10	37.2 (C)	37.7	-	-
11α	20.9 (CH_2)	21.4	1.28 m	-
11β			1.43 m	-
12α	25.2 (CH_2)	25.5	1.10 m	-
12β			1.67 m	-
13	38.0 (CH)	38.7	1.68 m	-
14	42.9 (C)	43.3	-	-
15α	27.4 (CH_2)	27.7	1.05 m	-
15β			1.62 m	-
16α	35.6 (CH_2)	35.9	1.48 m	-
16β			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	-	-
21α	29.9 (CH_2)	29.4	1.34 m	-
21β			1.94 m	-
22α	40.0 (CH_2)	39.8	1.22 m	-
22β			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)
29α	109.3 (CH_2)	108.8	4.59 (1H, br, s,)	4.58 (1H, s)
29β			4.72 (1H, br, s)	4.68 (1H, s)
30	19.3 (CH_3)	19.5	1.71 (3H, s)	1.75 (3H, s)

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 δC 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$_\alpha$-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 (Hα-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 ^{1}H NMR and ^{13}C NMR spectral data with that from literature the structure of compound was proposed to be a pentacyclic triterpene, betulin (Fig. 2) also known as lup-20(29)-ene-3β,28-diol [25, 26]. 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*.

Compound (EL3): The ^{1}H 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, H-27), 1.04 (3H, s, H-26), 0.88 (3H, s, H-25), 0.92 (3H, s, H-24), 0.79 (3H, s, H-28), 0.89 (3H, s, H-23). There is one isopropylidene methyl group ($MeC=CH_2$) present at δ_H 1.69 (3H, s, C-29) and a methyl methoxyl (-OCH_3) singlet at 3.91 (3H, s, H-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, H-30) and 4.57 (1H, d, *J* = 2.47 Hz, H-30). A proton signal was observed at δ_H 4.59 (1H, m, H-3) due to a deshielded methine proton on a carbon that has an oxygen atom attached to an ester. The HSQC spectrum (Appendix K9)

Established its placement at δ_C 80.9. Two doublets of 5-hydroxy ferulic acid signals due to coupled trans-substituted protons on the ArCH=CH- were observed at δ_H 7.53 (1H, d, *J* = 15.86 Hz, H-32$_\alpha$,) and 6.27 (1H, d, *J* = 15.86 Hz, H-33$_\beta$). Two doublet signals due to two aromatic methine protons appeared at δ_H 6.82 (1H, d, *J* = 1.56 Hz, H-2') and 6.65 (1H, *J* = 1.74 Hz, H-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, H-19). The above ^{1}H 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 9 (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′) and 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 ^{1}H and ^{13}C NMR, HSQC assignments were confirmed with COSY, HMBC and NOESY experiments (Appendix K10 & 11). 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$_\beta$ (H-30) showed cross peak correlation with the methyl protons δ_H 1.69 (H-29). The methane 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 1.40α and 2.05$_\beta$, showed cross peak correlation with a methine proton

Table 3. ^{1}H and ^{13}C NMR spectroscopic data for betulin (EL2) in $CDCl_3$ compared against literature values. Literature [26] [^{1}H 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	^{1}H NMR (500 MHz)	^{1}H NMR literature
1α	38.7 (CH_2)	38.7	0.9 m	-
1β			1.62 m	1.63 m
2α	27.0 (CH_2)	27.1	1.53 m	-
2β			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)
6α	18.3 (CH_2)	18.3	1.32 m	1.40 m
6β			1.53 m	-
7α	34.2 (CH_2)	34.3	1.32 m	-
7β			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	-	-
11α	20.8 (CH_2)	20.1	1.17 m	1.15 m
11β			1.38 m	-
12α	25.2 (CH_2)	25.2	1.64 m	1.51 m
12β			1.71 m	-
13	37.3 (CH)	37.3	1.61 m	1.51m
14	42.9 (C)	42.7	-	-
15α	27.4 (CH_2)	27.4	0.95 m	-
15β			1.54 m	1.56 m
16α	29.2 (CH_2)	29.2	1.19 m	-
16β			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	-	-
21α	29.7 (CH_2)	29.8	1.32 m	1.35 m
21β			1.98 m	-
22α	34.0 (CH_2)	34.0	1.33 m	-
22β			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)
28α	60.7 (CH_2)	60.6	3.26 (1H, d, *J* = 10.80 Hz)	3.31 (1H, d. *J* = 12.0 Hz)
28β			3.73 (1H, d, *J* = 10.80 Hz)	3.78 (1H, d, *J* = 12.0 Hz)
29α	109.9 (CH_2)	110.0	4.52 (1H, br, s)	4.56 (1H, s)
29β			4.62 (1H, br, s)	4.66 (1H, s)
30	19.1 (CH_3)	19.1	1.68 (3H, s)	1.66 (3H, s)

δ_H 1.26 and the methylene protons δ_H 1.69_{β} and 1.07_{α} (H-12). In the HMBC spectrum the olefinic methine proton δ_H 7.53 (H-32α) 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_{α}, 4.69_{β}, H-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-32α) with protons δ_H 6.82 (H-2′), 6.65 (H-6′) and 6.27 (H-33_β).

The olefinic proton δ_H 6.27 (H-33_β) was observed to correlate with protons δ_H 7.53 (H-32α), 6.82 (H-2′) and 6.65 (H-6′). The olefinic protons δ_H 4.57_α and 4.69_β (H-30) showed cross peaks with protons δ_H 2.37 (H-19), 1.37 (H-18) and 1.69 (H-29). The methyl protons δ_H 1.69 (H-29) correlated with olefinic protons δ_H (4.57_α, 4.69_β, H-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 3β-(-5-hydroxyferuloyl) lupen-20(30)-ene [27,28] (Fig. 2). The isolation of this compound from *E. latideus* is being reported for the first time.

3.3 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: (IC_{50}) 3D7: 38.21 ± 0.49 µg/mL (hexane extract); (28.07 ± 1.65 µg/mL) (dichloromethane extract) and (IC_{50}) Dd2: 38.93 ± 0.54 µg/mL (hexane) all displayed moderate activity. Promising activity was shown by crude extracts (IC_{50}) 3D7: (12.86 ± 1.86 µg/mL) (ethyl acetate extract) and Dd2: (13.97 ± 2.59 µg/mL) (methanol extract). High activity from this plant was displayed by the crude extracts: (IC_{50}) Dd2: 2.78 ± 0.02 µg/mL (dichloromethane extract); (4.37 ± 0.99 µg/mL) (ethyl acetate extract) and 3D7: (9.75 ± 1.47 µg/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. Antiplasmodial activities of crude extracts (Hex, DCM, EtOAc, MeOH) (2.78-38.93) µg/mL and pure compounds (1.64-25.14) µg/mL showed significant difference with the reference standards (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 *Plasmodium* compounds compared to the two standard controls (CQ and MQ) (Table 5).

E. latideus root crude extracts of hexane, dichloromethane, ethyl acetate and methanol had good activity (IC_{50}) 3D7: (9.75-38.21) µg/mL and Dd2: (2.78–38.93) µg/mL. The activities of the four extracts were classified as highly active and having moderate activity. Some antiplasmodial 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* reported antiplasmodial activity of 134 species [29]. 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 µg/mL while the actives of the DCM/MeOH extracts had IC_{50} between 4.6-11.0 µg/mL. Another investigation was carried out on *Euclea divinorum* [30]. In this investigation the crude extracts were screened against chloroquine (CQ susceptible and resistant strains of *P. falciparum* (D6 and W2, respectively). The CH_2C1_2 and EtOAc crude extracts obtained from the leaves showed high *in vitro* antiplasmodial activity in the range of IC_{50} = 6.12 ± 0.45 - 17.29 ± 1.44 µg/mL and 8.42 ± 1.06 - 12.09 ± 0.67 µg/mL against CQ-susceptible strain, respectively.

The activities of these previous investigations are within the range 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 against Dd2 strain respectively, which were slightly high. 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*, demonstrated antiplasmodial activity.

Table 4. ^{1}H and ^{13}C NMR spectroscopic data for 3β-(-5-hydroxyferuloyl) lupen-20(30)-ene (EL3) in $CDCl_3$ compared against literature values. Literature [28] [^{1}H NMR 400 MHz, ^{13}C NMR 100 MHz, $CDCl_3$, *J* in Hz.

Position of Carbon	^{13}C NMR (125 MHz)	^{13}C NMR literature	^{1}H NMR (500 MHz)	^{1}H NMR literature
1α	38.4 (CH_2)	38.4	1.02 m	-
1β			1.69 m	-
2α	23.8 CH_2)	23.8	1.69 m	-
2β			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	-
6α	18.2 (CH_2)	18.2	1.4 m	-
6β			1.5 m	-
7α	34.2 (CH_2)	34.2	1.02 m	-
7β			1.38 m	-
8	40.9 (C)	40.8	-	-
9	50.4 (CH)	50.3	1.26 m	-
10	37.1 (C)	37.1	-	-
11α	21.0 (CH_2)	20.9	1.21 m	-
11β			1.40 m	-
12α	25.1 (CH_2)	25.1	1.07 m	-
12β			1.69 m	-
13	38.1 (CH)	38.0	1.69 m	-
14	42.9 (C)	42.8	-	-
15α	27.4 (CH_2)	27.4	1.01 m	-
15β			1.69 m	-
16α	35.6 (CH_2)	35.6	1.37 m	-
16β			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	-	-
21α	29.8 (CH_2)	29.8	1.26 m	-
21β			1.91 m	-
22α	40.0 (CH_2)	40.0	1.20 m	-
22β			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)
30α	109.4 (CH_2)	109.3	4.57 (1H, d, *J* = 2.47 Hz)	4,58 (1H, br d, *J* = 2.4 Hz)
30β			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)

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 3β-(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 demonstrated moderate activity, IC_{50} 3D7; 23.91 ± 0.05 µg/mL and Dd2: 25.14 ± 0.01 µg/mL. This activity was low as compared to that of its ethyl acetate crude extract from which it was isolated, that was highly active on both strains of parasite (IC_{50} 3D7: 12.86 ± 1.86 µg/mL, Dd2: 4.37 ± 0.99 µg/mL). The crude also had a resistance index of 0.34 which low as compared to that of the pure compound that had 1.05. 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 isolated compound was also reported in previous studies [31]. In their study the isolated four diterpenes had (IC_{50}) D6: 14.3-24.4 µg/mL, W2: 17.5-18.4 µg/mL were less active compared to the chloroform crude extract that displayed (IC_{50}) D6: 7.89 µg/mL, W2: 8.74 µg/mL. Antiplasmodial activity screening of Lupeol is documented in previous investigations. Some of the studies were investigated, where on 3D7 and Dd2 parasitic strains [32]. Their findings 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* gave high activity of IC_{50} 5.0 µg/mL on *P. falciparum* K1 strain of parasites [33]. Also antiplasmodial screening of Lupeol isolated from the DCM/MeOH crude extract of the bark of *Albizia* zygia was carried out on *P. falciparum* K1 strain that gave IC_{50} of > 0.078 µg/mL [34]. Another report was given on the *in vitro* inhibitory activity of Lupeol against CQ-sensitive 3D7strain of *P. falciparum* [35]. Their study gave an IC_{50} value of 11.8 mg/mL which was shown to cause a human erythrocyte shape towards stomatocytes. From these findings there was difference in the antiplasmodial activity of Lupeol isolated from *E. latideus* and that from other plant species reported from previous investigations. However all plant species used demonstrated activity v that ranged from highly active to moderate activity.

The second compound betulin isolated from *E. latideus* displayed good antiplasmodial activity of (IC_{50}) 3D7: 1.64 ± 0.02 µg/mL, Dd2: 7.69 ± 1.21 µg/mL. It exhibited high activity compared to the ethyl acetate crude extract from which it was isolated. It also had a resistance index of 4.69 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 which the activity of the pure compounds was higher than the crude extracts conducted in an investigation [36]. 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 µg/mL and 3.6-4.9 µg/mL against CQ D6 and CQ W2 strains respectively. The ethyl acetate had 12.7-5.7 µg/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 against the *P. falciparum* CQ-susceptible strain (D10) [37]. The study was investigated on betulin isolated from the dichloromethane leaf crude extract of *Scheffleria umbellifera* which gave good activity of IC_{50} of 3.2 µg/mL. Betulin isolated from several plant families such as Rhamnaceae (*Ziziphus vulgaris*) and Labiatae (*Zataria multiflora*) showed moderate activity (IC_{50} < 12 µg/mL and < 27 µM, respectively) [38]. Another investigation was conducted on betulin isolated from *Ampelozizyphus amazonicus* [32]. Their findings gave IC_{50} values of 17.08 and 14.22 µM against 3D7 CQ sensitive and Dd2 CQ resistant parasites respectively. Another evaluation was also conducted on betulin isolated from *Uapaca nitida* which was found to be inactive with IC_{50} of 500 µg/mL [39, 40]. However a report on betulin isolated from *Croton argrophylloides* inhibited *P. falciparum* strains with an IC_{50} of 12 µg/mL [41]. The activities of betulin isolated from *E. latideus* was in agreement with the values documented in literature form the previous investigations.

3.4 Acute Toxicity of Crude Extracts

The present work on *in vivo* acute toxicity was approved by the Research Ethical Committee for

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)	
		3D7 strain (CQ Sensitive)	Dd2 strain (CQ Resistant)
E. latideus	Hex	38.21 ± 0.49 [a]	38.93± 0.54 (1.02)[b]
	DCM	28.07 ± 1.65[a]	2.78 ± 0.02 (0.10)[b]
	EtOAc	12.86 ± 1.86[a]	4.37 ± 0.97 (0.34)[b]
	MeOH	9.75 ± 1.47$_a$	13.97 ± 2.59 (1.43)[b]
Pure compounds	Lupeol (**EL1**)	23.91 ± 0.05 [56.13]*[a]	25.14 ± 0.01 [59.01]* (1.05)[b]
	Betulin (**EL2**)	1.64 ± 0.02 [3.71]*[a]	7.69 ± 1.21 [17.40]* (4.69)[b]
	3β-(5-hydroxyferuloyl)lup-20(30)-ene (**EL3**)	NT	NT
Reference	Chloroquine diphosphate (CQ)	0.0093 ± 0.0099[a]	0.0440 ± 0.0102 (4.73)[b]
Standards	Mefloquine hydrochloride (MQ)	0.0056 ± 0.0011[a]	0.0161 ± 0.0132 (2.88)[b]

*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 of IC_{50} CQ-resistant strain (Dd2 /IC_{50} CQ sensitive strain 3D7). NT – Not tested*

using animals at Makerere University, Department of Pharmacology (No.1250). The values for lethal dose (LD_{50}) determined for the crude extracts of *E. latideus* were > 5000 mg/kg. Based on this the plant was considered to be practically non-toxic. These extracts included that of hexane, dichloromethane, ethyl acetate and methanol. 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 were seen for the methanol crude extract at doses 500-2000 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 [8] 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 LD_{50} > 5000 mg/kg and therefore considered non-toxic.

4. CONCLUSION

The analyses revealed for the first time that compounds present in the crude extracts of *E. latideus* have good antiplasmodial activity**.** Besides, the resistance indices of the crude extracts were low showing that they were equally active against the CQ-resistant Dd2 parasite strain. This shows that the plant has promising antiplasmodial activity against CQ-resistant Dd2 strain of *P. falciparum*. The plant crude extracts were found to be practically non-toxic, which shows that the exhibited antiplasmodial activity of the crude extracts is not due to their toxicity, and they have a large safety margin.

Therefore, the medicinal plant can be considered to be safe for consumption in its crude form after clinical trials to offer prophylactic cure. 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

Authors have declared that no competing interests exist.

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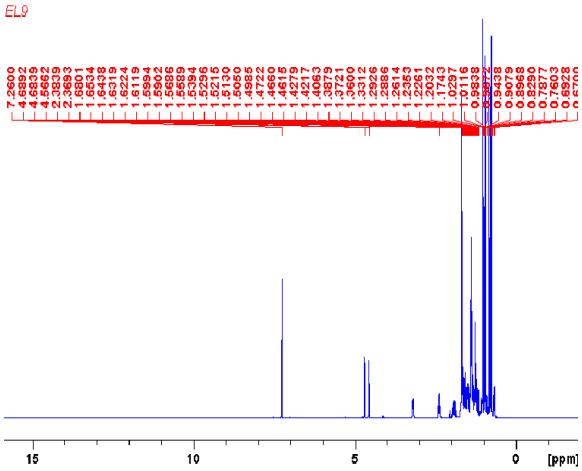
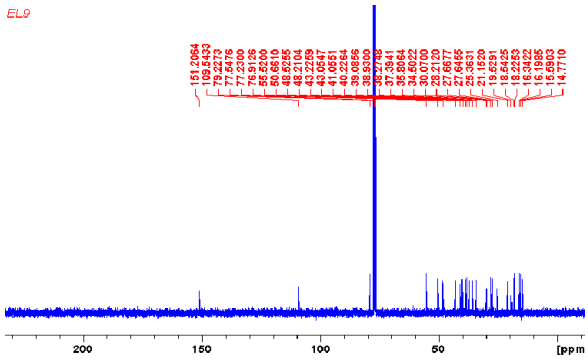
APPENDICES

K1



^{1}H NMR spectrum for compound EL1 in $CDCl_3$

K 2



^{13}C NMR spectrum for compound EL1 in $CDCl_3$

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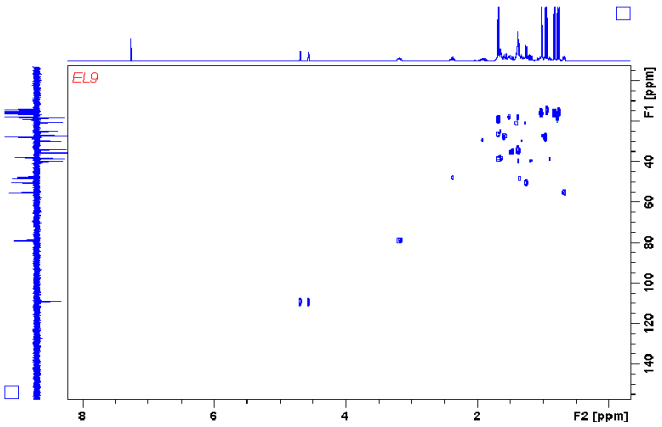
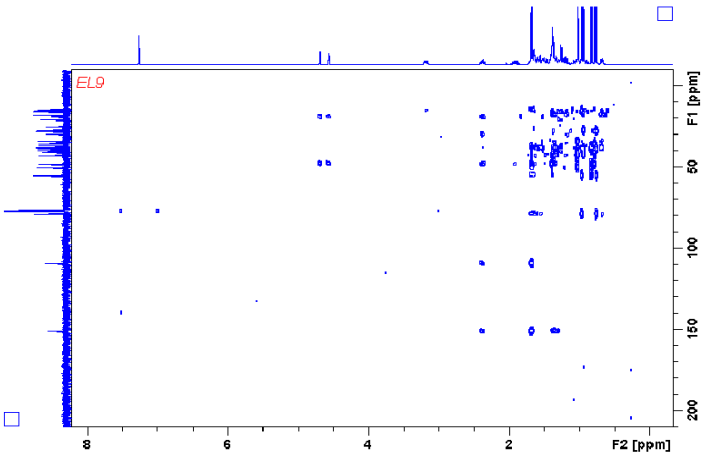


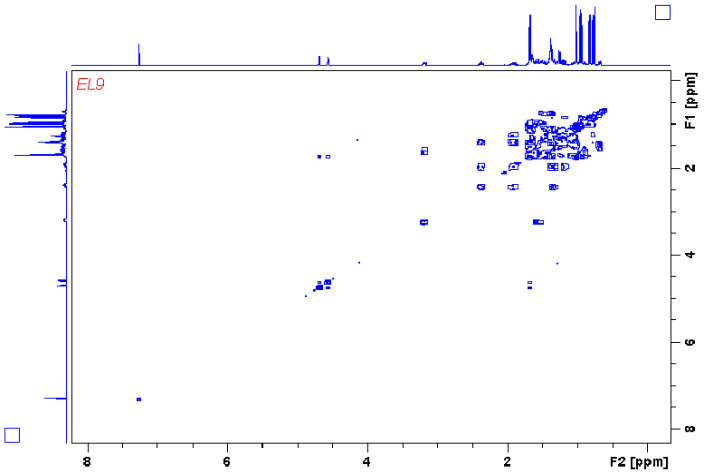
HSQC-DEPT spectrum for compound EL1 in $CDCl_3$

K4



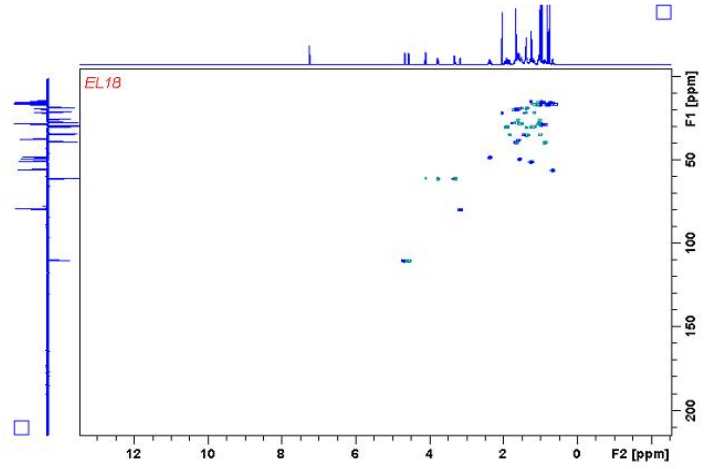
HMBC spectrum of compound EL1 in $CDCl_3$

K5



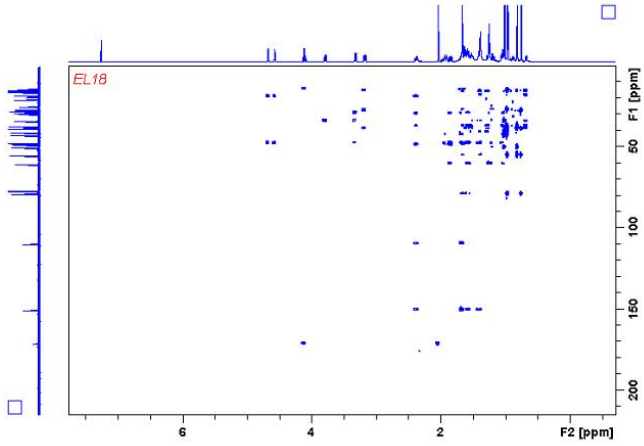
COSY spectrum for EL1 in $CDCl_3$

K6



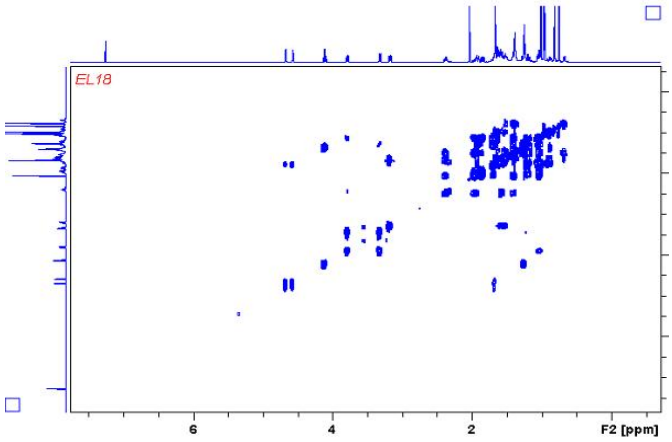
HSQC-DEPT spectrum for compound EL2 in $CDCl_3$

K7



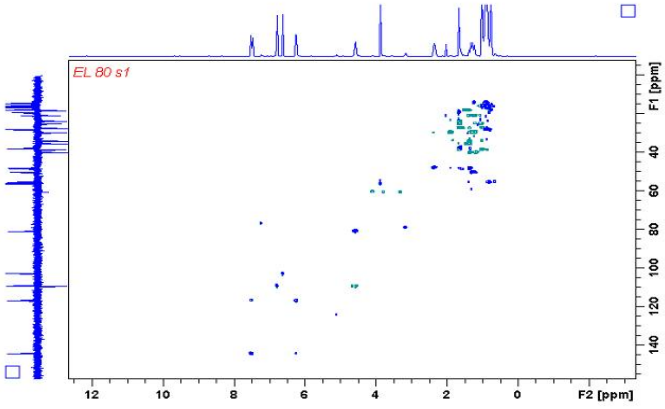
HMBC spectrum for compound EL2 in $CDCl_3$

K8

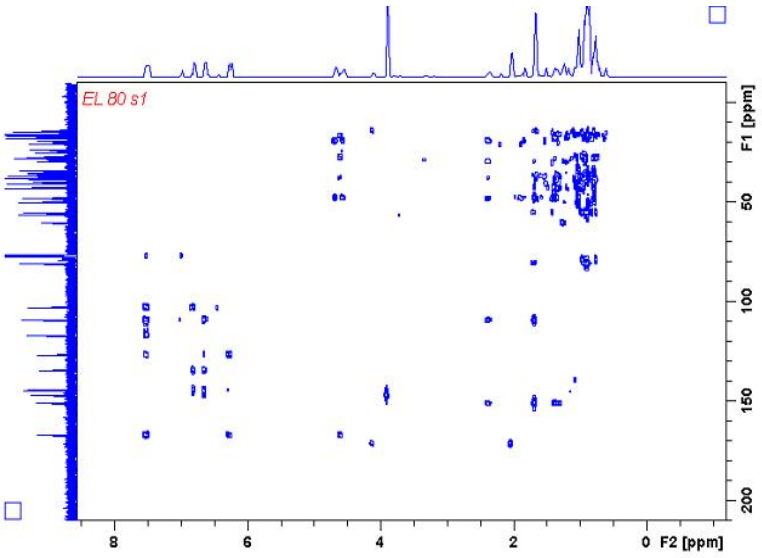


COSY spectrum for compound EL2 in $CDCl_3$

K9

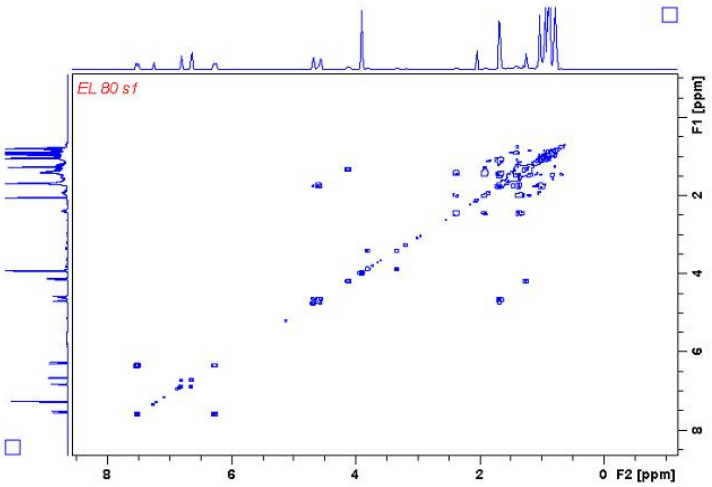


HSQC-DEPT spectrum for compound EL3 in $CDCl_3$
K10



HMBC spectrum for compound EL3 in $CDCl_3$

K11



COSY spectrum for compound EL3 in $CDCl_3$

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