BIOTEKNOLOGI
Volume 17, Number 1, May 2020
Pages: 1-13

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c170101

Antimicrobial and phytochemical screening of *Lannea schweinfurthii* (Engl.) Engl.

KIHAGI REGINA WAMUYU, ALEX K. MACHOCHO♥, ALPHONSE W. WAFULA
Department of Chemistry, Kenyatta University. P.O Box 43844-(00100 GPO), Nairobi, Kenya. Tel.: +254-20-8710901 ext. 57455, ♥email: machochoalex@hotmail.com, machocho.alex@ku.ac.ke

Manuscript received: 19 December 2019. Revision accepted: 4 April 2020.

Abstract. *Wamuyu KR, Machocho AK, Wafula AW. 2020. Antimicrobial and phytochemical screening of* Lannea schweinfurthii *(Engl.) Engl. Bioteknologi 17: 1-13.* Phytochemicals are currently receiving more attention because of their effectiveness in the treatment of infectious diseases as well as mitigating various side effects caused by conventional antimicrobials. Pharmacological studies of *Lannea schweinfurthii* (Engl.) Engl. Has reported an antimicrobial property of the plant, but the active ingredients in the plant are largely understudied. Here, we collected the stem bark of *L. schweinfurthii* (Engl.) Engl., then ground it into a fine powder. Sequential extraction was conducted with hexane, dichloromethane (DCM), ethyl acetate (EtOAc), and methanol (MeOH). The crude extracts were examined for bioassay screening for their antibacterial activity against Gram-positive *Staphylococcus aureus* and *Bacillus subtillis* and Gram-negative *Pseudomonas aeruginosa* and *Escherichia coli* and antifungal activities against *Candida albicans*. The EtOAc and MeOH crude extracts showed moderate and high activities, ranging from 11 to 21 mm inhibition zones against the microbes used except *P. aeruginosa*. Hexane and DCM crude extracts showed a mild activity of 7 mm. Tetracycline and nystatin were applied as positive controls for bacteria and fungi, respectively, and exhibited inhibition zones of 18 mm. The GC-MS analysis was carried out on the crude DCM, and MeOH extracts to give a preliminary hint of the class of compounds. The spectral data obtained from the crude extracts of DCM and MeOH indicated the presence of phenolic compounds, fatty acids; and their derivatives, polyketide derivatives, terpenoids, and steroids in the plant. Purification of crude extracts was performed using solvent partition and chromatography: CC, VLC, and PTLC. Structural elucidation and characterization were done using standard spectroscopic methods. The combined hexane/DCM extract produced two compounds, namely 3-(10'-tridecenyl) phenol (LS01) and di-(2'-ethylhexyl) ester phthalic acid (LS05). The extract of ethyl acetate yields three compounds, namely lupeol (LS02), β-sitosterol (LS03), and epicatechin (LS04). Bioassay of the isolated compounds showed that epicatechin had high activity against the *S. aureus* and *B. subtillis* and Gram-negative *E. coli*. Furthermore, epicatechin showed an activity of 14 mm against *C. albicans*. Here, we showed that the stem bark of *L. schweinfurthii* has bioactive chemical constituents. Further studies, such as cytotoxicity tests, should be done both on the crude extracts and isolated compounds to ascertain reported activity to use bioactive components either as antimicrobials or as templates in drug synthesis. On the other hand, measures should be put in place to conserve the plant species from extinction.

Keywords: Antibacterial, antifungal, *Lannea schweinfurthii*

INTRODUCTION

Traditional societies in Africa have formulated methods of providing every person in the society with essential healthcare; via acceptable and accessible means by the utilization of indigenous resources such as plants, animals, and minerals (Chhabra et al. 1987). Moreover, herbal medicine considers every country's socio-cultural background. Of late, despite emphasis being put into the research of synthetic drugs, a particular interest in medicinal plants has been reborn. The growing interest in medicinal plants is partly because many synthetic drugs are potentially toxic and related to many side effects on the host. Besides, the effectiveness of many herbal medicines is now an accepted fact (Thomson, 1978; Geddes, 1985). Further, herbal preparations constitute valuable natural resources from which chemicals of great potential interest for medicine, agriculture, industry, and other areas can be characterized and isolated. Useful drugs such as atropine (1), reserpine (2), quinine (3), and morphine (4) were discovered from traditional herbal remedies (Sneader 1985).

It is predicted that about 25% of the drugs prescribed worldwide are derived from plants, and 121 such active compounds are currently in use (Sahoo et al. 2010). Between 2005 and 2007, 13 drugs originated from natural products that were approved in the United States. Over 100 natural product-based drugs are in clinical studies (Li and Vederas, 2009). Among 252 drugs in the World Health Organization's (WHO) essential medicine list, 11% are exclusively of plant origin (Sahoo et al. 2010).

This fact has necessitated microbiologists all over the world to search for the formulation of novel antimicrobial agents and investigate of the efficacy of natural plant products as replacements for chemical antimicrobial agents (Pandian et al. 2006). Many microorganisms and plants produce secondary metabolites, compounds that are not related to the primary metabolism of the producing organism, as their defense mechanism. Many of these products play essential roles as therapeutics and stimulants feed additives, among others (Hans 1993). Medicinal plants are widely known natural sources for the treatment of various diseases since antiquity. The World Health

Organization (WHO) reports about 20,000 plant species are used for medicinal purposes (Gullece et al. 2006; Maregesi et al. 2008).

Natural products, in the form of compounds or as standardized plant extracts, provide unlimited opportunities for the discovery of new drugs because of the unmatched availability of chemical diversity (Cos et al. 2006). The vast range of antimicrobial agents from lower organisms and synthetic drugs sufficed in the control or treatment of infectious diseases. However, there is a problem of microbial drug resistance and an increase of opportunistic infections, particularly with acquired immune deficiency syndrome (AIDS) patients and individuals undergoing immunosuppressive chemotherapy. Many antifungal and antiviral drugs are of limited use because of their toxicity, while other viral diseases have not yet found a cure. These problems pose a need for searching for more new drug substances.

The objectives of the study were: (i) To investigate antibacterial and antifungal activity of stem bark crude extracts from *L. schweinfurthii* against selected strains of bacteria and fungi. (ii) To elucidate the chemical structures of compounds isolated from *L. schweinfurthii* using spectroscopic methods. (iii)To evaluate the antibacterial and antifungal activity of the purified compounds by *in vitro* assays.

MATERIALS AND METHODS

Laboratory equipment and instruments used

The plant materials were made into powder samples using a motor grinding laboratory mill (Christy and Norris Ltd., Chelmsford, England) then weighed. The uncorrected melting points of the pure compounds were calculated on a Gallen Kamp melting point apparatus. A ultraviolet lamp, ENF-240 C/F (Supertonics Corporation Westbury, UK) of long and short wavelength (365 nm and 254 nm, respectively) was utilized for visualizing the spots on a developed thin layer chromatography (TLC) plate.

Chromatographic materials and solvents

Analytical TLC pre-coated plastic sheets (polygram Sil G/UV 254) and aluminum sheets (Alugram Sil G/UV254) of 20 by 20 cm (Matcher-Nagel Gmbh and Co. Frankfurt, Germany) were utilized for the establishment of optimum solvent systems for separations, the complexity of the extracts and the purity of isolated compounds. Vacuum liquid chromatography (VLC) was done out using slurry packing with Kiesegel silica gel 60 G (0.04-0.6 mm Merck, Germany). The column chromatography was done using glass columns of internal diameter 3.0 cm and length 0.8 m. They were packaged with Kiesegel silica (Merck, 70-230 mesh/ 0.63-0.2 mm) using the slurry method. Further purification was conducted using Sephadex LH 20, as sieve material packed using the slurry method.

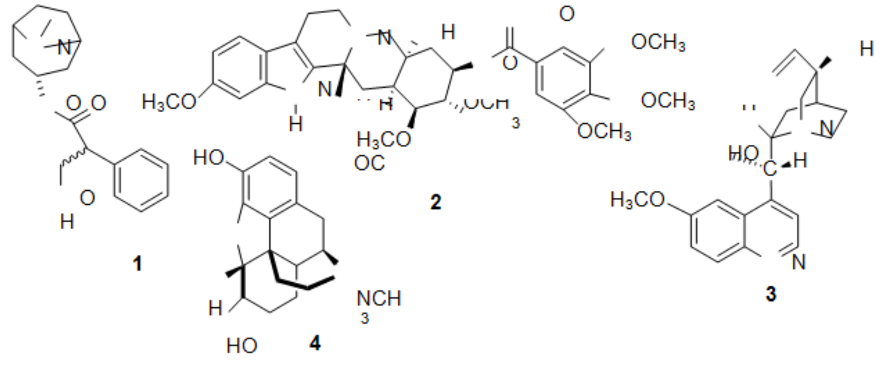
The organic solvents used here included hexane, dichloromethane (DCM), chloroform ($CHCl_3$), ethyl acetate (EtOAc), acetone (MeCOMe) and methanol (MeOH); these were of general-purpose grade (Kobian Kenya Ltd, Nairobi) and as a result, they were freshly distilled before use. Dimethylsulphoxide (DMSO) used was of analaR-grade (Kobian Kenya Ltd, Nairobi).

Spray reagents

The spray reagents for detection and visualization of the isolated compounds on the TLCs included: (i) Anisaldehyde-sulfuric acid prepared by mixing of *p*-anisaldehyde (0.5 mL), acetic acid (10 mL), MeOH (85 mL) and concentrated sulfuric acid (5 mL) (Krishnaswamy 2003); (ii) Sulfuric acid/MeOH mixture prepared by combining 5 mL of concentrated sulfuric acid and 95 mL of distilled MeOH (Krishnaswamy 2003); (iii) Ammonia vapor for flavonoids (Krishnaswamy 2003).

Detection of compounds

Detection of compounds was done by visualizing the plates under UV light at 254 nm and 365 nm for active compounds. This step was followed by developing in ammonia vapor (for flavonoids) or by spraying with detecting agents such as *p*-anisaldehyde or a 5% solution of sulphuric acid in methanol. Terpenoids reflected blue fluorescent in UV 365 nm and changed into purple when plates were sprayed with anisaldehyde and then heated at 110°C for 10 minutes. Nevertheless, most terpenoids did not fluoresce in UV. Flavonoids were observed using UV light at both 254 nm and 365 nm. Their spots changed to yellow or orange when sprayed with *p*-anisaldehyde and ammonia, and then heated at 110 °C for 10 minutes.



Nuclear magnetic resonance (NMR) spectroscopy

The NMR spectra were collected from Varian Gemini 75 and 400 MHz machines. ^1H NMR spectra were run in deuterated chloroform ($CDCl_3$), dimethylsulphoxide (DMSO), or deuterated methanol (CD3OD) depending on the solubility of the isolated compounds. Proton nuclear magnetic resonance (^1H NMR) spectra were documented in deuterated solvents with tetramethylsilane (TMS) as the internal standard. Chemical shifts were calculated in parts per million (ppm) relative to TMS. The peak multiplicities were shown by the symbol s (singlet), d (doublet), t (triplet), q (quartet), dd (double of doublet), bd (broad doublet) and m (multiplet). Coupling constants were measured in hertz (Hz). Carbon-13 nuclear magnetic resonance (^{13}C NMR) spectra were measured at between 75 and 100 MHz while ^1H NMR spectra were measured on the same instrument at 200 and 400 MHz, respectively. Structures of the compounds isolated were elucidated by interpreting the ^1H and ^{13}C NMR. Complete elucidation was done by using 2D NMR experiments, which included DEPT and COSY together with a comparison with published data.

Plant collection and identification

The samples were collected from Bondo, Siaya County, with the assistance of Dr. Amuka of Maseno University. Their identity was determined and authenticated by Lucas Karimi of the Department of Pharmacy and Complementary/ Alternative Medicine, Kenyatta University. Voucher specimen number: CHEM/RK/LS/ SB1 was stored at Kenyatta University Herbarium. The stem bark was separated then air-dried under the shade for twenty-one days. The dried samples were then ground to a fine powder using the grinding mill (Christy and Norris Ltd., Chelmsford, England). The resulting powdery substance was weighed using a top-loading analytical balance (Denver Instrument, Colorado, USA).

Extraction procedure of *L. schweinfurthii* stem bark

The powdered plant materials were sequentially extracted with solvents of increasing polarity, starting with hexane, dichloromethane, ethyl acetate, and methanol (as per Figure 1). The extraction was done by soaking the finely ground material in the extracting solvent and allowing it to stay for 48 hours in each solvent with occasional swirling. Afterwards, it was filtered under vacuum. The extract was later concentrated using a rotary evaporator at 45°C and low pressure. The extraction was done twice for each of the four solvents and the two portions mixed to form one extract. The combined, concentrated crude extracts were then carefully sealed, labeled, and kept in pre-weighed sample bottles at-20 °C in a deep freezer. The crude extracts were used for bioassay.

Bioassay of the plant extracts

Selected test strains

Antimicrobial assays of the plant species were done in two phases. First a screening of the crude extracts (primary assay) to detect the presence or absence of activity. Four genera of clinical isolates that were used included: *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Escherichia coli*, and *Bacillus subtilis*. *Candida albicans* was selected as a fungal strain. The second step was the examination of the pure compounds isolated from the plant species (secondary assay) to determine their potency toward the four bacterial strains and two fungal strains (*Candida albicans* and *Penicillium notatum*). The pure bacterial and fungal isolates were obtained from the Department of Microbiology of Kenyatta University (Table 1). Two standard antimicrobials, obtained from the Microbiology Department of Kenyatta University, Nairobi, Kenya were used as reference drugs (Table 2).

Antifungal screening tests

The bioassay of both the crude extracts and isolated compounds was carried out using the agar-well plate diffusion technique. *C. albicans* and *P. notatum* that affect human beings were taken from the Department of Microbiology, Kenyatta University (Table 1). Potato dextrose agar (PDA) powder (39 g, Himedia laboratories, Pvt. Ltd., Bombay) was homogenized in distilled water to make a liter of solution. This step was followed by steam sterilization in an autoclave at 121 °C and 15 psi pressure for twenty minutes. On cooling to approximately 50°C, 15 mL portions of this solution were dispensed into sterile Petri dishes under sterile conditions then allowed to solidify. This solid agar provided the medium for growing the fungal spores.

Preparation of the antifungal drugs

One mg of each crude extract was weighed and dissolved in DMSO (50 µL), and the solution meshed up to 1 mL using methanol to give a stock solution of 1,000 ppm for the initial test. Pure cultures of the two fungi of interest were made on the PDA surface in the Petri dishes from the stock cultures and incubated at 30°C for seven days to produce a good crop of spores as mentioned by Clinical and laboratory standards institute (2013).

Table 1. Bacterial and fungal strains used in the bioassay.

Name	ATCC number	Type
Staphylococcus aureus (*Sa*)	25923	Gram positive
Bacillus subtilis (*Bs*)	202638	Gram positive
Escherichia coli (*Ec*)	25922	Gram negative
Pseudomonas aeruginosa (*Pa*)	10622	Gram negative
Candida albicans	10231	Fungi
Penicillium notatum	9478	Fungi

Table 2. Standard antimicrobials used as reference drugs.

Standard drug name	Abbreviations	Weight µg/discs
Tetracycline	TET	25
Nystatin	NY	10

Note: µg/discs-Amount of the drug in the disc

The fungal inoculum was made by harvesting the spores with a bent spore-harvesting needle in a sterile environment and then moved in a sterile tube containing sterile distilled water (Radovanović et al. 2009). The spore suspension was pipetted on to the PDA medium. The plate was then tilted a few times to spread the inoculum before being left still for 10 minutes. Four agar wells were made in the inoculated PDA medium using a sterile cork borer (6 mm). The drug of known concentration (0.1 mL) was pipetted into each of the four wells in triplicates. Finally, the Petri dishes were then covered, sealed, and incubated aerobically at 30 °C for 72-96 hours. The diameter of the incubation zone occurred around the agar wells were measured with a transparent laboratory line ruler for another three consecutive days (Chhabra and Uiso,1991). Sterile distilled water and the solvent mixture in the ratios used to prepare the drugs being tested were employed as a negative control. Nystatin (10 mg) was used as a standard for the comparative purposes for fungi (NCCLS 2003).

Antibacterial screening tests

Table 1 listed all the pathogenic bacterial strains used in this study. *In vitro* antibacterial activity was determined using the agar diffusion method. The test organisms were cultured in nutrient broth medium from stock cultures and later when needed, transferred on the nutrient agar in Petri dishes (NCCLS 2003).

Preparation of media and growth of bacterial cultures

Agar media (28 g, Oxoid Ltd., Basingstoke, England) was prepared as described elsewhere (Chhabra and Uiso 1991). Portions of the sterilized nutrient agar medium were poured into 90 mm diameter pre-sterilized Petri dishes to make a uniform depth of 4 mm under septic conditions and allowed to cool at room temperature. They were then incubated at 37-39°C for 24 hours in an inverted position to test their sterility. Bacteria cultures from stock cultures were inoculated and spread on the nutrient agar surface with three-fold dilutions and incubated aerobically at 37-39°C for 24 hours. The suspension was spread on another freshly prepared agar surface using a sterile cotton swab (Radovanović et al. 2009).

Nutrient broth powder (13 g, Oxford Ltd., Basingstoke, England) was homogenized in distilled water to make 1,000 mL of solution. 25 mL portions of the solution were poured into bijou bottles then autoclaved. On cooling, one loopful of the bacterial strain from the 24-hour culture was mixed to the nutrient broth medium and incubated at 37-39°C for 24 hours in a rotator shaker (NCCLS 2003). The broth bacteria culture (0.1 mL) was transferred into the nutrient agar media in the Petri dishes and spread using a sterilized L-shaped glass rod.

Introduction of the plant extract in the inoculated Petri dishes

The evaluation of antimicrobial activities of the crude extracts was based on the disc diffusion method (NCCLS, 1999). A steady air current was applied to dry plant extracts for 24 hours in a pre-weighed sample bottle. A solution of each extract was made by dissolving 50 mg of the extract in 1 mL of DMSO. 10 µL of the solution was poured onto sterile adsorbent filter paper discs made from Whatman filter No.1 to a concentration of 500 µg/mL per disc.

Other discs were also merged in 5% DMSO and sterile normal saline to serve as a negative control. The impregnated discs were re-sterilized under ultraviolet (UV) light for one hour to expel the solvent. Sterile impregnated discs were then placed on the inoculated plates using sterile forceps. They were then pressed down with slightly to confirm complete contact with the inoculated agar surface. The plates were left to stand for one hour to let diffusion to take place, and then incubated aerobically at 37 °C in an inverted position. The inhibition zones were calculated (in mm) after 24 and 48 hours in triplicates. Tetracycline (25 mg) served as a standard for comparing the plant extracts by examining their activity against the bacterial strains used (Chhabra and Uiso 1991).

Gas chromatography linked with mass spectroscopy

Analysis of crude extracts GC-MS was performed on both the crude methanol and DCM extracts as a preliminary test to give a hint of the class of compounds present in the plant species. The composition of the crude extracts of MeOH and DCM was determined using the GC system (GC-800 series), with a fused capillary column (15 m length, 0.25 mm internal diameter and 0.25 µm film thickness). Static phase methyl silicone (SE-30) was directly coupled to quadruple M/S (Hewlett Packard 5973). Electron Impact Ionization was carried out at an energy of 70 eV. Helium was used as a carrier gas. The injector and detector were maintained at 200 and 250°C, respectively. The analytical conditions were set as follows: oven temperature was isothermal at 60°C for 2 min, and then 60 to 240°C at a rate of 15°C per min held isothermal for 6min. The instrument was scanned at a mass range from 60 to 400 atomic mass units (a.m.u).

Fractionation of the extracts

The step of separation and purification of the crude plant extracts was carried out using both column chromatography and VLC on silica gel. The VLC column was dry-packed with silica gel (Kiesegel 60G, Merck, Germany) and consolidated by administrating hexane to the packing and then sucking using a vacuum to dry to effect proper preparation. The preparation was uniform and created an evenly flat surface. The crude extracts of hexane and dichloromethane showed homogeneity and were constituted into one extract, then mixed with silica gel. The mixture was then applied as a dry powder onto the top of the packed VLC. Separation and elution were achieved using 1000 mL portions of different solvent systems in increasing polarity, starting with 100% hexane. Dichloromethane (DCM) was then added in parts of 1000 mL with a 5% increase in polarity up to 100%, and finally, MeOH was mixed until the addition of 10% MeOH in DCM.

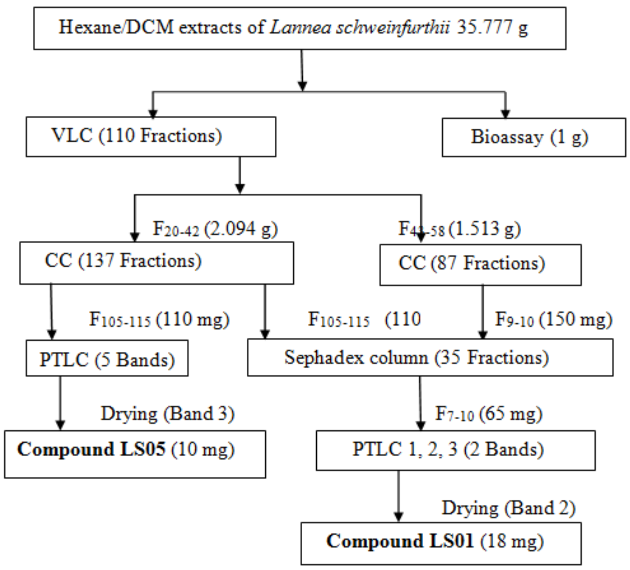


Figure 1. Isolation of compounds LS01 and LS05 from hexane/DCM extract.

The MeOH extract was re-extracted with ethyl acetate, and the extract obtained (20.500 g) combined with EtOAc extract obtained from sequential extraction (65.403 g). The EtOAc extracts (85.903 g) were separated by packing them in column chromatography. The column chromatography was packed with silica gel (Kiesegel silica 240G, Merck, Germany). Elution was conducted in portions of 1000 mL, starting with 100% hexane. The polarity of the eluting solvent mixture was gradually increased by introducing 5% of EtOAc, up to 100%. Finally, polarity was further strengthened by adding MeOH up to a volume of 10% MeOH in EtOAc.

Fifty (50) mL fractions were collected for each solvent system. The portions were later subjected to column chromatography and other chromatographic techniques. Finally, the pure compounds were acquired either through further purification by using a column packed with Sephadex LH 20 (a sieve gel method) or using a series of preparative TLC. The purity was monitored at every step by using TLCs. Glass teat pipettes were used to spot the samples on thin-layer chromatography plates. The loaded plates were developed at room temperature in glass jars by the ascending solvent technique, then visualized for the detection of compounds. Fractions that showed homogeneity were mixed and concentrated at the same time to give pure or partially pure compounds for further purification. Purification and isolation of the five compounds were based on the procedures in Figure 1.

Purification and isolation of compounds

Purification and isolation of compounds from hexane/DCM extract

The hexane and DCM crude extracts were found to have overlapping spots on the TLC plates, indicating that they were homogeneous. They were mixed to form one extract and loaded in a VLC column and eluted with 1000 mL portions of a solvent system whose polarity was increased gradually, starting with 100% hexane. 5% of DCM was introduced gradually until 100% had been combined. Finally, 5% of MeOH was meshed up to 10% MeOH in DCM. A total of 110 fractions of 50 mL each were obtained, and they were plotted on a TLC plate to establish the optimum solvent system for the separation in which a 0.5% MeOH/DCM solvent system was characterized.

Fractions F_{20-42} and F_{43-58} were loaded in two columns separately and eluted with 500 mL portions of a solvent system whose polarity was varied gradually by increasing the more polar solvent by 5%, starting 100% hexane, and then incorporating DCM up to 100% and finally MeOH up to 10% MeOH in DCM. The columns gave 137 and 87 fractions, respectively, out of which fractions $F_{105-115}$ and F_{9-10} were seen to be homogeneous and were pooled and loaded on a Sephadex column followed by a series of preparative TLCs to make a compound coded as RK/DCM; Hex/LS/SB/002a. The mixture had one spot on TLC chromatogram with an R_f of 0.56 (75% DCM/Hexane) and was an oily liquid serialized as compound LS01.

Another portion of fractions $F_{105-115}$ was purified separately using preparative TLC to yield five bands out of which the third band coded as RK/DCM; Hex/LS/SB/003 showed an R_f of 0.61 in 60% DCM in hexane and was a colorless oily liquid, serialized as compound LS05. Figure 1 outlines the purification and isolation of compounds LS01 and LS05 from hexane/DCM extract.

Purification and isolation of compounds from EtOAc extract

The EtOAc crude extract (85.903 g) was loaded in a column and eluted with 1000 mL portions of a solvent system whose polarity was varied gradually by incremental addition of 5% of EtOAc, starting with 100% hexane. EtOAc was introduced until 100% had been mixed, followed by MeOH, which was added up to 10% MeOH in EtOAc. A total of 286 fractions were plotted on the TLC plate to establish the optimum solvent system for the separation in which a 0.5% MeOH/EtOAc solvent system was identified.

The fractions $F_{177-210}$ were constituted and isolated with DCM/MeOH in the ratio of 1:1, then loaded into a column and eluted with Hexane/EtOAc solvent systems of varying polarity starting with 40:1 ratio then finally adding up to 100% EtOAc. A total of 81 fractions of 5 mL was collected. Fractions F_{9-29} were seen to be homogeneous and were constituted, and a series of four preparative TLCs was conducted to yield two bands. The upper band RK/EtOAc/LS/SB/008a showed one spot on TLC chromatogram with an R_f of 0.57 in 50% DCM in hexane and was a white needle-like solid serialized as compound LS02.

The fractions F_{38-67} were constituted and isolated with DCM/MeOH in the ratio of 1:1, then loaded into a column and eluted with 60 mL of Hexane/EtOAc solvent systems starting with 40:1 ratio then finally 100% EtOAc. A total of 81 fractions of 5 mL were taken. Fractions F_{9-29} were constituted, and a series of four preparative TLCs was done to produce two bands. The lower band RK/EtOAc/LS/SB/006a showed one spot on TLC chromatogram with an R_f of 0.56 in 100% DCM and was a white crystalline solid assigned as compound LS03.

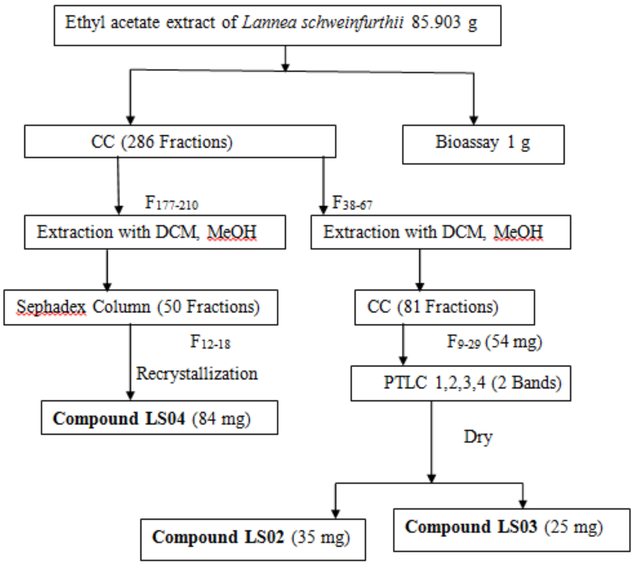


Figure 2. Isolation of compounds LS02, LS03, and LS04 from EtOAc extract.

The fractions F$_{177-210}$ were constituted and extracted with DCM/MeOH in the ratio of 1:1 then loaded into a Sephadex column in which they were eluted with 1000 mL of DCM/MeOH solvent system in the proportion of 1:1 to give a total of 50 fractions, every 20 mL. Fractions F$_{12-18}$ were homogeneous, and they were pooled and recrystallized to make RK/EtOAc/LS/SB/007. The compound appeared as yellow crystalline solid and showed one spot on TLC chromatogram with an R$_f$ of 0.62 in 70% acetone in MeOHl, which was serialized as compound LS04. Figure 2 describes the purification and isolation of compounds LS02, LS03, and LS04.

The isolated five compounds, LS01, LS02, LS03, LS04, and LS05**,** were packed in pre-weighed sample bottles then taken for bioassay and NMR analysis.

Physical and spectroscopic data of the isolated compounds

The physical data measured for compounds LS01, LS02, LS03, LS04, and LS05**,** included the uncorrected melting point for the solids, the appearance, as well as the retention factor R$_f$ while the spectral data determined included the ^{13}C NMR and ^{1}H NMR spectra. Other spectral data obtained were as described in the GC-MS profile of the crude DCM and methanol extracts.

Compound LS01

Colorless liquid (hexane/DCM); R$_f$ of 0.56 (75% DCM in hexane); yield (18 mg); (^{1}H NMR CDCl$_3$, δ (ppm), 400 MHz) δ 7.13 (1H, t, J=7.7 Hz), 6.75 (1H, bd, J=7.5 Hz), 6.65 (1H, bs), 6.63 (1H, bd, J=7.6 Hz), 5.40 (2H, m), 2.55 (2H, m), 1.98 (m), 1.59 (m), 1.29 (m), 1.25 (m) and 0.96 (3H, t, J=7.2 Hz); ^{13}C NMR (CDCl$_3$-δ, 100 MHz). δ 14.0 (C-13’), 29.2, 29.3, 29.5, 29.5, 29.6, 29.7, 25.6 (C-12'), 35.8 (C-1'), 31.3 (C-2'), 32.6 (C-9'), 112.4 (C-6), 115.3 (C-2), 121.0 (C-4), 129.4 (C-5), 129.4 (C-11'), 131.9 (C-10’), 145.0 (C-3), 155.4 (C-1).

Compound LS02

White needle like crystals (EtOAc); Mp 126-128°C; R$_f$ of 0.57 (50% DCM in hexane); yield (35 mg); ^{1}H NMR (CDCl$_3$, δ (ppm), 400 MHz) δ (4.67, 4.58, 3.16, 2.30, 1.66, 1.01, 0.95, 0.92, 0.81, 0.77, 0.74); ^{13}C NMR (CDCl$_3$-δ, 100 MHz). δ 14.6 (C-27), 15.4 (C-24), 16.0 (C-26), 16.1 (C-25), 18.0 (C-28), 18.3 (C-6), 19.3 (C-30), 20.9 (C-11), 25.1 (C-12), 27.4 (C-2), 27.4 (C-15), 28.0 (C-23), 29.8 (C-21), 35.6 (C-16), 34.3 (C-7), 37.2 (C-10), 38.1 (C-13), 38.7 (C-1), 40.0 (C-22), 38.9 (C-4), 40.8 (C-8), 42.8 (C-14), 43.1 (C-17), 48.0 (C-19), 48.3 (C-18), 50.4 (C-9), 55.3 (C-5), 79.0 (C-3), 109.3 (C-29), 150.8 (C-20).

Compound LS03

White crystalline solid (EtOAc); Mp 128-130 °C; R$_f$ of 0.56 (100% DCM); yield (25 mg); ^{1}H NMR (CDCl$_3$, δ, 200 MHz) δ 5.32 (1H, d, J=7.2 Hz), 3.50 (1H, m), 1.03 (3H, s), 0.94 (3H, d, J=8.4 Hz), 0.86 (9H, m), 0.79 (3H, s); ^{13}C NMR (CDCl$_3$, 75 MHz) 11.9 (C-29), 12.0 (C-18), 18.8 (C-26), 19.0 (C-19), 19.4 (C-21), 19.8 (C-27), 21.1 (C-11), 23.1 (C-28), 24.3 (C-15), 26.1 (C-23), 28.2 (C-16), 31.7 (C-7), 29.2 (C-25), 31.9 (C-22), 31.9 (C-2), 34.0 (C-8), 36.5 (C-10), 39.8 (C-12), 36.5 (C-20), 37.3 (C-1), 42.3 (C-4), 42.3 (C-13), 45.9 (C-24), 50.2 (C-9), 56.8 (C-14), 56.1 (C-17), 71.8 (C-3), 121.7 (C-6), 140.8 (C-5).

Compound LS04

A yellow crystalline solid (EtOAc); Mp 119-221 °C; R$_f$ of 0.62 (70% Acetone/ MeOH); yield (84 mg); ^{1}H NMR (DMSO, δ (ppm), 200 MHz) δ 6.87 (1H, s), 6.64 (1H, t, J=8.8 Hz), 5.87 (1H, d, J=1.4 Hz), 5.69 (1H, d, J=1.4 Hz), 4.65 (1H, d, J=4.6 Hz), 3.99 (1H, m), 2.66 (1H, dd, J=16.6, 4.4 Hz), 2.44 (1H, dd, J=16.6, 3.2 Hz); ^{13}C NMR (DMSO-δ, 75 MHz) δ 28.9 C-4), 78.7 (C-2), 65.6 (C-3), 94.7 (C-6), 95.7 (C-8), 99.2 (C-4a), 115.4 (C-2’), 115.6 (C-5’), 118.6 (C-6’), 131.3 (C-1’), 145.2 (C-4’), 145.1 (C-3’), 156.4 (C-8a), 156.9 (C-7), 157.2 (C-5).

Compound LS05

A colorless oily liquid (hexane/DCM); R$_f$ of 0.61 (60% DCM: Hexane); yield (10 mg); (^{1}H NMR CDCl$_3$, δ (ppm), 400 MHz) δ 7.71 (2H, dd, J=8.7, 5.4 Hz), 7.53 (2H, dd, J=8.7, 5.4 Hz) 4.22 (4H, m), 1.68 (2H, m), 1.42 (m), 1.32 (m), 1.25 (m), 0.91 (m); ^{13}C NMR (CDCl$_3$-δ, 100 MHz) δ 11.1, 38.9, 14.2, 23.1, 23.9, 29.1, 30.5, 68.3, 128.9 (C-3, 6), 131.0 (C-1, 2), 132.6 (C-4, 5) and 167.9 (C=O).

RESULTS AND DISCUSSION

Crude extract yields

The dry powdered stem bark of *L. schweinfurthii*, which weighed 4.3 kg, were subjected to sequential. The number of crude extracts obtained, and percentage yields were recorded and tabulated in Table 3.

Table 4. Inhibition zones of the crude extracts of *L. schweinfurthii* stem bark.

	S. aureus	***B. subtilis***	***P. aeruginosa***	***E. coli***	***C. albicans***
Hexane	7.00±0.20b	7.00±0.80a	6.00±0.10a	7.00±0.10b	7.00±0.10b
DCM	7.00±0.50b	7.00±0.60a	6.00±0.30a	7.00±0.10b	7.00±0.10b
EtOAc	20.00±0.70e	19.00±0.70c	6.00±0.20a	16.00±0.20c	11.00±0.20c
MeOH	13.00±0.10c	15.00±0.60b	6.00±0.10a	21.00±0.20e	14.00±0.50d
(-ve) Control	6.00±0.40a	6.00±0.70a	6.00±0.10a	6.00±0.10a	6.00±0.40a
(+ve) Tet.	18.00±0.20d	18.00±0.40c	16.00±0.40b	18.00±0.10d	-
(+ve) Ny.	-	-	-	-	18.00±0.60e
p-value	<0.001	<0.001	<0.001	<0.001	<0.001

Note: Mean values followed by the same small letter within the same column do not differ significantly from one another (One-Way ANOVA,SNK test,α =0.05). Tet. for Tetracycline (bacteria); Ny. for Nystatin (Fungi); (-ve) Control-DMSO

Table 3. Masses of sequential extraction of *L. schweinfurthii* stem bark and percentage yields.

Extraction solvent	**Mass in grams**	**% Yield**
Hexane	9.269	0.216
DCM	26.508	0.616
EtOAc	65.403	1.521
MeOH	145.744	3.389

MeOH extract resulted in the highest percentage yield, while hexane extract had the least. The results demonstrated that the percentage yield increased with an increase in polarity of the solvent used. This could be attributed to the characteristic of MeOH being more polar than the solvents used dissolved all the other remaining compounds, including sugars and other highly polar compounds like glycosides.

Antibacterial and antifungal assay of crude extracts

All of the four crude extracts were examined against four bacterial strains and one fungus to determine their antimicrobial activity. The pathogens used were *B. subtilis* (ATCC 2*02638)* and *S. aureus* (ATCC 259223) (Gram-positive bacteria), E. coli (ATCC 25922) and P. aeruginosa (ATCC 10622) (Gram-negative) and *C. albicans* (ATCC 10231) (fungus). The inhibition zones of bacterial and fungal growth (in mm) were calculated after 48 hours and seven days, respectively. The measurements were performed from the end of the growth of one side of the disc to the end of growth of the other hand, including the diameter of the disc (Chhabra and Uiso, 1991) (Table 4).

There was a significant difference between the variety of extracts and the controls in all the bacteria and fungi used. DCM and hexane extracts showed mild activity (inhibition of 7 mm), and their inhibition zones did not differ significantly. MeOH and EtOAc showed high activity (inhibition of 13 mm and above) against the microbes used. The antibiotics served as positive control, and showed inhibition zones of 18 mm against all microbes except for *P. aeruginosa*, which exhibited inhibition of 16 mm. The results demonstrate the challenging of the Gram-negative bacteria to be inhibited by many kinds of antibiotics (Tait-Kamradt et al. 2000). The inhibition zones for the antibiotics were relatively higher compared to those given by crude extracts. However, EtOAc crude extract exhibited significantly higher inhibition zones of 20 and 19 mm against the *S. aureus* and *B. subtilis*, respectively, compared to other extracts and controls. The methanol extract showed a significantly higher inhibition zone of 21 mm against the *E. coli* bacteria, compared to the standard antibiotics. Furthermore, the most upper inhibition zone was shown by EtOAc extract, which gave an inhibition zone of 21 mm.

The crude extracts showed mild and moderate activity (inhibition of between 7 and 14 mm) against *C. albicans* fungus. The fact that the crude extracts exhibited activity against both the bacteria and fungi was evidence of their potency, thus, support the use of *L. schweinfurthii* plant as a traditional medicine against bacterial and fungal infections. However, cytotoxicity tests should be carried out to ascertain the activity observed.

GC-MS data for the crude DCM and MeOH extracts

GC-MS was performed for the crude DCM, and MeOH extracts to give a preliminary idea of the class of compounds in *L. schweinfurthii*. The compounds detected were serialized based on their retention time. Most compounds present in the plant species were fatty acids and their derivatives, phenolic compounds, polyketide derivatives, steroids, and terpenoids, which were given in Tables 5 and 6.

Plant phenolics that were analyzed comprised of shikimic acid derivatives such as coumarates and phenylpropanoids, phenols, and polyketide derivatives. Further, terpenoids measured from both DCM and methanol crude extracts were monoterpenoids, diterpenoids, sesquiterpenoids, triterpenoids, and their derivatives.

Also, there were several fatty acids and their derivatives that were analyzed, which included alcohols, hydrocarbons, acetylenes, and isoprene fatty acids, while the steroids present in the extracts included sitosterol and androsterone.

Table 5. Number of compounds detected in crude DCM extract of *L. schweinfurthii.*

Class of compounds	Number of compounds
Fatty acids and derivatives	27
Terpenoids	33
Phenolic compounds	6
Polyketide derivatives	2
Steroids	1

Table 6. Number of compounds detected in crude MeOH extract of *L. schweinfurthii.*

Class of compounds	Number of compounds
Fatty acids and derivatives	13
Terpenoids	7
Phenolic compounds	6
Polyketide derivatives	4
Steroids	1

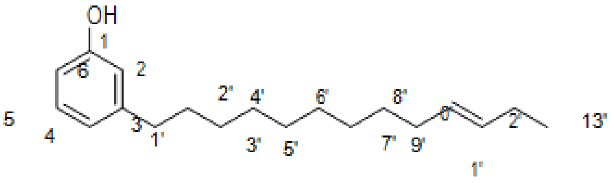
Structure elucidations

Compound LS01

The compound was obtained from hexane/DCM extract as a colorless liquid. On analytical TLC, the compound showed an Rf of 0.56 in 75% DCM in hexane. On spraying with anisaldehyde, the compound had a red spot that faded slightly but remained red, suggesting a phenolic compound (Dewick 1998).

The ^{1}H NMR spectrum showed the presence of aromatic, olefinic, and aliphatic protons. The signal at δ 7.13, 6.75, 6.65, and 6.63 was assigned to four aromatic protons in different chemical environments indicating that the ring was di-substituted with the two groups being at meta-positions with each other. A more in-depth analysis of the spectrum showed a very strong signal at δ 1.29 and a triplet integrating into three protons at δ 0.96, corresponding to a methyl group. This indicated the presence of a long alkyl chain. The signal shown as a multiplet at δ 5.4 stated the presence of olefinic protons and thereby the existence of a double bond in the side chain.

Furthermore, the multiplet at δ 1.98 was characteristic of allylic protons and was assigned to the two methylene protons at a position adjacent to the double bond. The attachment of the double bond in the chain was deduced from ^{1}H-^{1}H COSY experiment with correlation data given in Table 7, in which correlations were detected between the olefinic proton δ 5.40, the proton multiplet at δ 1.98 and triplet at δ 0.96. The interactions between the multiplet at δ 1.98 of allylic protons and terminal methyl protons at δ 0.96, while at the same time interaction with the multiplet at δ 5.40 of olefinic protons brought to the conclusion that the double bond occurs between the third and the fourth carbons from the tail. Similarly, there were correlations found between the benzylic protons δ 2.55 and the adjacent methylene protons at δ 1.59. Other signals were identified as methylene proton pooled at position 3’ to 8’ for δ 1.29 (m) (Dewick 1998).



LS01

Table 7. ^{1}H NMR (400 MHz, CDCl3) and ^{13}C NMR (100 MHz) data for compound LS01.

Position	^{1}H NMR (δ)	Multiplicity/ integration/ J values(Hz)	COSY ^{1}H-^{1}H	^{13}C NMR (δ)
1	-	-	-	155.4
2	6.65	bs (1H)	-	115.3
3	-	-	-	145.0
4	6.75	bd (1H) J=7.5	-	121.0
5	7.13	t (1H) J=7.7	-	129.4
6	6.63	bd (1H) J=7.6	-	112.4
1’	2.55	t (2H)	H-2’	35.8
2’	1.59	m (2H)	H-1’, 3’-8’	31.3
3’-8’	1.29-1.25	m	H-9’	29.7-29.1
9’	1.98	m	H-3’-8’	32.6
10’	5.40	m (1H)	H-9’	131.9
11’	5.40	m (1H)	H-12’	129.4
12’	1.98	m	H-13’,11’	25.6
13’	0.96	t (3H) J=7.2	H-12’	14.0

In the ^{13}C NMR spectrum, there were nineteen peaks, which indicates chemical shifts assignable to either aromatic or olefinic carbons between δ 155.4 and 112.4. The other signals were detectable between δ 35.8 and 14.0 were assigned to aliphatic carbons. Further, the signal at δ 155.4 was recognized as an aromatic hydroxylated carbon atom, and together with the signal at δ 145.0 represented the only two quaternary carbons in the compound, as seen in DEPT experiment. The signals at δ 131.9 and 129.4 were identified to the carbon atoms of the double bond. The four protonated aromatic carbon atoms showed their signals at δ 129.4, 121.0, 115.3, and 112.4.

The signal at δ 35.6 was determined as benzylic carbon (C-1'), while that at δ 14.0 was identified as the terminal methyl carbon (C-13'). The remaining signals δ 32.6 to 25.6 were recognized as the methylene carbons in the chain. ^{13}C NMR spectrum was instrumental in measuring the overall structure of the compound whose name is 3-(10'-tridecenyl) phenol. The extraction of phenolic compounds LS01 is a close group to LS01 from the plant species described previously (Okoth, 2014). Nevertheless, the compound LS01 was isolated for the first time from *L. schweinfurthii*.

Compound LS02-Lupeol

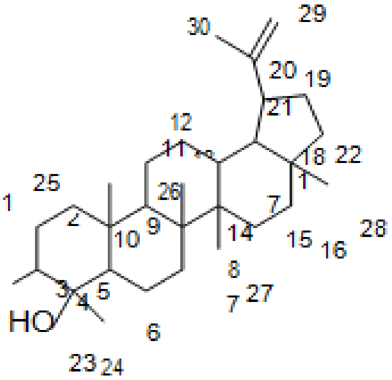
Hexane/DCM extract yielded LSO2 compound as a needle-like crystal with a melting point range of 126-128 °C. On analytical TLC, the compound showed an Rf of 0.57 in 50% DCM in hexane. When the chromatogram was sprayed with anisaldehyde, it changed into purple, indicating that the compound is a terpenoid. The ^{1}H NMR spectrum showed protons in three regions, namely aliphatic, hydroxylated, and allylic; which strongly indicated that the compound was a triterpenoid. Two downfield signals found at δ 4.80 and 4.68, existing as broad singlets, indicated the existence of a terminal double bond (Satomi et al. 2002). The signal found at δ 3.35 was identified to be the proton of a hydroxylated carbon at location 3. The presence of seven singlets, each integrating into three protons at δ 0.99, 1.00, 1.13, 1.26, 1.27, 1.29, and 1.82, clearly indicated pentacyclic triterpenoids. The result agreed with the lupeol type structure of a triterpenoid (Satomi et al. 2002)(Table 8).

^{13}C NMR spectrum for compound 5 recognized 30 carbons, which further confirms as a pentacyclic structure. The two downfield signals found at δ 151.0 and 109.3 reflected the olefinic carbons with the downfield signal being that of the quaternary carbon. The signal seen at δ 79.0 was representative of the hydroxylated carbon at position 3. The seven methyl carbon atoms were found at δ 28.1, 16.1, 15.4, 14.6, 16.0, 18.1, and 19.3. They are nearly the same compared to that of lupeol; pentacyclic triterpenoids reported from various plant sources (Satomi et al. 2002).

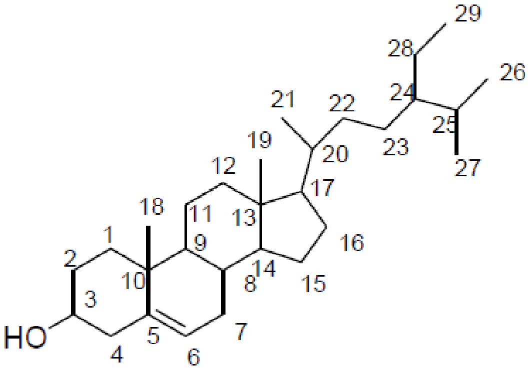
Compound LS03-β-Sitosterol

Compound LS03 was extracted from EtOAc as a white crystalline solid with a melting point of 128-130°C. The compound showed an R_f of 0.56 in 100% DCM. When the chromatogram was sprayed with anisaldehyde spray, the spot changed purple and later green, thus, suggesting that the compound comprises of a terpenoid (Dey and Harborne 1991). ^{1}H NMR spectrum of the compound showed three regions, namely aliphatic, hydroxylated, and allylic part on the spectrum signals and firmly suggested a terpenoid structure. A signal found at δ 5.32 (^{1}H, d J=8 Hz) indicated the presence of a double bond at a quaternary carbon atom. A multiplet centered found at δ 3.50, characteristic of a proton germinal to a hydroxyl group at C-3 in terpenoids, was also found. Six signals reflecting the methyl groups were also found at δ 1.03 (3H, s), 0.94 (3H, d, J=8.4 Hz), 0.86 (9H, m) and 0.70 (3H, s), which is characteristic of a modified terpenoid (Dey and Harborne 1991)(Table 9).

^{13}C NMR spectrum detected a total of twenty-nine peaks. The signals involving the double bonds were determined by the peaks at δ 140.8 and 121.7 in which the former was a quaternary carbon atom. The signal at δ 71.8 reflected a hydroxylated carbon atom at C-3. Methyl



LS02



LS03

Table 8. ^{13}C NMR (100 MHz, CDCl3) data of compound LS02 and lupeol (Satomi et al. 2002).

Carbon	Compound LS02	Lupeol	Carbon	Compound LS02	Lupeol
1	40.0	40.3	16	35.6	35.8
2	27.4	27.8	17	43.0	43.2
3	79.0	78.2	18	48.3	48.6
4	39.1	39.6	19	48.0	48.3
5	55.3	55.9	20	151.0	151.1
6	18.3	18.8	21	30.1	30.2
7	34.3	34.6	22	39.0	39.3
8	41.0	41.2	23	28.1	28.7
9	50.5	50.8	24	16.1	16.6
10	37.2	37.5	25	15.4	15.4
11	21.1	21.2	26	14.6	14.8
12	28.0	28.3	27	16.0	16.2
13	38.1	38.3	28	18.1	18.2
14	42.8	43.1	29	109.3	110.0
15	30.1	30.1	30	19.3	19.5

Table 9. ^{13}C NMR (100 MHz, CDCl3) data of compound LS03 and β-sitosterol (Dey and Harborne 1991; Mahato and Kundu 1994).

Carbon	Compound LS03	β-sitosterol	Carbon	Compound LS03	β-sitosterol
1	37.3	37.2	16	28.2	28.2
2	31.9	31.9	17	56.1	56.0
3	71.8	72.0	18	12.0	11.8
4	42.3	42.2	19	19.0	19.2
5	140.8	140.7	20	36.5	36.1
6	121.7	121.6	21	19.4	18.9
7	31.7	31.8	22	31.9	32.1
8	34.0	33.8	23	26.1	25.7
9	50.2	50.1	24	45.9	45.8
10	36.5	36.4	25	29.2	29.1
11	21.1	21.4	26	18.8	18.7
12	39.8	39.7	27	19.8	19.7
13	42.3	42.5	28	23.1	23.0
14	56.8	56.7	29	11.9	11.9
15	24.3	24.2			

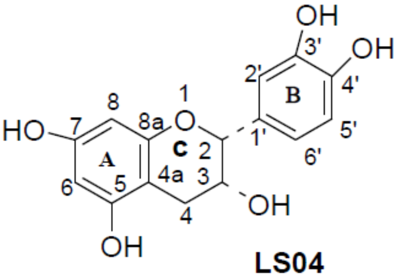
groups were showed by the signals at δ 11.9, 12.0, 18.8, 19.0, 19.4, and 19.8. The overall spectrum of compound LS03 compared carefully to that of β-sitosterol, a sterol that has been described from various plant sources (Dey and Harborne 1991). The ^{13}C NMR data for compound LS03 compared relatively well with that reported by Dey and Harborne (1991) of β-sitosterol represented in Table 7.

Compound LS04-Epicatechin

Compound LS04 was taken from a fraction of EtOAc as a yellow crystalline solid with melting point 219-221°C. On analytical TLC data, the compound had an R_f of 0.62 in 70% acetone in MeOH. When the chromatogram was sprayed with anisaldehyde, the spot changed into red, which later turned yellow, indicating that the compound was a flavonoid (Harborne 1998). ^{1}H NMR spectrum displayed chemical shifts values in three distinctive areas of the spectrum; the aromatic, hydroxylated (oxygenated), and aliphatic protons.

The peak at δ 6.87, a doublet of doublets centered at δ 6.64 and a doublet at δ 6.64, represented protons of the tri-substituted benzene ring. It should be noted, however, that the peaks at δ 6.64 were the protons at carbon 5' and 6' were overlapping owing to the least difference in chemical shifts. The overlapped data resulted in a triplet instead of the common doublet. Further, two doublets centered at δ 5.8 and δ 5.69 suggested the existence of another benzene ring that was tetra-substituted. The relatively small coupling constant (J=1.4 Hz) between the two protons might indicate a meta configuration with respect to each other. The pattern of the above-mentioned protons firmly suggested a flavonoid skeleton with hydroxyl groups at positions 5 and 7 of ring A and 3’ and 4’ of ring B (Harborne 1998).

The doublet at δ 4.65 was assigned to the methine proton at position 2 in ring C. The signal at δ 3.99, appearing as a multiplet, was assigned to the proton at location three of ring C of a flavonoid, attached to a carbon atom that is oxygenated, preferably a hydroxyl group. Such a proton occurred as doublets of the doublet, suggesting that the proton is coupled by three other protons (Harborne 1998). Nevertheless, the peak appeared as a doublet, perhaps due to the spatial orientation of the hydroxyl group. The other two signals are seen as doublets of a doublet at δ 2.66 and δ 2.44 suggested geminal protons at position four and were assignable to the axial and equatorial protons, respectively (Harborne 1998). Ring C did not possess a carbonyl group and was substituted by the methylene protons represented by the geminal protons mentioned above. This indicated that the compound had a flavan skeleton. In the ^{13}C NMR spectrum, there were fifteen signals supporting the flavonoid skeleton. The peaks found at δ 157.2, 156.9, 156.4, 145.2, and 145.1 agree with the existence of five oxygen-linked aromatic carbons in the proposed structure. The absence of peaks at δ 170-190 showed the lack of carbonyl carbons in the compound, supporting the earlier proton assignment. The signal at δ 28.9 reflected the methylene carbon identified position four substituting the carbonyl carbon. This was further supported by the DEPT experiment, which showed the presence of one methylene carbon at δ 28.9 ppm. The signal at δ 65.7 reflected hydroxylated carbon at position three, while the signal at δ 78.7 suggested aa carbon at location two, which appeared more downfield than the one at location three. This is attributed to the carbon being a benzylic one as opposed to an oxygenated carbon atom (Harborne 1998). The signal at 99.2 was identified as the quaternary at position 4a of ring A. The spectral data for compound LS04 agreed with published studies for epicatechin (Tables 10 and 11) (Harborne 1998). Based on the spectroscopic data, compound LS04 was identified as epicatechin.



LS04

Table 10. ^{1}HNMR (200 MHz, DMSO) data for compound LS04 and Epicatechin (Harborne 1998).

Proton	**Chemical shift (δ) forLS04**	**Multiplicity/ Integration**	**J values (Hz)**	**Chemical shift (δ) for Epicatechin**
2’	6.87	s (^1H)	-	6.90
5’	6.64	t (^1H)	8.8	6.66
6’	6.64	t (^1H)	8.0	6.66
8	5.87	d (^1H)	1.4	5.89
6	5.69	d (^1H)	1.4	5.72
2	4.65	d (^1H)	4.6	4.74
3	3.99	m (^1H)	-	4.01
4 (ax.)	2.66	dd (^1H)	4.4, 16.6	2.79
4 (eq.)	2.44	dd (^1H)	3.2, 16.6	2.48

Table 11. ^{13}C NMR data (75 MHz, DMSO) for compound LS04 and Epicatechin (Harborne 1998).

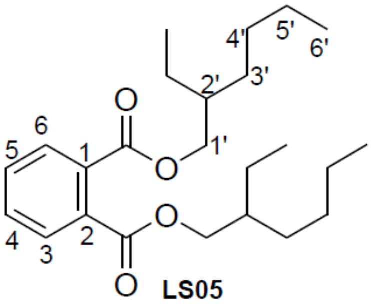
Carbons	**Compound LS04 (δ)**	**DEPT**	**Epicatechin (δ)**
C-4	28.9	CH_2	29.3
C-3	65.6	CH	67.5
C-2	78.7	CH	79.9
C-6	94.7	CH	95.9
C-8	95.7	CH	96.4
C-4a	99.2	C	100.1
C-2’	115.4	CH	115.4
C-5’	115.6	CH	115.9
C-6’	118.6	CH	119.5
C-1’	131.3	C	131.9
C-4’	145.1	C	145.8
C-3’	145.2	C	146.0
C-8a	156.4	C	157.4
C-7	156.9	C	157.7
C-5	157.2	C	158.0

Epicatechin is known for its antioxidant and anti-carcinogenic activities; it is also an anti-atherogenic and antitumor agent (Xu et al. 2004). The compound was initially named *kakaool* attributed to the fact that it is found in large quantities in cacao beans (Freudenberg et al. 1932). There are several studies on the isolation of this compound from various plant species (Rao et al. 1997; de Carvalho et al. 2008; Reddy et al. 2008). Okoth (2014) demonstrated the isolation of epicatechin for the first time from *Lannea schweinfurthii*.

Compound LS05

Compound LS05 was taken from a fraction of DCM/ Hexane as a colorless oily liquid with an R_F of 0.61 in 60% DCM in Hexane. When sprayed with anisaldehyde, the spot changed into blue, suggesting that the compound comprises a fatty acid derivative (Harborne 1998).

^{1}H NMR spectrum displayed chemical shifts values in three different regions of the spectrum; the aromatic, oxygenated, and aliphatic protons. The double of doublets centered at δ 7.71 and at 7.53 represented protons of di-substituted benzene rings. Also, the splitting patterns in the aromatic region indicated two sets of equivalent protons from an ortho-disubstituted benzene ring. The signal at δ 4.22, appearing as a multiplet, was determined as the methylene group geminal to the ester alcohol group. Also, the peaks at δ between 1.68 and 0.91 were assigned to aliphatic protons of the side chains. The downfield signal at 1.68 was identified as the methine proton of the aliphatic chain suggesting the existence of a branch in the alkyl chain (Rao et al. 2000).



LS05

In the ^{13}C NMR spectrum, there were 12 signals representing carbon atoms in three different chemical environments. The peak at δ 167.9 displayed the presence of a carbonyl carbon of an ester group supporting the earlier proton assignment. Additionally, the three peaks found at δ 132.6, 131.0, and 128.9 were identified as the three sets of equivalent carbon atoms in the aromatic ring coming from an ortho-substituted benzene ring with similar side chains. The signal found at δ 68.3 suggested the presence of oxygen-linked aliphatic carbons and was identified as the ether carbon in the proposed structure. The signals between δ 38.9 and 11.1 reflected the aliphatic carbons in the side chains, with the downfield signal being identified methine carbons in the chain (Rao et al. 2000).

The overall assignment of the structure of LS05 was performed using GC-MS. It should be noted that the spectral data of compound LS05 was strictly correlated to that of di-(2-propylpentnyl) ester phthalic acid (145) and dibutyl phthalate (83) observed in the crude methanol and DCM extracts of *L. schweinfurthii* using GC-MS. It was evident that the compound possessed a benzene ring with two similar ester groups that were ortho to each other. Furthermore, the ester group contained a branch in the aliphatic chain. The deductions were conclusive in characterizing the compound, the proposed structure of compound LS05 given, and the name was given di-(2'-Ethylhexyl) ester phthalic acid. To the best of our knowledge, (2'-Ethylhexyl) ester phthalic acid was isolated for the first time from the plant species (Tables 12 and 13).

Bioassay of isolated compounds

Antibacterial test for the isolated compounds

The compounds LS01, LS02, LS03, LS04, and LS05 proceeded to antibacterial tests against *S. aureus*, *B. subtilis*, *E. coli*, and *P. aeruginosa* (Table 14). There was a significant difference between the various isolated compounds and the controls in all the bacteria used. The purified compound extracted from *L.schweinfurthii* stem bark coded LS01 had mild activities against the two Gram-positive bacteria. However, the compound had no activity against Gram-negative bacteria. An inhibition zone of 9 and 7 mm was shown by lupeol against the *S. aureus* and *B. subtilis*, respectively, and 8 mm against the *E. coli*, but no activity was detected against the other two Gram-negative bacterial strains. β-sitosterol showed mild activity against the Gram-positive bacteria but had no activity against the Gram-negative bacteria.

Table 12. ^{1}HNMR (400 MHz, CDCl3) data for compound LS05.

Proton	Chemical shift (δ)	Multiplicity/ Integration	J-values (Hz)
3, 6	7.71	dd (2H)	5.4, 8.7
4, 5	7.53	dd (2H)	5.4, 8.7
1’	4.22	m (4H)	
2’	1.68	t (2H)	
	1.42	m	
	1.32	m	
	1.25	m	
6’	0.91	m	

Table 13. ^{13}C NMR (100 MHz, CDCl3) data for compound LS05.

Carbon	Compound LS05 (δ)	Carbon	Compound LS05 (δ)
C=O	167.9	CH_2	29.1
4,5	132.6	CH_2	29.8
1,2	131.0	CH_2	23.9
4,5	128.9	CH_2	23.1
C-O	68.3	CH_3	14.2
CH	38.9	CH_3	11.2
CH_2	30.5		

Table 14. Inhibition zones of the isolated compounds after 24hrs; 500 µg/mL per disc.

	S.aureus	***B. subtilis***	***E. coli***	***P. aeruginosa***
LS01	8.00±0.10c	7.00±0.30b	6.00±0.50a	6.00±0.40a
LS02	9.00±0.20d	7.00±0.10b	8.00±0.20c	6.00±0.10a
LS03	8.00±0.20c	9.00±0.10c	6.00±0.10a	6.00±0.10a
LS04	15.00±0.50e	14.00±0.20d	10.00±0.50d	9.00±0.40b
LS05	7.00±0.10b	7.00±0.10b	7.00±0.30b	6.00±0.10a
Control (-ve)	6.00±0.20a	6.00±0.40a	6.00±0.20a	6.00 ±0.30a
Control (+ve)	18.00±0.30f	18.00±0.30e	18.00 ±0.20e	16.00±0.40c
P-value	<0.001	<0.001	<0.001	<0.001

Note: Mean values followed by the same small letter within the same column do not differ significantly from one another (One-Way ANOVA,SNK test,α =0.05). Control-ve-DMSO; +ve Control-Tetracycline

Table 15. Inhibition zones (in mm) of isolated compounds in the diffusion method assay after 7 days; 1000 µg/mL per disc.

Compound	***C. albicans***	***P. notatum***
LS01	6.00±0.20a	6.00±0.40a
LS02	6.00±0.20a	8.00±0.20c
LS03	6.00±0.10a	9.00±0.10d
LS04	13.00±0.30b	12.00±0.20e
LS05	6.00±0.10a	7.00±0.50b
Control (-ve)	6.00±0.20a	6.00±0.20a
Control (+ve)	18.00±0.40c	18.00±0.50f
P-value	<0.001	<0.001

Note: Mean values followed by the same small letter within the same column do not differ significantly from one another (One-Way ANOVA,SNK test,α =0.05). Control-ve-DMSO; Control +ve-Nystatin

The inhibition zones against *S. aureus* and 9 mm against *B. subtilis* were 8 mm. Epicatechin had a significantly higher inhibition zone against the test strains used compared to the other compounds and the negative control. The inhibition zones of epicatechin were 10 and 9 mm against *E. coli* and *P. aeruginosa*, respectively, 14 mm against *B. subtilis* and 15 mm against *S. aureus*. All the isolated compounds exhibited some activity against the Gram-positive but no activity against the Gram-negative bacteria *P. aeruginosa*, except for epicatechin. The findings could explain, to some extent, the usage of *L. schweinfurthii* in many communities as herbal remedies. Other tests, such as in vitro cytotoxicity studies, should be carried out to ascertain the observed activity.

Antifungal test for the isolated compounds

The isolated compounds proceeded to antifungal assay against two fungi, which included *C. albicans* and *P. notatum* (Table 15). There was a significant difference between all the isolated compounds and the controls in the two fungi used. The results further showed that the activities of the isolated compounds were smaller than those of the crude extracts from which the compounds were obtained. The activity against fungi was lower than against bacteria used. Nevertheless, some activity was noted from some of the isolated compounds. Epicatechin had the highest activity against the fungi used. It displayed an inhibition zone of 13 mm against *C. albicans* and 12 mm against *P. notatum*. All the other compounds isolated from *L. schweinfurthii* had mild activity against *P. notatum* but no activity against *C. albicans*.

The bacteria strains used in the study are known to cause diseases such as gastrointestinal infections. The fungi used in this study are known to cause ailments such as candidiasis, athlete foot, vaginal thrush, and skin abscess (Johns et al. 1995). The activity demonstrated by the crude extract and the isolated compounds from *L. schweinfurthii* compares well with previously reported study (Okoth, 2014).

Conclusions

Phytochemicals are useful in the effective control of infection caused by bacteria and fungi. This study focused on antibacterial, antifungal, and phytochemical screening of *L. schweinfurthii*. The percentage yields of the extracts became higher from hexane to DCM to EtOAc to MeOH, indicated that the stem bark of *L. schweinfurthii* is very rich in both non-polar and polar metabolites. Hexane/DCM crude extract of *L. schweinfurthii* had mild activity towards the Gram-positive, Gram-negative, and the fungi used. The MeOH extract had the highest activity towards *E. coli*. The EtOAc extract had the highest activity against the *S. aureus*. TLC analysis of the crude extracts showed that the compounds extracted are not the only compounds present in the plant material. The percentage by mass of the isolated compounds is remarkably low because the plant material contains many compounds of almost equal polarity, making the isolation process complex. This finding shows that the stem bark of *L. schweinfurthii* contains fatty acids, terpenoids, steroids, and plant phenolics, among other compounds. Some of these compounds include 3-(10'-tridecenyl) phenol, lupeol, β-sitosterol, epicatechin, and di-(2'-Ethylhexyl) ester phthalic acid. Their structures were elucidated using NMR data. (viii) Three compounds 3-(10'-tridecenyl) phenol, epicatechin, and di-(2'-Ethylhexyl) ester phthalic acid was isolated for the first time from the plant species. Epicatechin exhibited high antibacterial activity against the Gram-positive bacteria with inhibition zones of 15 and 14 mm for *S. aureus* and *B. subtilis*, respectively, and against the fungus *C. albicans*.

Recommendations and further studies

The crude and purified extracts of *Lannea schweinfurthii* need to be subjected to further tests on other disease-causing microbes; both bacteria and fungi. Further, investigation on the synergic effects among the crude extracts, isolated compounds, and conventional antibiotics should be carried out. Also, there is a need to carry out phytochemical studies methanolic extracts and all other fractions that were not analyzed in this study. More work should be carried out on isolated compounds to determine both pharmacological and biological activities and on the use of crude blends. The isolated compounds that displayed the highest activity could be subjected to more studies such as cytotoxicity tests to be used as antimicrobials or as templates for the synthesis of drugs applied in the treatments of infectious diseases caused by bacteria and fungi.

REFERENCES

Chhabra SC, Mahunnah RLA, Mshiu EN. 1987. Plants used in traditional medicine in Eastern Tanzania. I. Pteridophytes and angiosperms (Acanthaceae and Canellaceae). J Ethno-Pharmacol 21: 253-277.

Chhabra SC, Uiso FC. 1991. Antibacterial activity of some Tanzanian plants used in traditional medicine. Fitoterapia 62: 499-504.

Clinical and Laboratory Standards Institute. 2013. Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. CLSI document M100-S23. Wayne 33 (1): 100-102.

Cos P, Vlietinck AJ, Berghe DV, Maes L. 2006. Anti-infective potential of natural products: how to develop a stronger in vitro ‘proof-of-concept’ J Ethno-Pharmacol 106 (3): 290-302.

de Carvalho MG, Suzart LR, Cavatti LC, Kaplan MAC. 2008. New flavonoids and other constituents from *Ouratea hexasperma* (Ochnaceae). J Braz Chem Soc 19: 1423-1428.

Dewick P. 1998. Medicinal Natural Products, A Biosynthetic Approach. John Wiley and Sons Ltd, United Kingdom.

Dey PM, Harborne JB. 1991. Methods in plant Biochemistry. Academic Press, New York.

Freudenberg K, Cox RFB, Braun E. 1932. The catechin of cacao bean. J Am Chem Soc 54: 1913-1917.

Geddes AM. 1985. Prescribers’ needs for developed and third world. In: Greenwood, F.O.O.’ Grady (ed.), The Scientific Basis of Antimicrobial Chemotherapy, vol. 1, Cambridge University Press, Cambridge.

Gullece M, Aslan A, Sokmen M, Sahin F, Adiguzel A, Agar G, Sokmen. 2006. Screening of the antioxidant and antimicrobial properties of the lichens *Parmelia saxatilis*, *Platismatia glauca*, *Ramalina pollinaria*, *Ramalina polymorpha* and *Umbilicaria nylanderian*. Phytomedicine 13: 515-521.

Hans GS. 1993. General Microbiology. 7th ed. Cambridge University Press, New York.

Harborne JB. 1998. Phytochemical Methods: A guide to modern technique of plant analysis. Chapman and Hall, London.

Johns T, Faubert GM, Kokwaro JO, Mahunnah RLA, Kimanani EK. 1995. Anti-giardial activity of gastrointestinal remedies of the Luo of East-Africa. J Ethno-Pharmacol 46: 17-23.

Krishnaswamy NR. 2003. Chemistry of natural products: A Laboratory Handbook. Orient Blackswan Publisher. Hyderabad, India.

Li J, Vederas JC. 2009. Drug discovery and natural products: End of an era or an endless frontier? Science 325: 161-165.

Maregesi SM, Pieters L, Ngassapa OD, Apers S, Vingerhoets R, Cos P, Berghe DAV, Vlietinck AJ. 2008. Screening of some Tanzanian medicinal plants from Bunda district for antibacterial, antifungal and antiviral activities. J Ethno-Pharmacol 119: 56-66.

National Committee for Clinical Laboratory Standards (NCCLS). 1999. Performance Standards Antimicrobial susceptibility testing. 9th International Supplement. M100 S9, Wayne PA.

National Committee for Clinical Laboratory Standards (NCCLS). 2003. Standardization of antimicrobial susceptibility testing by disc diffusion: Standard approved, 8th ed., Wayne PA.

Okoth DA. 2014. Phytochemistry and bioactive natural products from *Lannea alata, Lannea rivae, Lannea schimperi* and *Lannea schweinfurthii* (Anacardiaceae). [Dissertation]. KwaZulu-Natal University, South Africa.

Pandian MR, Banu GS, Kumar G. 2006. A study of antimicrobial activity of *Alangium salviifolium*. Indian J Pharmacol 38: 203-204.

Radovanović A, Radovanović BB, Jovančićević B. 2009. Free radical scavenging and antibacterial activities of southern Serbian red wines. J Food Chem 117: 326-331.

Rao GN, Kumar PM, Dhandapani VS, Krishna TR, Hayashi T. 2000. Constituents of Cassia auriculata. Fitoterapia 71: 82-83.

Rao KV, Sreeramulu K, Venkata RC, Gunasekar D, Martin MT, Bodo. 1997. Two new biflavonoids from *Ochna obtusata*. J Nat Prod 60: 632-634.

Reddy BAK, Reddy NP, Gunasekar D, Blond A, Bodo B. 2008. Biflavonoids from *Ochna lanceolata*. Phytochemistry 1: 27-30.

Sahoo N, Manchikanti P, Dey S. 2010. Herbal drugs: Standards and regulation. Fitoterapia 81 (6): 462-471.

Satomi F, Naomichi T, Masateru O, Alaa MN, Toshihiro N, Hiroyuki S, Shima D, Hideo Y. 2002. Two novel long chain alkanoic acid esters of lupeol from *Alecrim propolis*. Chem Pharm Bull 50: 439-440.

Sneader W. 1985. Drug discovery: The evolution of modern medicine, John Wiley and Sons, New York.

Tait-Kamradt A, Davies T, Appelbaum PC, Depardieu F. 2000. Two new mechanisims of macrolide resistance in clinical strain of *Streptococcus pneumoniae* from Eastern Europe and North America. J Antimicrob Agents 44: 337-340.

Thomson WAR. 1978. Healing Plants. McGraw-Hill Book Co. Ltd, London.

WHO. 2008. Traditional Medicine. Fact sheet No. 134. WHO, Geneva.

Xu JZ, Yeung SYV, Chang Q, Huang Y, Chen Z. 2004. Comparison of antioxidant activity and bioactivity of tea epicatechins with their respective epimers. Br J Nutr 91: 873-881.