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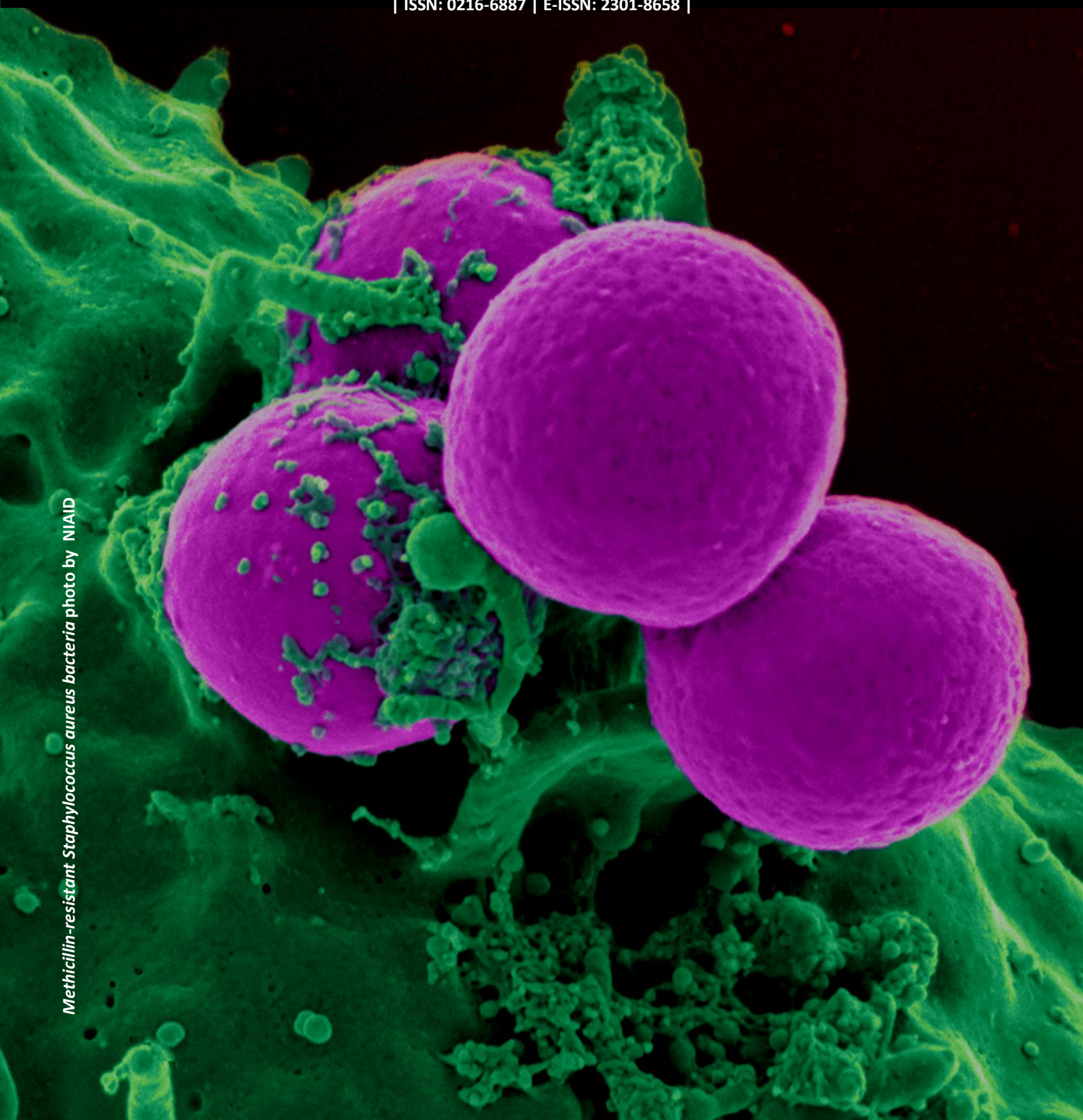

Methicillin-resistant Staphylococcus aureus bacteria photo by NIAID

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Abstract should not be more than 200 words. **Keywords** is about five words, covering scientific and local name (if any), research theme, and special methods which used; and sorted from A to Z. All important **abbreviations** must be defined at their first mention. **Running title** is about five words. **Introduction** is about 400-600 words, covering the background and aims of the research. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains for why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgments** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

Figures and Tables of maximum of three pages should be clearly presented. Title of a picture is written down below the picture, while title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; chart is preferred to use black and white images. Author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned source. **There is no appendix**, all data or data analysis are incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

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Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

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

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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.

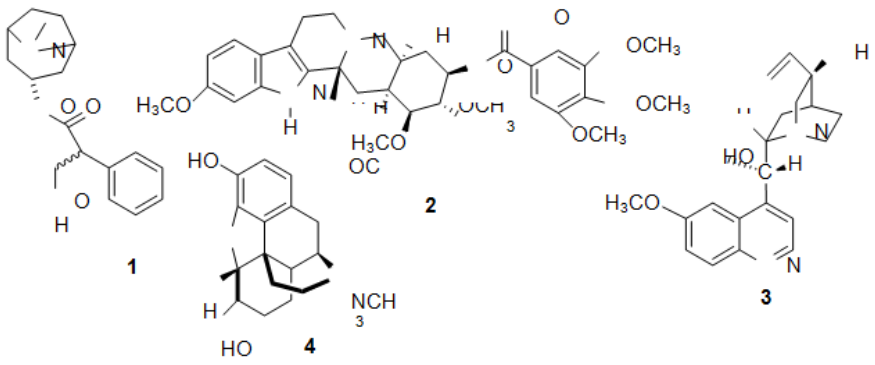
The organic solvents used here included hexane, dichloromethane (DCM), chloroform ($CHCl_3$), ethyl acetate (EtOAc), acetone (MeCOMe) and methanol (MeOH); these were of general-purpose grade (Kobian Kenya Ltd, Nairobi) and as a result, they were freshly distilled before use. Dimethylsulphoxide (DMSO) 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. ^{1}H 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 (^{1}H 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 ^{1}H NMR spectra were measured on the same instrument at 200 and 400 MHz, respectively. Structures of the compounds isolated were elucidated by interpreting the ^{1}H 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.

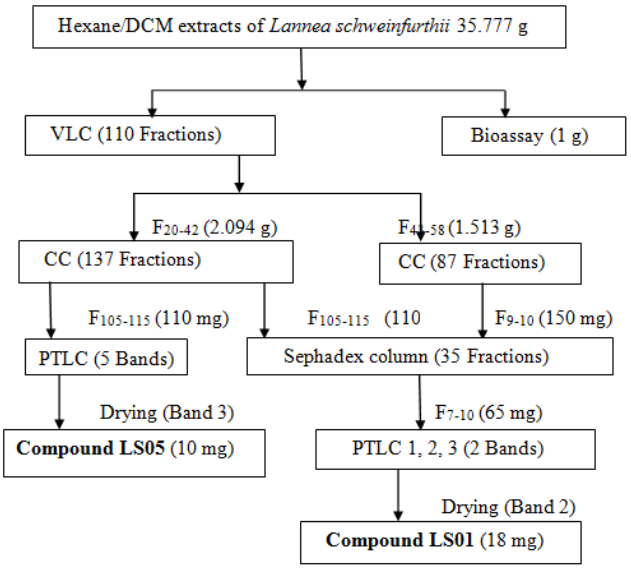


Figure 1. Isolation of compounds LS01 and LS05 from hexane/DCM extract.

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 $F_{20\text{-}42}$ and $F_{43\text{-}58}$ 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 $F_{105\text{-}115}$ and $F_{9\text{-}10}$ 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 $F_{105\text{-}115}$ 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 $F_{177\text{-}210}$ 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 $F_{9\text{-}29}$ 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 $F_{38\text{-}67}$ 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 $F_{9\text{-}29}$ 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.

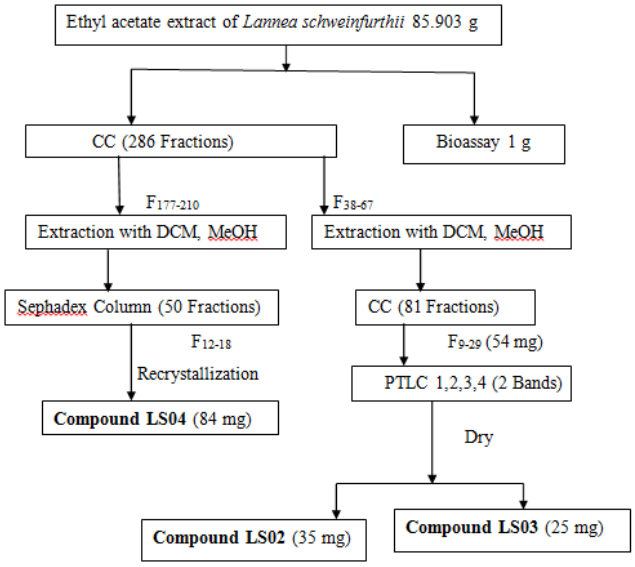


Figure 2. Isolation of compounds LS02, LS03, and LS04 from EtOAc extract.

The fractions F177-210 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 F12-18 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 Rf 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 Rf 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); Rf 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; Rf 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; Rf 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; Rf 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); Rf 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

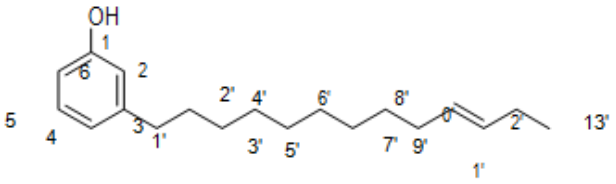
Structure elucidations

Compound LS01

The compound was obtained from hexane/DCM extract as a colorless liquid. On analytical TLC, the compound showed an R_f 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 R_f of 0.57 in 50% DCM in hexane. When the chromatogram was sprayed with anisaldehyde, it changed into purple,

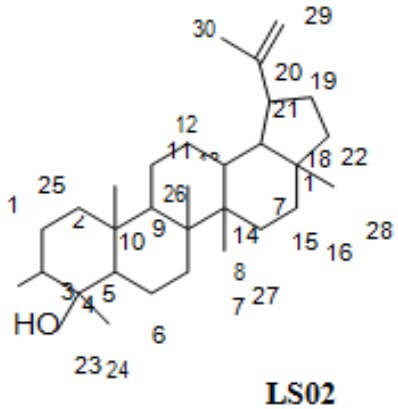
indicating that the compound is a terpenoid. The ^{1}H NMR spectrum showed protons in three regions, namely aliphatic, hydroxylated, and allylic; which strongly indicated that the compound was a triterpenoid. Two downfield signals found at δ 4.80 and 4.68, existing as broad singlets, indicated the existence 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).

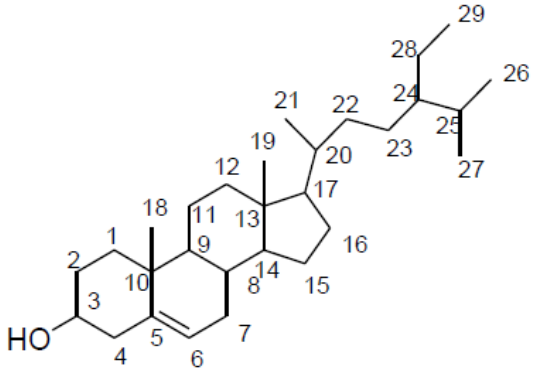
Compound LS03-β-Sitosterol

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			

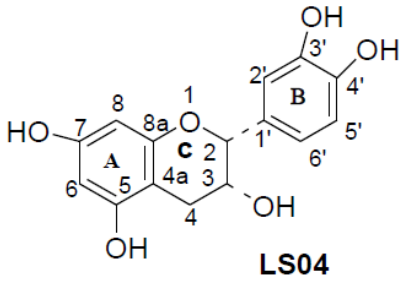
groups were showed by the signals at δ 11.9, 12.0, 18.8, 19.0, 19.4, and 19.8. The overall spectrum of compound LS03 compared carefully to that of β-sitosterol, a sterol that has been described from various plant sources (Dey and Harborne 1991). The ^{13}C NMR data for compound LS03 compared relatively 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	(^{1}H)	-	6.90
5'	6.64	t	(^{1}H)	8.8	6.66
6'	6.64	t	(^{1}H)	8.0	6.66
8	5.87	d	(^{1}H)	1.4	5.89
6	5.69	d	(^{1}H)	1.4	5.72
2	4.65	d	(^{1}H)	4.6	4.74
3	3.99	m	(^{1}H)	-	4.01
4 (ax.)	2.66	dd	(^{1}H)	4.4, 16.6	2.79
4 (eq.)	2.44	dd	(^{1}H)	3.2, 16.6	2.48

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

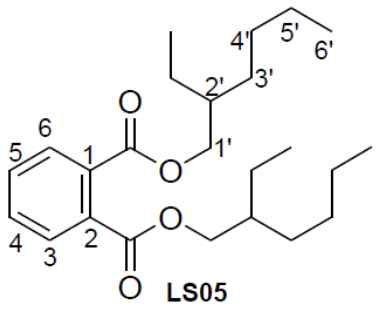
Carbons	Compound LS04 (δ)	DEPT	Epicatechin (δ)
C-4	28.9	CH_2	29.3
C-3	65.6	CH	67.5
C-2	78.7	CH	79.9
C-6	94.7	CH	95.9
C-8	95.7	CH	96.4
C-4a	99.2	C	100.1
C-2'	115.4	CH	115.4
C-5'	115.6	CH	115.9
C-6'	118.6	CH	119.5
C-1'	131.3	C	131.9
C-4'	145.1	C	145.8
C-3'	145.2	C	146.0
C-8a	156.4	C	157.4
C-7	156.9	C	157.7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

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Antibacterial activity of *Plantago major* leaves against *Streptococcus pyogenes* ATCC 19615 as a cause of tonsilitis

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Abstract. *Astuti AD, Etikawati N, Pangastuti A. 2020. Antibacterial activity of Plantago major leaves against* Streptococcus pyogenes *(ATCC 19615) as a cause of tonsilitis. Bioteknologi 17: 14-21.* Lymphatic organs are organs that play a role in the immune system. One such lymphatic organ is tonsils which are in the esophagus. Tonsils are for the body's defense against infection. Tonsils can become inflamed from bacteria or viruses. One of bacteria that causes tonsillitis is *Streptococcus pyogenes*. The prevalence of tonsillitis in various countries, especially developing countries, is high. Therefore, prevention and treatment must be carried out appropriately with the use of natural materials for treatment continues to be developed. *Plantago major* L. is a weed that has antibacterial bioactivity, so it potentially can become a tonsillitis drug. This study aims to determine the effect of variations in the concentration of *P. major* ethanol extract on the growth of *S. pyogenes* ATCC 19615 bacteria and the class of chemical compounds of *P. major* ethanol extract. The antibacterial activity test used a completely randomized design (CRD) with 7 treatment groups, namely 5 variations in the concentration of *P. major* ethanol extract, a positive control group, and a negative control group with three repetitions. Variations in concentration of *P. major* ethanol extract, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL as much as 30 µl with positive control of Bacitracin disc and negative control of DMSO 10% 30 µl. The method used was the well method and the clear zone was measured using a caliper. Data analysis was carried out qualitatively and quantitatively using the Analysis of Variance One-Way Anova using SPSS. Based on the One-Way Anova test and continued with Duncan's test, a significance value of 0.000 <0.05 was obtained, thus it was said to be significant. The ethanolic extract of *P. major* was able to inhibit the growth of *S. pyogenes* ATCC 19615 at concentrations of 250 mg/mL, 500 mg/mL, and 750 mg/mL, and 1000 mg/mL. Based on the thin layer chromatography test, the ethanolic extract of *P. major* contains a class of flavonoid compounds, alkaloids, steroids, phenols, tannins, and terpenoids.

Keywords: Extract, *Plantago major*, *Streptococcus pyogenes*, tonsillitis

INTRODUCTION

Lymphatic organs are organs that play a role in the immune system, one of which is the tonsils located in the esophagus (Schedlowski and Tewes 1999). Tonsillitis is inflammation of the tonsils caused by a bacterial or viral infection. Chronic tonsillitis, which occurs frequently in children, is caused by improper antibiotic treatment (Kurien et al. 2003). Tonsillitis sufferers in Indonesia numbered roughly 23% in 2012, according to data from the Ministry of Health of the Republic of Indonesia. While ENT patients in 2012 were 3.8% for chronic tonsillitis, according to epidemiological data (Zuhdi et al. 2020).

According to WHO, Acute Respiratory Infection (ARI) is one of the diseases that can cause death, especially in children in developing countries. The prevalence of ARI in Indonesia is very high, around 21.6%, especially in urban areas. From 2000 to 2003, the number of children under five with ARI remained stable even though the ARI eradication program had been launched (Wahyuningsih et al. 2017). There is a prevalence of tonsillitis from different countries. In 1998-2007 there were 15,067 cases with a prevalence of 22% in Islamabad, Pakistan (Awan et al. 2009). The prevalence of chronic tonsillitis in the United States in 1995 was 0.7% (Novel 2010). Research on chronic tonsillitis in Russia conducted at the age of 1-15 years obtained data that as many as 84 (26.3%) were diagnosed with chronic tonsillitis and of whom had a history of tonsillectomy (Khasanov et al. 2006).

Streptococcus pyogenes is a common cause of bacterial infection in humans. When the body's defensive system weakens, these bacteria target the throat and skin, causing diseases including pharyngitis, scarlet fever, and impetigo. Invasive illnesses caused by *S. pyogenes* include muscle inflammation, bone infections, meningitis, and endocarditis (Cunningham 2000). Penicillin, beta-lactam, tetracycline, and macrolide antibiotics are all resistant against *S. pyogenes* (Pires et al. 2009).

Tonsillectomy and tonsillotomy are surgeries for treating tonsillitis that induce pain and bleeding, making them less safe, because blood volume is still low and harmful for the respiratory system at a young age (Stelter 2014). Antibiotics can be beneficial when used correctly, but if they are not used correctly (irrational prescribing), it can result in health and economic losses (Utami 2012). If the first line of antibiotics fails, the second line, third line, and so on must be used instead, which are still expensive but are more likely to result in microorganism immunity, making treatment difficult (Apua 2011). This is the basis for the need to explore natural ingredients with medicinal properties.

The broad-leaved plantain (*Plantago major* L.) is one of the medicinal plants. This plant is found in tropical and mountainous areas (Pangemanan 1999; Padua et al. 1999). The roots, stems and leaves are used as cough medicine, dysentery medicine, wound dressing, urinary stones, diabetes, gallstones, leprosy, and abdominal pain (Anon 1995). Van Stenenis-Kruseman (1963), explained that *P. major* extract was used to treat whooping cough. Research from Sharifa et al. (2008), showed that the ethanol and methanol extracts of *P. major* had antibacterial activity for both gram-negative and gram-positive bacteria. Research on the antibacterial activity of the ethanolic extract of *P. major* on *S. pyogenes* ATCC 19615 has not been carried out. Therefore, scientific research is needed to prove the antibacterial activity of *P. major*.

MATERIALS AND METHODS

Plant material

The plant material needed was *P. major*; samples collected were from plants which had flowered. This plant was obtained from Ampel Gading Hamlet, Kenteng Village, Bandungan District, Semarang Regency, Central Java. The leaves were the part of the plant that was used.

Experiment design

This study used a completely randomized design (CRD) with seven treatment groups, each with three replicates, including five different concentrations of *P. major* L ethanol extract, a positive control group, and a negative control group. Variations in the concentration of *P. major* ethanolic extract were 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL, each 30 l, with a positive control of Bacitracin antibiotic disc and a negative control of DMSO 10% 30 µl. Determination of concentration variations was based on the results of research by Razik et al. (2012).

Extraction

As much as 2.35 kg of *P. major* leaves were dried in a 50°C oven for 24 hours (Irianti et al., 2018). To make powder, the leaves were pulverized in a blender (simplicia). *P. major* leaves and stems, weighing up to 200 grams, were macerated for 5 days in 1000 cc of 96% ethanol (1:5). During the 5-day period, the solvent was changed only once. After that, it was filtered with Whatman No. 42 paper and evaporated at 50°C with a rotary evaporator (Dewi et al. 2019). At 40°C, the extract was thickened in a water bath (Irianti et al., 2018). The extract was placed in an evaporating dish and kept at 4°C in the refrigerator (Razik et al. 2012).The thick extract was weighed, and the yield was calculated using the formula:

$$\text{Yield} = \frac{\text{thick extract weight (g)}}{\text{sample weight (g)}} \text{ x } 100\ \% \text{ (Dewatisari } et\ al.\text{, 2017)}$$

Antibacterial activity test

Making Mueller Hinton blood agar media

The Nutrient Agar (NA) media was weighed to 10 grams and 2.5 grams of NaCl was added. This was then dissolved in 500 mL of distilled water, for the pH was measured to show the number 7. After that it was boiled before the solution was sterilized using an autoclave at a temperature of 121°C and a pressure of 1 atm for 15 minutes. After the media was sterile and the temperature drops to about 50°C, 20-25 mL of sheep's blood was added. Media was then poured into a petri dish (Haerazi et al. 2014).

Rejuvenation of Streptococcus pyogenes *ATCC 19615*

Mueller-Hinton Media Sterile blood agar was prepared in a petri dish and then a single loop of *S. pyogenes* ATCC 19615 was taken and streaked on the media using the streak plate method. After that, it was incubated for 24 hours at 37°C (Wirdia et al. 2017).

Antibacterial activity test of Plantago major *leaves against Streptococcus pyogenes ATCC 19615*

The antibacterial activity test was carried out in vitro with the well method and then the zone of inhibition of bacterial growth was determined. The concentration of *P. major* extract was varied, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL with DMSO 60% as a solvent (Razik et al. 2012). The wells with a size of 6 mm were dripped with 30 l of extract solution. The positive control used Bacitracin discs, while the negative control used 60% DMSO. The next stage was incubation for 24 hours at 37°C. Bacterial growth was observed and the clear zone was measured using a caliper (Octaviani et al. 2019).

Phytochemical screening of ethanol extract of *Plantago major* leaves

The active compound content of the ethanolic extract of *P. major* was carried out using the Thin Layer Chromatography (TLC) method. The stationary phase was a silica gel plate G60 F254 with a length of 10 cm and a width of 2 cm. Silica gel was washed with 96% ethanol then activated in an oven at 100ºC for 60 minutes. The extract was dissolved in 96% ethanol until it was watery and then it was spotted on the stationary phase (Yuda et al. 2017).

Flavonoid test

The mobile phase used was n-hexane:ethyl acetate:methanol (4:5:1) with $AlCl_3$ as a stain. A positive reaction is indicated by the presence of a yellow to greenish-yellow stain. This indicates the presence of flavonoid compounds (Mabruroh et al. 2019; Ahmad et al. 2015).

Alkaloid test

The mobile phase used was chloroform: ethanol (24:1) with Dragendorff's stain. After being sprayed with Dragendorff, it could shows yellow or bluish spots. This

indicates the presence of alkaloid compounds (Pratita 2017).

Steroid test

The mobile phase used was chloroform: methanol (9:1) with Liebermann-Burchard reagent stains visible and then heated at 105ºC for 5 minutes. A positive reaction is indicated by the presence of a blue green stain (Kristanti et al. 2008).

Phenolic test

The mobile phase used was n-hexane:ethyl acetate (3:7) with 5% $FeCl_3$ stain. After that, it was observed with UV light at a wavelength of 254 nm and UV 366 nm. The presence of phenolic compounds would be indicated by a change in the color of the stain to blue-black or bluish-green (Fajriaty et al. 2018).

Terpenoid test

The mobile phase used was n-hexane:ethyl acetate (6:4) with a Liebermann-Burchard stain. After that, it was heated for 5 minutes at 105ºC. The presence of terpenoids would be indicated by the formation of a blue-violet or red-violet color (Hanani 2015).

Tannin test

The mobile phase used was methanol: ethyl acetate (7:3) with 5% $FeCl_3$ stain. The presence of tannin compounds would be indicated by a blue-black color and condensed tannins by the formation of a green-brown stain (Banu and Nagarajan 2014).

Observation and data collection

Observation of the antibacterial activity test was in the form of the diameter of the inhibition zone around the well after 24 hours of incubation. In the characterization of each class of chemical compounds, the color change of each spot was observed after the reagent was sprayed. In the phenol compound test, after spraying the reagent, the spot was observed with UV light at a wavelength of 254 nm and UV 366 nm.

Data analysis

The data obtained was analyzed using quantitative and qualitative analysis. Qualitative analysis consisted of data on chemical compound content of *P. major* ethanol extract and contact bioautography test, which were analyzed descriptively with data in the form of tables and figures. Quantitative analysis is data on bacterial inhibition zones. In the quantitative test, the data was analyzed using the Analysis of Variance One-Way Anova using SPSS. If there was a difference, it is continued with further testing using Duncan's test with an α=0.05 (Haerazi et al. 2014).

RESULTS AND DISCUSSION

Extraction

Plantago major samples for extraction (Figure 1) were obtained from Ampel Gading Hamlet, Kenteng Village, Bandungan District, Semarang Regency with an altitude of approximately 1300 meters above sea level (masl). The criteria for selecting *P. major* plants are those that have flowered. The parts used for extraction was all parts of the leaf. The *P. major* extraction method used the maceration method with 96% ethanol solvent. In this study, ethanol solvent was used because it is relatively safe and can attract most of the active compounds in plants so that the optimal amount of active ingredients is produced (Sulastri and Oktaviani 2015). The viscous extract obtained from the extraction process using the maceration method was 16.098 grams, while the yield in the ethanol extraction process of the leaves and stems of *P. major* was 8.049%.

Antibacterial activity test of *Plantago major* ethanol extract against *Streptococcus pyogenes* ATCC 19615

In the antibacterial activity test, 5 variations of the concentration of *P. major* ethanol extract were made, namely 125 mg/mL, 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL with Bacitracin positive control and 60% DMSO negative control. The results of the inhibition are shown in Table 1 and Figure 2.

Table 1. The average diameter of the inhibition zone of the ethanolic extract of *Plantago major* against *Streptococcus pyogenes* ATCC 19615 with an incubation time of 24 hours.

Concentration (mg/mL)	Average diameter of inhibition zone (mm)
125	0
250	7,14
500	8,48
750	8,85
1000	10,57
Positive control (Bacitracin)	16,61
Negative control (DMSO 60%)	0

Figure 1. *Plantago major* L. growing in Ampel Gading Hamlet (Private collection 2020).

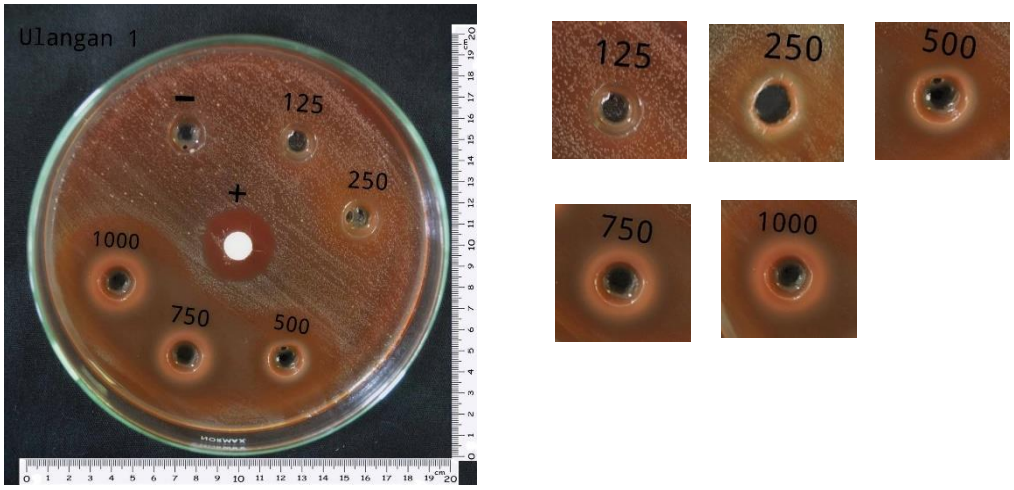

Figure 2. The results of the inhibition zone of *Plantago major* ethanol extract against *Streptococcus pyogenes* ATCC 19615 with an incubation time of 24 hours, the inhibition zone at a concentration of 125 mg/mL, the inhibition zone at a concentration of 250 mg/mL, the inhibition zone at a concentration of 500 mg/mL, the inhibition zone at a concentration of 750 mg/mL, the inhibition zone at a concentration of 1000 mg/mL.

Based on the results of One-Way Anova data analysis, there were significant differences in the diameter of the inhibition zone between the treatment groups. Data analysis continued with Duncan's test and the results showed that the negative control group (DMSO 60%) and the concentration of the ethanol extract of *P. major* 125 mg/mL were not significantly different, which indicated that the concentration had no activity to inhibit the growth of *S. pyogenes* ATCC 19615. Concentrations of 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL had inhibitory activity against the growth of *S. pyogenes* ATCC 19615, but the zone of inhibition was not as large as the positive control group (Bacitracin).

Bacitracin, according to Zaidi et al. (2020), has good antibacterial efficacy against gram-positive bacteria like *Streptococcus mutans*. The process works by preventing the formation of biofilms, which causes bacterial shape to change and cell wall synthesis to be inhibited. Bacitracin has the potential to destroy nucleic acids, hence it is frequently used for antimicrobial therapy (Ciesiolka et al. 2014).

Previous research conducted by Razik et al. (2012), showed methanol extract of *P. major* showing a zone of inhibition in gram-positive bacteria *Lactobacillus* sp. at a concentration of 125 mg/mL-1000 mg/mL and *Staphylococcus aureus* at a concentration of 250 mg/mL-1000 mg/mL. Based on the results of the study, the higher the concentration of the ethanol extract of *P. major*, the greater the zone of inhibition. Supported by the statement of Haerazi et al. (2014), that the higher the concentration of the extract, the higher the content of chemical compounds and more diffuse in the bacterial culture.

The ethanol extract of *P. major* is thought to be able to inhibit *S. pyogenes* ATCC 19615 because it contains flavonoid compounds, phenols, tannins, and terpenoids that work synergistically. Supported by research by Dewi et al. (2019), that the phenolic compounds, flavonoids, tannins, and saponins contained in the ethanol extract of *P. major* work synergistically when combined so that they are more effective at inhibiting bacteria. Sharifa et al. (2012), stated that the ethanolic extract of *P. major* caused cell shrinkage, indentation, and cell shrinkage in gram-positive (*Streptococcus aureus*) and gram-negative (*Eschericia coli*) bacteria. Supported by research by Wijesundara and Rupasinghe (2019), phytochemical compounds such as the flavonoid group, namely isoflavonoids, flavones, and phenolic compounds from licorice ethanol extract have antibacterial activity against *S. pyogenes*. Nzeako et al. (2006), also reported that clove leaf extract (*Syzygium aromaticum*) containing terpenoids, flavones, and phenolic compounds was also reported to have antibacterial activity against *S. pyogenes*.

The mechanism of inhibition of flavonoid compounds (isoflavonoids), flavones, and phenolic compounds begins with the entry of compounds through the peptidoglycan of the *S. pyogenes* bacteria; which can affect the cytoplasmic membrane resulting in structural changes in the membrane, including changes in fluidity, changes in the outer surface of the cell wall due to leakage of cytosolic fluid. This leak resulted in morphological changes to the death of *S. pyogenes* bacterial cells (Wijesundara and Rupasinghe 2019). Limsuwan et al. (2012), also mentioned that cell leakage was caused by weakening of the cell wall due to osmotic pressure during incubation.

Phytochemical screening of ethanol extract of *Plantago major* leaves

Phytochemical screening of the ethanol extract of the leaves and stems of *P. major* L. showed that the extract contained alkaloids, flavonoids, steroids, phenols, tannins, and terpenoids. According to research by Dewi et al. (2019), based on the UV-Vis Spectrophotometry test, the ethanolic extract of *P. major* L. contains phenols and flavonoids (flavones, flavanols, and aurons). While based on the analysis of phytochemical compounds, *P. major* L.

contains phenols, flavonoids, saponins, and tannins. According to research Adom et al. (2017), groups of chemical compounds in *P. major* L. are flavonoids, alkaloids, terpenoids, phenols, iridoid glycosides, fatty acids, polysaccharides, and vitamins.

Alkaloid test

A group of alkaloid compounds contained in the ethanol extract of *P. major* was detected with Dragendorff's reagent. The results of spraying reagents can be seen in Figure 3.

The mobile phase used was chloroform: ethanol (24:1). After spraying Dragendorff's reagent, spot C (Rf 0.71) changed color from yellow to bluish which indicates that the ethanol extract of *P. major* contains alkaloids. Based on the results of the elution, there were four spots (spots), namely spot A (Rf 0.23), spot B (Rf 0.34), spot C (Rf 0.71), and spot D (Rf 1.03).

The color change in the spot occurs because the alkaloid compound has a nitrogen group and there is one pair of free electrons, so that this compound can bind positively charged metal ions and then form complex compounds. Dragendorff's reagent for the alkaloid test was made using nitrogen to form a coordinate covalent bond with K+ which is a metal ion so that the stain color changes to yellow or bluish (Figure 4) (Marliana et al, 2005). The group of alkaloid compounds has biological activities, namely antimalarial, anticancer, antimicrobial, antioxidant, and anti-inflammatory (Ernawati et al. 2018).

Since the alkaloid compound has a nitrogen group and one pair of free electrons, the spot changes color. This allows the molecule to attach positively charged metal ions and build complex compounds. Dragendorff's reagent for the alkaloid test uses nitrogen to establish a coordinating covalent bond with K^+, a metal ion, causing the stain to turn yellow or bluish in color (Figure 4) (Marliana et al. 2005). Antimalarial, anticancer, antibacterial, antioxidant, and anti-inflammatory properties are among the biological activities of alkaloid substances (Ernawati et al. 2018).

Flavonoid test

The group of flavonoid compounds contained in the ethanol extract of *P. major* was detected with $AlCl_3$ reagent. The results of spraying reagents can be seen in Figure 5.

The mixture of n-hexane:ethyl acetate:methanol was utilized as the mobile phase (4:5:1). The color of spot E (Rf 1.01) changed to yellow after spraying with $AlCl_3$ reagent, showing that the ethanol extract of *P. major* contains a family of flavonoid chemicals. There were six areas identified based on the elution results: spot A (Rf 0.25), spot B (Rf 0.5), spot C (Rf 0.6), spot D (Rf 0.66), spot E (Rf 1, 01), and spot F (Rf 1, 01). (Rf 1.05).

The color change in the spot occurs due to a chemical reaction with AlCl3 reagent. This reagent will react with flavonoids to form complex compounds. The AlCl3 reagent detects the presence of an o-hydroxy group on the C-4 atom and an o-hydroxy ketone group on the C-3 or C-5 atom (Figure 6), therefore there will be a color change in the stain to yellow (Azizah et al. 2014). According to Saravanakumar et al. (2009), flavonoids have pharmacological, antimicrobial, antioxidant, cytotoxic, and antiproliferative activities.

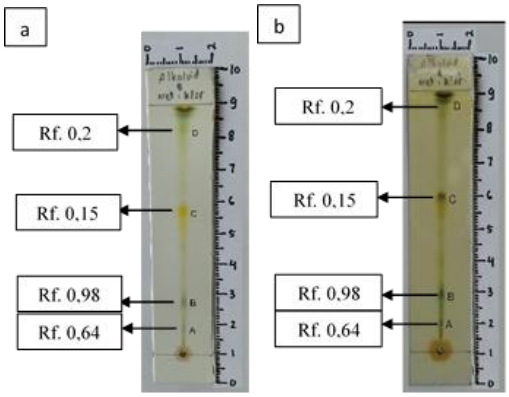

Figure 3. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and chloroform: ethanol (24:1) mobile phase, a. Before spraying Dragendorff's reagent, b. After spraying Dragendorff's reagent.

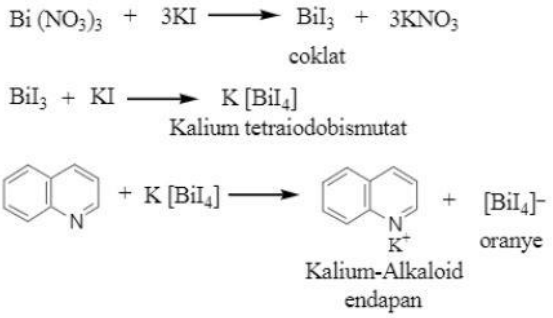

Figure 4. Dragendorff reagent reaction (Marliana et al. 2005).

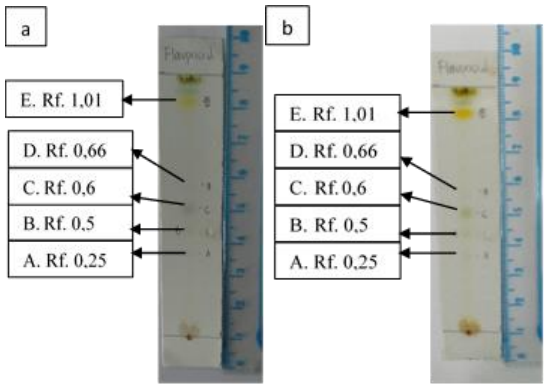

Figure 5. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and n-hexane:ethyl acetate:methanol (4:5:1) mobile phase, a. Before spraying $AlCl_3$ reagent, b.After spraying $AlCl_3$ reagent.

Steroid test

The group of steroid compounds contained in the ethanol extract of *P. major* was detected with Liebermann-Burchard reagent, accompanied by heating at a temperature of 105ºC for 5 minutes. The results of spraying reagents can be seen in Figure 7.

The mobile phase used was chloroform: methanol (9:1). The color change in the spot occurs due to the extension of the conjugation caused by the release of the hydrogen group when the steroid compound reacts with the Liebermann-Burchard reagent (Siadi 2012). Based on the results of the elution, there were six spots (spots) namely spot A (Rf 0.16), spot B (Rf 0.46), spot C (Rf 0.71), spot D (Rf 0.8), E (Rf 0.86), and F (Rf 0.92). The steroid compound group has antibacterial, antifungal, and antidiabetic activity (Hidayah et al. 2016).

Phenolic test

The group of phenolic compounds contained in the ethanol extract of *P. major* was detected with 5% $FeCl_3$ reagent. The results of spraying reagents can be seen in Figure 8.

The mobile phase used was n-hexane:ethyl acetate (3:7). After being sprayed with 5% FeCl3 reagent, there was a color change in spots B (Rf 0.35) and C (Rf 1.01) from yellow to blackish blue, indicating that the ethanol extract of *P. major* contains a group of phenolic compounds. The color change occurs due to the formation of complex compounds between phenol and Fe, which are metal elements (Wardhani and Sulistyani 2012).

Based on the results of the elution, there were four spots (spots), namely spot A (Rf 0.35), spot B (Rf 0.5), spot C (Rf 1.01) and spot D (Rf 1.06). After being observed with UV light at a wavelength of 254 nm, the spot shows a blue-black color while at a wavelength of 366 nm, the spot is not visible. According to Gandjar et al. (2007), stains will appear at wavelengths of 254 nm and 366 nm because these wavelengths interact with the fluorescence indicator of the TLC plate. This plate component has light emission that is emitted when electrons move from low energy to higher energy then return to their original state by releasing energy.

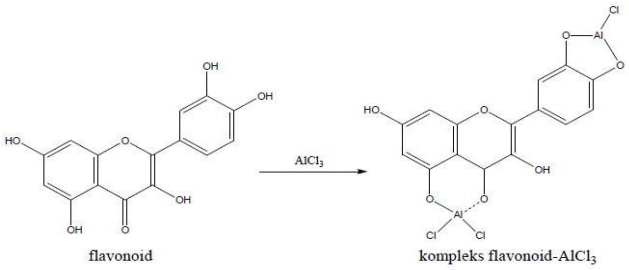

Figure 6. Reaction for the formation of complex compounds between flavonoids and $AlCl_3$ (Dewi et al. 2018).

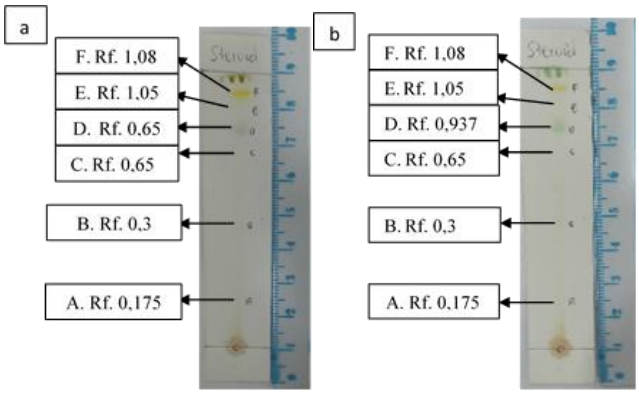

Figure 7. Chromatogram of *Plantago major* ethanolic extract with silica gel plate G60 F254 as stationary phase and chloroform: ethanol (24:1) mobile phase, a. Before spraying Liebermann-Burchard reagent accompanied by heating at a temperature of 105°C for 5 minutes, b. After spraying Liebermann-Burchard reagent then heated at a temperature of 105°C for 5 minutes.

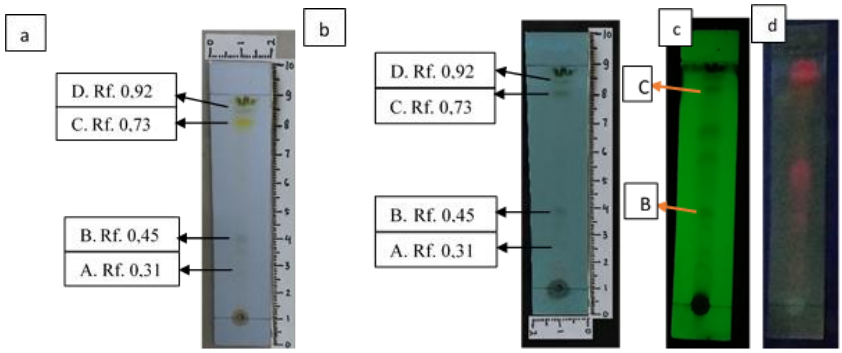

Figure 8. Chromatogram of *Plantago major* ethanol extract with a stationary phase of silica gel plate G60 F254 and a mobile phase of n-hexane: ethyl acetate (3:7), a. Before spraying 5% $FeCl_3$ reagent b. After spraying 5% $FeCl_3$ reagent, c. Fluorescence results at a wavelength of 254 nm after being sprayed with 5% $FeCl_3$ reagent, d. Fluorescence results at a wavelength of 366 nm after being sprayed with 5% $FeCl_3$ reagent.

A spot that glows under UV light at 254 nm shows the presence of two conjugated double bonds in the molecule, according to Alen et al. (2017). Spots that glow under 366 nm UV light have a longer conjugated double bond or chromophore (an organic molecule with a conjugated double bond that absorbs color) and an auxochrome group. Phenolic chemicals, according to Carolia and Noventi (2016), act as antibacterials by denaturing proteins and bacterial cells.

Tannin test

The group of tannin compounds contained in the ethanol extract of *P. major* was detected with 5% $FeCl_3$ reagent. The results of spraying reagents can be seen in Figure 9. The mobile phase used was methanol: ethyl acetate (7:3). After being sprayed with 5% FeCl3 reagent, there was a color change in spot B (Rf 0.81) and C (Rf 0.96) from yellow to blackish, indicating that the ethanol extract of *P. major* contains a class of tannin compounds. Based on the results of the elution, there are three spots (spots): spot A (Rf 0.71), spot B (Rf 0.81), and spot C (Rf 0.96).

The color change in the spot occurs because the $FeCl_3$ reagent reacts with the hydroxyl group on the tannin compound, resulting in hydrolysis of the tannin group and produces a blue-black color change, while the condensed tannin changes color to blackish green (Pardede et al. 2013). According to Sa'adah (2010), tannin and $FeCl_3$ compounds form complex compounds with Fe^{3+} ions. According to Ergina et al. (2014), if the test results for phenol compounds are positive, it is possible that one of the compounds is tannin. This is because tannins are polyphenolic compounds. Maharani et al. (2017) mentioned that tannins have antibacterial, antioxidant, and anti-diarrheal activities.

Terpenoid test

The terpenoid compounds contained in the ethanol extract of *P. major* were detected with Liebermann-Burchard reagent and then heated at 105 ºC for 5 minutes. The results of spraying reagents can be seen in Figure 10.

The mobile phase used was n-hexane:ethyl acetate (6:4). After being sprayed with Liebermann-Burchard reagent and then heated at 105 ºC for 5 minutes, there was a color change in spot E (Rf 0.94), spot F (Rf 1), and spot G (Rf 1.11) from yellow to red-violet. which showed that the ethanol extract of *P. major* contained a class of terpenoid compounds. The color change in the spot occurs due to the extension of the conjugation caused by the release of the hydrogen group when the terpenoid compound reacts with the Liebermann-Burchard reagent (Siadi 2012).

Based on the results of the elution, there were seven spots (spots), namely spot A (Rf 0.54), spot B (Rf 0.65), spot C (Rf 0.7), spot D (Rf 0.84), spot E (Rf 0.94), spot F (Rf 1), and spot G (Rf. 1.11). According to Wu et al. (2020), terpenoids have biological activities such as anti-inflammatory, antimicrobial, anticancer, antioxidant, and immunomodulatory. Irianti et al. (2018), stated that the class of terpenoid compounds affects the permeability of bacterial cell membranes.

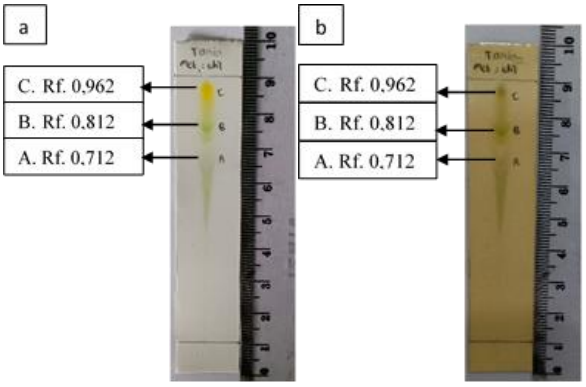

Figure 9. Chromatogram of *Plantago major* ethanol extract with silica gel plate G60 F254 as stationary phase and methanol as mobile phase: ethyl acetate (7:3), a. Before spraying 5% $FeCl_3$ reagent b. After spraying 5% $FeCl_3$ reagent.

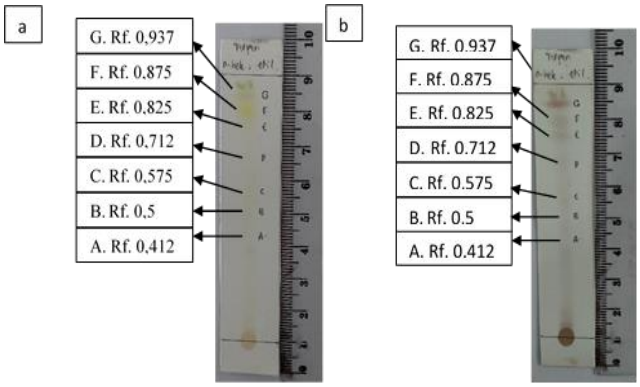

Figure 10. Chromatogram of *Plantago major* ethanol extract with G60 F 254 stationary phase and n-hexane as mobile phase: ethyl acetate (6:4), a. Before spraying Liebermann-Burchard reagent then heated at 105oC for 5 minutes, b.After spraying Liebermann-Burchard reagent then heated at 105ºC for 5 minutes.

The conclusion of this study is that the ethanolic extract of *P. major* at concentrations of 250 mg/mL, 500 mg/mL, 750 mg/mL, and 1000 mg/mL had an inhibitory effect on the growth of *S. pyogenes* ATCC, while at concentrations of 125 mg/mL and DMSO 60% negative control had no effect. Groups of chemical compounds in the ethanol extract of *P. major* based on thin layer chromatography tests are flavonoids, alkaloids, terpenoids, tannins, steroids, and phenols.

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Biogas production from POME (Palm Oil Mill Effluent) with the addition of EPOB compost (Empty Palm Oil Bunches)

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Abstract. *Ramadhani GH, Mahajoeno E, Susilowati A. 2020. Biogas production from POME (Palm Oil Mill Effluent) with the addition of EPOB compost (Empty Palm Oil Bunches). Bioteknologi 17: 22-26.* Palm oil mill effluent (POME) is mostly produced by palm oil factories, resulting in environmental pollution. One strategy is by processing waste into a biogas product. The purpose of this research is (i) to make compost from EPOB to produce good compost; (ii) to determine the level of biogas (CH_4) produced from various combinations of POME substrates. Composting was carried out with six treatments. Each treatment consisted of a composition of materials such as EPOB, mushroom seeds [*Volvariella volvacea* (Bulliard ex Fries) Singer], and POME with different concentrations. The method used in composting is the Berkeley method. The mixed material is put in a trash bag and stirred periodically for two weeks in the composting process. The four treatments conducted the experimental design to determine the biogas (CH_4) level. Each treatment consisted of POME, activated sludge, and compost with different concentrations. The process begins with mixing the material into the digester, then every 10^{th}, 20^{th}, and 30^{th} day the gas is taken to measure the CH_4 content. CH_4 levels were obtained by taking gas with a syringe needle on the cover of the digester, then transferred to a flacon bottle. The flacon bottle containing the gas was measured by the Gas Chromatography and Mass Spectroscopy (GCMS) method. The GCMS gas detector is equipped with an FID to detect gas levels and gas type. The results will be displayed in the form of GCMS output and can be seen from the residence time range to determine the type of gas and concentration. The study resulted in the best compost in treatment five, which looks good from a physical point of view, with a 48% water content and 18.25% C/N ratio. The biogas content (CH_4), which produces the highest gas, is 36.206% in treatment 2 (T2) with a composition of 70% POME, 20% activated sludge, and 10% compost.

Keywords: Biogas, compost, empty palm oil bunches, methane, POME (Palm Mill Oil Effluent)

INTRODUCTION

Palm oil is one of the main commodities of Indonesia (Arifiyanto et al., 2017; Faizal and Emdi, 2017). The palm oil industry in Indonesia shows rapid development, resulting in environmental problems. These environmental problems lead to pollution originating from the palm oil industry waste. Palm oil mill waste is very abundant. It is estimated that palm oil mill waste in Indonesia reaches 28.7 million tons of liquid waste/year and 15.2 million tons of solid waste/year (Pardamean 2017).

One type of liquid waste from the palm oil industry is POME (Palm Mill Oil Effluent). In addition, solid waste originating from the palm oil processing process consists of empty oil palm bunches (EPOB), shells, fibers, mud, and cake. One of the solid wastes used in this research is EPOB which is composted and is expected to increase biogas production. The addition of activated carbon helps increase the C/N ratio, improving the anaerobic digestion process and obtaining optimum conditions for CH_4 gas to increase production (Ritonga and Masrukhi 2017). Compost is made with the main raw materials from EPOB, POME, and mushroom [*Volvariella volvacea* (Bulliard ex Fries) Singer] seeds. The addition of edible mushroom seeds belonging to the WRF (White Rotting Fungus) group plays a role in degrading lignin and the main polymers such as cellulose and hemicellulose in EPOB (Nasrul and Maimun 2009).

Anaerobic fermentation to produce biogas is carried out in 4 stages: hydrolysis, acidogenesis, acetogenesis, and methanogenesis (Gijzen 1987). The results of this biogas production can be in the form of gases such as methane (CH_4) (50-75%), CO_2 (25-45%), and small amounts of H_2, N_2, H_2S, CO, H_2O, O_2, dust (Hosseini et al. 2015). This research only focused on producing methane gas using the GCMS gas chromatography method, and it can be seen how much methane gas content is produced. The results of the methane gas produced can later be used for further research on the use of methane gas as alternative energy.

MATERIALS AND METHODS

Research site

This research was conducted in a palm oil mill, namely PT. Swasti Siddhi Amagra, Libo Jaya Village, Kandis Sub-district, Siak District, Riau, Indonesia. Then, continued research in the laboratory located at the factory. The research was carried out in February-March 2019.

Ingredient

This study used the main raw materials in POME, activated sludge, EPOB compost, and *V. volvacea* mushroom seeds obtained from Solo Mushroom, Karanganyar, Central Java, Indonesia. The chemicals used were a solution of H_2SO_4, H_3PO_4, H_3BO_3 4%, HCL, ammonium iron (ii) sulfate, distilled water, NaF solution 4% diphenylamine indicator.

Research design

The experimental design used in this study uses a completely randomized design (CRD) to prove that different substrate compositions produce the best results in producing biogas or methane concentrations. Completely randomized design (CRD) with 4 treatments and 2 replications including control treatment i.e., 100% POME (C), first treatment, i.e., 80% POME, 15% activated sludge and 5% compost (T1), second treatment, i.e., 70% (POME), 20% activated sludge and 10% compost (T2), the third treatment, i.e., 60% (POME), 25% activated sludge and 15% compost (T3).

Procedure

This study uses POME as the main medium for biogas products. The source of inoculum comes from sludge (activated sludge) and the addition of compost. In this study, a simple digester with a volume of 1 liter was used, i.e., 850 mL of the digester volume, which was used as the working volume, and the remaining 15% was used for the air volume.

Composting. Compost was made using the Berkeley method from EPOB fiber, mushroom seeds (*V. volvacea*), and POME with six different treatments. They were put into sacks or trash bags and then covered and perforated. Measurements were made on physical characteristics and temperature on the 3rd, 5th, 7th, 10th, and 14th days. The mature compost after 14 days was measured in water content and C/N ratio. Compost was made into six treatments, namely compost I consisting of 900 grams of EPOB, 0 grams of mushroom seeds, and 270 mL of POME; compost II consisting of 900 grams of EPOB, 30 grams of mushroom seeds, and 270 mL of POME; compost III consisting of 900 grams of EPOB, 60 grams of mushroom seeds, and 270 mL of POME; compost IV consisting of 900 grams of EPOB, 0 grams of mushroom seeds, and 450 mL of POME; compost V consisting of 900 grams of EPOB, 30 grams of mushroom seeds, and 450 mL of POME, and compost VI consisting of 900 grams of EPOB, 60 grams of mushroom seeds, and 450 mL of POME.

The process of inserting materials. The characteristic measurement of POME includes the C/N ratio as a first step to producing good biogas (20-30%). Then insert the material into a simple digester circuit.

Measurement of biogas levels. The gas is taken using a syringe needle and then put into a flacon, then closed with an airtight rubber cover and wrapped in plastic wrap. Gas samples were measured using the gas chromatography method with a GCMS device equipped with FID as a detection sensor. The gas that has been detected can be seen on the monitor in the form of an output containing peak, area, and residence time to find out what type of gas and what concentration.

RESULTS AND DISCUSSION

EPOB compost results

Composting that has been carried out for 14 days can be seen in terms of physical characteristics, C/N ratio, and water content according to SNI. From the six compost treatments, the best compost is selected. The best compost will be selected as an additional substrate for making biogas. The compost results on the 14th day can be seen in Table 1.

Based on SNI 19-7030-2004, mature compost, in terms of physical characteristics, has an earthy odor (odorless), dark brown, easy to crush, and a temperature similar to groundwater (about 25-29ºC). Meanwhile, the C/N ratio is 10-20%, and the water content does not exceed 50%. It can be concluded that the compost, according to SNI, is compost V. Compost V is categorized as the best compost and is used to add substrate for biogas production). Compost V showed a faster temperature increase than other composts from day 3rd to day 7th, around 29-40ºC. The increasing temperature results in the development of thermophilic bodies known to have the ability to remodel recalcitrant compounds such as lignin. Then the temperature decreases again, and the composting process enters the maturation phase (Strom 1985).

Table 1. The physical character of compost on day 14th

Compost	Physical character	Temperature (ºC)	C-Organic	Total N	Water content
I	Musty smell, not easy to crumble, light brown	35	35,67%	1,48	58%
II	Musty smell, not easy to crumble, brown	31	35,00	1,55	54.2%
III	Smells like earthy odor, crumbles easily, dark brown	28	32,02	2,00	58%
IV	Musty smell, not easy to crumble, light brown	33	27,56	1,50	51.8%
V	Earthy odor, crumbles easily, dark brown	29	26,62	1,46	45%
VI	Earthy odor, not easy to crumble	34	25,13	1,55	46.4%

The C/N ratio in Table 1 indicates that compost containing white-rot fungus media (*V. volvacea*) at a concentration of 60 grams in compost III and VI can effectively reduce the C/N ratio, as Nasrul and Maimun (2009) stated that the effect of adding white-rot fungi causes the C/N ratio to decrease more rapidly, indicating that the composting process was proceeding normally, show by the decomposition of organic materials into compost with a low C/N ratio. The use of edible mushroom (*V. volvacea*), which belongs to the white-rot fungus (WRF) division of Basidiomycetes, could produce ligninolytic enzymes extracellularly that would degrade and reduce lignin levels (Kaal et al. 1995).

Compost IV that was not given with white-rot fungi could reduce the C/N ratio even though the ratio of C/N produced was not as low as that of compost V and VI, which were given with mushrooms. It may be because the addition of mushrooms was less influential. After all, the composition of POME was too much, namely 450 mL, and in its contents, some microorganisms play a role in decomposing cellulose in the EPOB composting process, namely *Bacillus* sp. and the genus *Aspergillus* (Rajagukguk 2018). Although compost IV, in terms of the C/N ratio, is per SNI, in terms of physical characteristics, it does not guarantee that compost IV is ripe because of the smell, which is still very pungent and musty. The condition of the compost is still moist.

Biogas production and methane concentration

The process of biogas formation in the anaerobic fermentation of organic matter is carried out by the microorganism Archaea methanogens to produce combustible gas because it contains methane (CH_4). The reshuffle process was carried out using a simple digester on a laboratory scale for 30 days of observation time, and data were taken every 0th, 10th, 20th, and 30th day. The average volume of biogas is presented in Figures 1 and 2.

Figure 1 has an increase in volume between treatments in producing biogas. The variation of the biochemical properties of the substrate causes the biogas production also to vary (Irvan et al., 2012). Several organic materials can be used together with some suitable gas production or normal growth requirements of methane bacteria. Some of the properties of these organic materials have a significant impact on the gas production rate. The increase in biogas volume is due to the different substrates of each treatment. Treatment 2 (T2) had a higher increase on day 30th than other treatments, i.e., 593 mL. It was in line with the methane formation, i.e., 36.21%. The organic matter with a concentration equal to the inoculum degrades faster. It facilitates the diffusion of dissolved materials, resulting in faster gas formation. Mahajoeno et al. (2008) research revealed that a 20% concentration of activated sludge inoculum with 15 liters of substrate produced the most gas compared to other concentrations. In addition to the volume of biogas formed, the methane concentration results after being tested by GCMS gas chromatography can determine the content of methane gas. The following is a graph of the concentration of methane gas presented in Figure 2.

The methane gas produced is also not too large, only 36.21% at T2. In this study, only a small-scale digester was used, temperature stability was not maintained, and periodic shaking was not carried out. However, in Sarono et al.'s (2016) research, the concentration of methane gas from the main ingredient POME produced by high-temperature treatment of 55°C could produce a methane concentration of 65.44%. Data on biogas volume and methane concentration were also statistically tested with SPSS using GLM repeated measures independently. Hypothesis testing was used to determine whether there was a significant difference in the volume of biogas and methane concentration on the 10th, 20th, and 30th days of extraction. The hypothesis testing was carried out to determine whether there was a statistically significant difference on the 10th, 20th, and 30th day of extraction and obtained a sig value of 0.02 for the volume of biogas and 0.014 for the concentration of methane. Because the sig value was less than 0.05, it was concluded that the data of biogas volume and methane concentration with extraction time on the 10th, 20th, and 30th days are statistically significant differences.

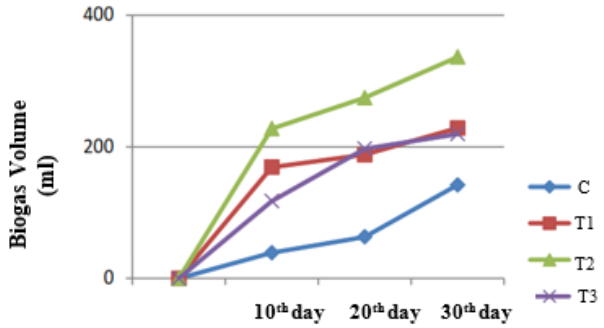

Figure 1. Volume of biogas formed up to day 30th. C: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; T3 POME 60% + Activated sludge 25% + Compost 15%

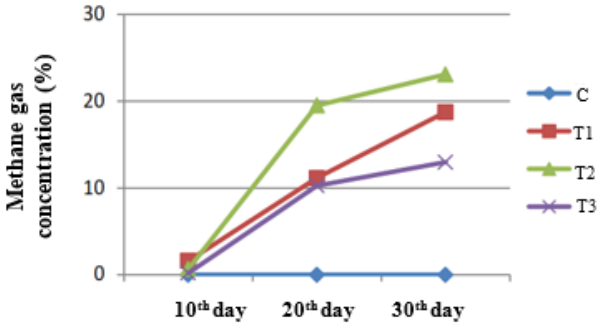

Figure 2. Concentration of methane formed up to day 30th. K: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; T3 POME 60% + Activated sludge 25% + Compost 15%

COD and BOD concentration

The Chemical Oxygen Demand (COD) and Biochemical Oxygen Demand (BOD) values are important parameters determining the overhaul or degradation of waste organic matter. The effect of the substrate concentration can also affect the value of COD and BOD resulting from the process of overhauling organic matter in the waste. The COD values are presented on average in Figure 3.

The decrease in COD value is due to the hydrolysis process, organic material being remodeled as nutrients for organisms and then converted into simpler compounds (Kresnawaty 2008). The highest COD reduction or reduction occurred on days 10th to 30th, 17.60% in T2. The lowest occurred on days 0th to 10th, 0.25% in the control treatment (C). The resulting BOD value is also shown in the decrease in the BOD value in Figure 4.

The highest degradation efficiency value of BOD reduction was 5.95% in T2, and the lowest was 0.15% in the control treatment. It shows that microorganisms that work optimally degrade because the longer residence time provides a longer contact opportunity between the substrates; thus, the degradation efficiency process is better than at other times. There was no inoculum in the form of activated sludge in the control treatment, a source of microorganisms that can help break down organic material into simple molecules. Based on COD and BOD data. The longer the residence time will increase the efficiency of the degradation itself. The longer the contact time between organic waste and biomass, the degradation process of organic pollutants can last longer and make the substrate COD and BOD concentration lower (Munazah and Soewando 2008). An increasingly alkaline pH value can cause the efficiency of COD and BOD degradation to decrease in efficiency in several treatments, which makes the decomposition process not reach the perfect methanogenic stage. At this stage, there is a process of reshuffling organic matter into acid, then acetic acid, and forming methane gas (CH_4), resulting in a decrease in COD and BOD values (Adekunle and Okolie 2015).

TS and VS concentrations

Changes in the properties of waste can also be seen from changes in Total Solid (TS) and Volatile Solid (VS) values. The decreasing value of TS and VS means that microorganisms carry out the decomposition of organic matter. The TS value is seen in Figure 5.

The Total Solid value in T3 has a higher concentration than in other treatments. It is due to the concentration of the substrate being too dense or the organic material in so much quantity. Still, overall, the TS value has decreased due to the degradation process carried out by microorganisms. In addition, the value of VS is also presented in Figure 6.

Total solid and final solid volatile indicate a decline in value, although not so drastically. It is due to the decomposition process of the material by decomposing bacteria. This decrease indicates an increase in the levels of methane gas produced. The decrease in VS shows the degradation of organic compounds by non-methanogenic microorganisms (Yahya et al., 2017).

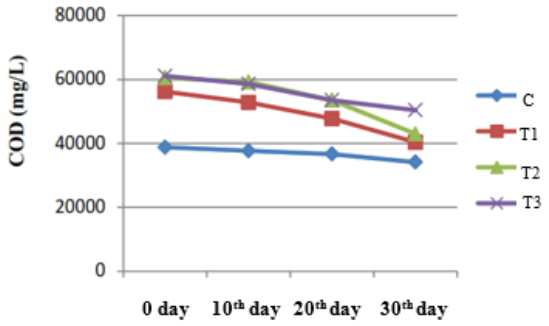

Figure 3. COD values formed up to day 30th. C: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; P3POME 60% + Activated sludge 25% + Compost 15%

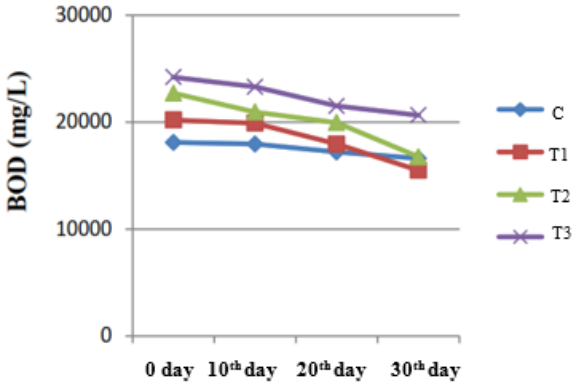

Figure 4. BOD values formed up to day 30th. C: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; T3 POME 60% + Activated sludge 25% + Compost 15%

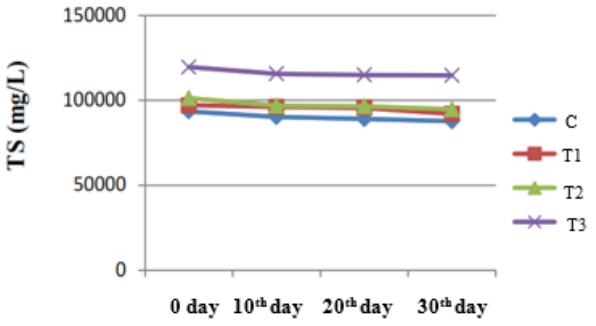

Figure 5. TS values formed up to day 30th. C: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; T3 POME 60% + Activated sludge 25% + Compost 15%

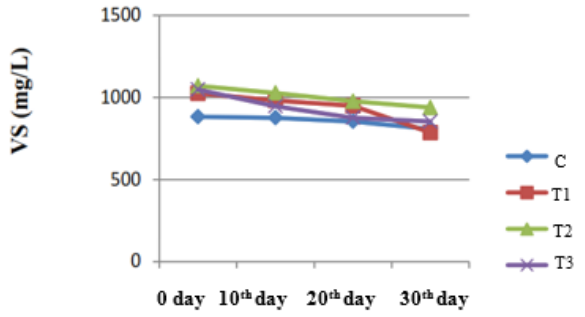

Figure 6. VS values formed up to day 30th. C: 100% POME; T1: POME 80% + Activated sludge 15% + Compost 5%; T2: POME 70% + Activated sludge 20% + Compost 10%; T3 POME 60% + Activated sludge 25% + Compost 15%

Concentration, temperature, and pH

At the beginning of the study, the pH tended to be acidic in each treatment. On the 10th to 30th day, the pH continued to increase. The highest pH was 6.82 in treatment 2 (T2). Increasing pH can accelerate the decomposition process, thereby accelerating the overhaul and indirectly accelerating biogas production (Metcalf and Eddy 2003). The increase in pH that occurs is one of the processes of biogas formation. Microorganisms continue to break down organic matter so that the pH increases (acid pH is close to neutral), thus increasing the pH to neutral conditions that are good for biogas production. A lot of biogas production is produced. The optimum pH for methanogenic bacteria or methane-producing bacteria is acid, which tends to be neutral, around 6.8-7.5 (Shah et al. 2014). The temperature obtained from day 0th of 57th OC shows the actual temperature of the POME itself, which is hot. This research does not use temperature variations, decreasing due to room temperature.

In conclusion, According to SNI, the fifth compost (V) is the best compost, with a C/N ratio of 18.35%. The volume of biogas produced is 593 ml. It is in line with the methane gas formed through the GCMS Gas chromatography technique, producing 36.21% methane gas in treatment 2 (T2). Meanwhile, for COD values of 17.60% and BOD 5.95%, which experienced the highest degradation efficiency in treatment 2, a high level of COD BOD reduction would result in a large amount of gas accumulation. The highest TS reduction value of 6.52% and VS 23.90% also occurred in treatment 2. This decrease indicated an increase in methane gas content; as evidenced in this study, the highest biogas volume and methane concentration were obtained in treatment 2.

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Isolation and identification of lipolytic bacterial from the digestive tract of ricefield eel (*Monopterus albus*)

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Abstract. *Berlian IN, Susilowati A, Pangastuti A. 2020. Isolation and identification of lipolytic bacterial from the digestive tract of ricefield eel (*Monopterus albus*). Bioteknologi 17: 27-36.* Lipolytic bacteria are bacteria that produce lipase enzymes. These bacteria can be isolated from various sources containing fat. Lipid is one of the nutrients needed by rice field eel (*Monopterus albus* Zuiew, 1973) to live and grow. In the digestive system of fish, typhoid bacteria are used to hydrolyze lipids and oils into fatty acids and glycerol, which are needed in metabolic processes. This study aims to obtain isolates of lipolytic bacteria from the digestive tract of rice field eels and to determine the identity of lipolytic bacteria that can be isolated from the digestive tract of rice field eels. The obtained rice field eels were rested and fasted for 8 hours. Then, the eels were dissected. The digestive tracts were cleaned and used. Isolation of bacteria used minimal media enriched with olive oil. Lipolytic bacteria screening used Rhodamine B Agar. Macroscopic morphological characterizations are colony shape, edge, color, and elevation. Microscopic morphology is cell shape, cell size, and gram of bacteria. The hemolysis test aimed to determine hemolysis activity using Blood Agar (BA). Molecular characterization was based on the sequence of genes encoding 16S rRNA. The results of lipolytic bacterial DNA sequences were analyzed using the NCBI BLAST Nucleotide Website (www.blast.ncbi.nlm.nih.gov/blast.cgi). Based on the isolation results, 63 isolates were obtained with 11 isolates of positive lipolytic bacteria. Positive lipolytic bacteria were orange glow on the colony when exposed to UV light at 350 nm. Based on morphological characterization, 4 isolates were cocci cells, and 7 isolates were bacilli cells. In addition, 8 gram-negative isolates and 3 gram-positive isolates were identified. The identified isolates were *Staphylococcus saprophyticus* strain L29 98%, *Stenotrophomonas maltophilia* 99%, *Acinetobacter junii* F-27 100%, *Paenibacillus lactis* PF4J 1-2 96%, *Aeromonas dhakensis* WWi303 99%, *S. saprophyticus* P0081Karwar 99%, *Aeromonas caviae* strain R25 - 6 98%, *Acinetobacter pittii* strain BJ6 99%.

Keywords: Digestive tract, isolation and identification, lipase, lipolytic bacteria, *Monopterus albus*, rice field eel

INTRODUCTION

Enzymes are very effective biocatalysts because they can significantly increase the speed of specific chemical reactions (Lehninger 1995). In addition, enzymes have a role in transforming various types of energy (Winarno 1986). Nearly 400 enzymes have been identified, and about 200 enzymes are used commercially. Since 1960, the commercial use of enzymes has experienced rapid development due to the increasing public understanding of the use of enzymes, especially in the industrial sector (Sharma et al. 2001). Some of the commercialized enzymes include lipase, xylanase, and protease.

Lipases (*triacylglycerol acyl hydrolases*, EC 3.1.1.3) are enzymes that naturally catalyze the hydrolysis of triacylglycerol (fats/oils) into fatty acids, monoacylglycerols, diacylglycerols, and glycerol. Based on its physiological function, lipase plays an important role in the hydrolysis of fats and oils into fatty acids and glycerol, which are needed in metabolic processes. In addition, a lipase acts as a catalyst for esterification of hydrolysis reactions and transesterification, which is useful in the oleochemical and biodiesel industries (Gupta et al. 2011).

Lipases can be used as biocatalysts to produce useful biodegradable compounds, such as 2-ethyl-1-hexyl ester, which was obtained from enzymatic transesterification of rapeseed oil fatty acids, and 1-butyl oleate, which was obtained from the direct esterification of butanol and oleic acid and used to reduce the viscosity of biodiesel (Linko et al. 1998). Lipase that has been mobilized using immobead 150 from the fungus *Rizhomucor miehei* has been successfully marketed in 2010 by *Sigma Aldrich*, with the enzyme activity produced by the fungus of 381 U/g and is sold at a price of around 270 million per kilogram (Kurnia 2010). Lipase has been used for industrial activities such as the food industry, dairy industry, paper industry, textile industry, leather industry, wastewater treatment, chemical production, medicine, and cosmetics. The detergent industry uses 1,000 tons of lipase per year (Jaeger et al. 1999). The need for large enzymes encourages the exploration of these enzymes from various sources.

Currently, lipases have been isolated and purified from various sources, such as bacteria, fungi, plants, and animals (Hasan et al. 2006). Lipases from bacteria are more in demand than lipases from other organisms because they are more stable, selective, and specific on a wide range of substrates (Veerpagu et al. 2014). In addition, lipase from bacteria is more beneficial because it can work in various catalytic processes, has higher yields, genetic engineering can be carried out, is not disturbed by weather fluctuations, and has faster growth in cheap media (Hasan et al. 2006). Lipase from bacteria can also be found in the digestive tract

of animals, such as the stomach and pancreas (Saktiwansyah 2001).

Koven et al. (1994) stated that an alternative source of lipase was microbial symbiosis in the digestive tract of mammals and fish utilizing fat as a carbon source. Intestinal microbes producing lipase play a role in increasing the residence time of fat in the intestine. Bairagi et al. (2002) stated that microorganisms isolated from the digestive tract of fish could produce lipases that were beneficial in the digestive process of fish. In a study conducted by Bairagi et al. (2002), it was also found that lipolytic bacteria in the digestive tract of some fish (bacterial population per gram of the digestive tract), including *Catla catla* of $1.3x10^3$, *Labeo rohita* of $0.8x10^3$, *Cirrhinus mrigala* of $0.3x10^3$, *Hypophthalmichtys molitrix* of $5.0x10^3$, *Ctenopharyngodon idella* of $1.0x10^3$, *Cyprinus carpio* of $4.3x10^3$, *Oreochromis mossambica* of $1.6x10^3$, *Clarias batrachus* of $0.7x10^3$, and *Channa punctatus* of $0.3x10^3$. In addition, according to Dhage (1968), lipase activity in *C. mrigala* and *L. rohita* was concentrated in the anterior intestine. Lipase is the main enzyme involved in digesting triglycerides in all vertebrates. Some information states that lipase from fish intestines comes from endogenous sources (Bairagi et al. 2002).

Monopterus albus (Zuiew, 1973) is a type of fish that is easy to find, especially in rice fields, easy to cultivate, and relatively inexpensive. *M. albus* is a carnivorous fish with a hormonal and enzymatic digestive system (Roy 2009). Fat is one of the nutrients needed by *M. albus* to live and grow. Carnivorous fish can digest and utilize fat more efficiently than omnivorous and herbivorous fish (Buwono 2000). The fat needed by eels is 3-4% in the feed. The high fat consumed by fish and which is not used as an energy source is stored as body fat (Haryati 2011). In the study of Li et al. (2011), the fat content in *M. albus* (1.71 ± 0.04 per 500 grams) was higher than the fat content in *Monopterus cuchia* (0.695 ± 0.05% per 100 ± 7.25 grams). Therefore, the digestive tract of *M. albus* allows the presence of lipolytic bacteria that have the potential as a source of lipase production. This enzyme can be used as a feed probiotic for *M. albus.* From this, efforts to isolate lipolytic bacteria need to be carried out, especially for biotechnology applications (Mubarak et al. 2011). In addition, efforts to identify the obtained bacteria are important to obtain information about these bacteria. Previously, Ridwan et al. (2019) found lactic acid bacteria, *Lactococcus lactis*, from the digestive tract of *M. albus.*

The aims of this study were (i) to obtain isolates of lipolytic bacteria from the digestive tract of *M. Albus*; (ii) to obtain the identity of the type of lipolytic bacteria isolated from the digestive tract of *M. albus.*

MATERIALS AND METHODS

Research time and place

This research was conducted in April-August 2018 at the Biology Laboratory, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret, Surakarta, Central Java, Indonesia.

Materials

The lipolytic bacteria isolated and identified in this study were sourced from the digestive tract of *M. albus* with body lengths ranging from 30-40 cm taken from 2 places, namely the cultivation of *M. albus* in Bendosari, Sukoharjo, which is an artificial habitat and the rice fields of Waru Village, Baki, Sukoharjo, Central Java, Indonesia, which are natural habitats.

Procedure

The obtained *M. albus* were rested and fasted for 8 hours to minimize the work of enzymes and to minimize food entering the digestive tract. After that, the eel was dissected by cutting the lower part of the abdomen from the anterior of the body to the ventral fin, then cutting towards the dorsal eel to the lateral line, and then cutting towards the anal part of the fish. Next, the stomach and intestines are taken, then the contents of the intestine are removed to maximize the yield of obtained enzymes. Next, the stomach and intestines were cut, then mashed and homogenized by adding sterile 0.85% NaCl then vortexed to produce a homogenate (Kurniawan 2016).

Lipolytic bacteria isolation

One gram of homogenate was taken, crushed, and dissolved in 9 mL of Physiological Saline Solution 0.85%. Next, the solution is vortexed evenly, and the solid is allowed to settle. The dilution was carried out up to a dilution series of 10^{-4}, but for isolation, samples were taken with a dilution series of 10^{-3} and 10^{-4}. Then the microorganism was planted on minimal agar enriched with olive oil using the spread plate method. It was done by inserting 0.1 mL of the microorganism source in each petri dish and then leveling it using a drigalsky with two repetitions of each dilution series (Susanty et al. 2013). The resulting isolates were incubated for 48-72 hours at a temperature of 27^0C. Finally, each colony with different morphology was selected as a candidate for lipolytic bacteria and stored in agar slanted at 4^0C (Gayathri et al. 2013).

Lipolytic bacteria screening

According to Ray et al. (2012), bacterial screening of pure bacterial isolates was carried out by taking 1 ose of isolate and then streaking it on Rhodamine B agar media. Then it was incubated at 27^0C for 48 hours. Positive results were seen in the hydrolysis of olive oil into fatty acids, which then interacted with Rhodamine B so that pink colonies appeared and could glow when irradiated using an Ultra Violet lamp with a wavelength of 350 nm.

Colony morphology characterization of lipolytic bacteria

Morphological observation

The morphological observation of lipolytic bacteria was by observing the colonies growing on NA media. The morphological characterizations of the colonies included color, shape, margins of bacterial colonies, and elevation. Based on the morphological character of each bacterial isolate that has lipolytic activity, it was identified using *Bergey's Manual of Systematic Bacteriology Second*

Edition Volume Two, The Proteobacteria (Garrity 2009) and *Bergey's Manual of Systematic Bacteriology Second Edition Volume Three, The Firmicutes* (Whitman 2009).

Gram stain

One ose of the bacterial isolate was taken and scratched on the surface of a sterile preparation and then fixed. One drop of crystal violet was added to the surface of the preparation containing the bacterial layer and allowed to stand for one minute. After one minute, the preparations were rinsed using distilled water until the dye faded. The preparations were dried over the fire of denatured alcohol. Then one drop of iodine solution was added to the surface of the preparations and allowed to stand for one minute. After one minute, the preparations were rinsed using distilled water. The preparations were rinsed with 96% alcohol until all the dye had faded. Then the preparations were rinsed using distilled water. The preparations were dried over the fire of denatured alcohol, and then one drop of safranin was added to the surface of the preparations and allowed to stand for 45 seconds. Finally, the preparations were washed using distilled water and dried. Furthermore, the preparations were observed using a light microscope with a magnification of 1000x to observe cell shape and determine the gram in bacteria (Pratita and Putra 2012).

Hemolysis test

Isolates were tested to determine their pathogenicity by hemolysis test. The isolates were grown on Blood Agar media with the addition of 5% sheep blood and then incubated for 18-24 hours at 37^0C. The appearance of a clear zone around the colony after 18 hours of incubation at 37^0C was considered a positive result of hemolysin production (Osek 2004).

Characterization of lipolytic bacteria with sequences of gene encoding 16S rRNA

DNA extraction

Extraction of lipolytic bacterial genomic DNA obtained from the screening process was carried out using the Presto™ Mini gDNA Bacteria Kit.

Amplification

Amplification of the gene encoding 16s rRNA for lipolytic bacteria used 63 forward primer (63f: 5'CAGGCCTAACACATGCAAGTC-3'); and 1387 reverse primer (1387r:5'-GGGCGGAWGTGTACAAGGC-3'). The PCR reaction was carried out by mixing 1 µL of 63 forward primers with a concentration of 10 pmol, 1 µL of 1387 reverse primer with a concentration of 10pmol, 25 µL of MyTaq™ HS Red Mix 2x, and 2 µL of DNA template, and 21 µL of ddH_2O. Pre-denaturation was carried out at 95^0C for 3 minutes. One PCR cycle of 30 cycles consisted of denaturation at 95^0C for 15 seconds, annealing at 56^0C for 15 seconds, elongation at 72^0C for 30 seconds, and finalizing at 72^0C for 2 minutes. Next, the PCR was stopped, and the PCR amplification product was stored at 4^0C. The PCR amplification products were transferred using gel electrophoresis (Marchesi et al. 1998).

Sequencing

Sequencing the PCR amplification of the gene encoding 16S rRNA for lipolytic bacteria was carried out at 1st Base Singapore.

Data analysis

The lipolytic bacterial isolates were obtained by morphological observations. They included the observations of macroscopic morphology (colony shape, color, margins, and elevation) and microscopic morphological observations (cell shape, gram bacteria, cell size). The hemolysis test results were analyzed descriptively. The identity of lipolytic bacterial DNA sequences resulting from the extraction process was analyzed using bioinformatics techniques with the BLAST Nucleotide device on the NCBI website (www.blast.ncbi.mlm.nih.gov/blast.cgi).

RESULTS AND DISCUSSION

Isolation and screening of lipolytic bacteria from the gastrointestinal tract of *M. albus*

In this study, the sources of lipolytic bacteria were the digestive tract of wild and cultivated eels. Therefore, they were taken in three repetitions. The repetition was done to obtain varied results so that various species of lipolytic bacteria could be compared and obtained. In this study, 63 bacterial isolates were obtained, consisting of 33 bacterial isolates from the digestive tract of wild eels (Table 1) and 30 bacterial isolates from the digestive tract of cultivated eels (Table 2). The isolate code consisted of BL or BB, which indicated that BL was from wild *M. albus* and BB was from cultivated *M. albus*; the number beside BL/BB indicates that the isolate was done at week 1, week 2, or week 3; the numbers next to it indicated the length of the isolated eel, and the letter at the end of the code indicates the sequence of isolates in one eel of the same size in the same week.

Vishwanath et al. (1998) stated that the fat content in the digestive tract of *M. albus* ranged from 10.74 ± 0.48% DWB. Lipolytic bacteria can be found in an environment containing fat or oil because that environment provides a good substrate for the growth of lipolytic bacteria (Swandi et al. 2015). According to Haryati (2011), fat consumed by fish but not used as an energy source will be stored as body fat so that it can trigger the growth of lipolytic bacteria.

Isolation of lipolytic bacteria in this study was carried out using minimal media enriched with olive oil. Olive oil acts as a carbon source needed for the growth of lipolytic bacteria. Therefore, the minimal media was chosen so that the bacteria only got a carbon source from olive oil so that lipolytic-specific bacteria were obtained. Furthermore, olive oil was chosen because it is easy to obtain and is a substrate that is easy to use by bacteria (Carissimi et al. 2007).

Table 1. Bacterial isolates from the digestive tract of wild *M. albus* obtained from each sampling

Sample length (cm)	Number of isolates	Isolate code
38	-	-
42	1	BL.1.42
38	8	BL.2.38.A; BL.2.38.B; BL.2.38.C; BL.2.38.D; BL.2.38.E; BL.2.38.F; BL.2.38.G;BL.2.38.H;
35	6	BL.2.35.A; BL.2.35.B; BL.2.35.C; BL.2.35.D; BL.2.35.E; BL.2.35.F
34.5	5	BL.3.34.5.A; BL.3.34.5.B; BL.3.34.5.C;BL.3.34.5.D; BL.3.34.5.E
36.5	13	BL.3.36.5.A; BL.3.36.5.B; BL.3.36.5.C; BL.3.36.5.D; BL.3.36.5.E; BL.3.36.5.F; BL.3.36.5.G; BL.3.36.5.H; BL.3.36.5.I; BL.3.36.5.J; BL.3.36.5.K; BL.3.36.5.L;BL.3.36.5.M
Total isolates		33

Table 2. Bacterial isolates from the digestive tract of cultivated *M. albus* obtained from each sampling

Sample length (cm)	Number of isolates	Isolate code
32.5	1	BB.1.32.5
33.5	2	BB.1.33.5.A; BB.1.33.5.B
27.5	16	BB.2.27.5.A; BB.2.27.5.B; BB.2.27.5.C; BB.2.27.5.D; BB.2.27.5.E; BB.2.27.5.F; BB.2.27.5.G; BB.2.27.5.H; BB.2.27.5.I; BB.2.27.5.J; BB.2.27.5.K; BB.2.27.5.L; BB.2.27.5.M; BB.2.27.5.N; BB.2.27.5.O;BB.2.27.5.P
27	-	-
31	8	BB.3.31.A; BB.3.31.B; BB.3.31.C; BB.3.31.D; BB.3.31.E; BB.3.31.F; BB.3.31.G; BB.3.31.H
29	3	BB.3.29.A; BB.3.29.B; BB.3.29.C
Total isolates		30

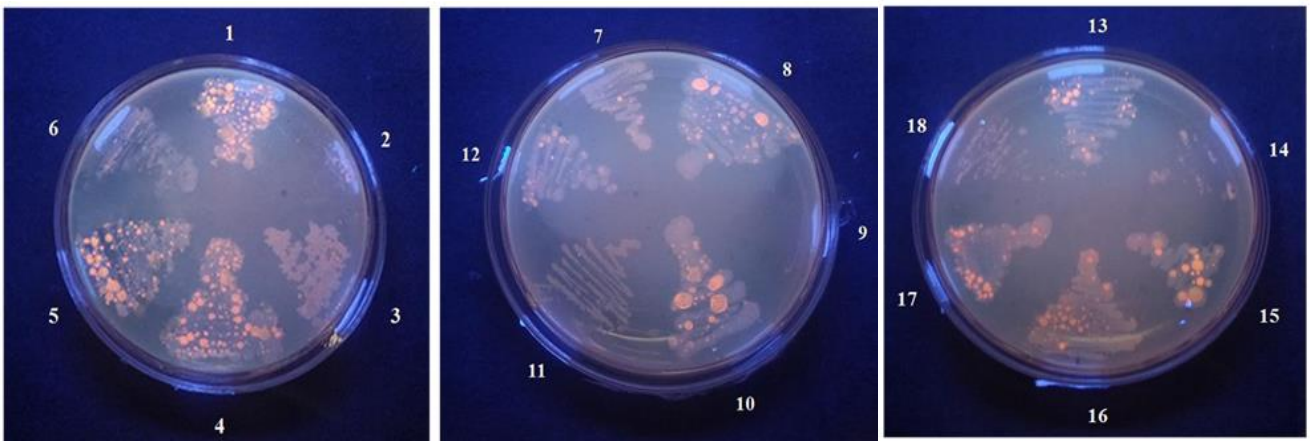

Figure 1. Colonies of lipolytic bacteria on Rhodamine B agar media under exposure to 350 nm UV lamp. Lipolytic positive isolates (BB.1.33.5.A (1); BB.1.32.5.A (4); BL.2.38.F (5); BB.2.27.5.C (7); BB.3.31. A (8); BB.3.31.D (10); BB.3.31.F (12); BL.3.36.5.A (13); BL.3.36.5.D (15); BL.3.36.5 .J (16); BL.3.34.5.A (17)) were shown by fluorescent orange under a 350 nm UV lamp

The lipolytic bacteria screening used Rhodamine B Agar media. Lipolytic activity was indicated by the presence of isolates that glowed orange when exposed to UV light with a wavelength of 350 nm (Figure 1). Rhodamine B Agar was chosen because it is a sensitive fluorescent indicator for lipase. A fluorescent indicator in the form of lipase-producing bacteria will produce an orange complex on Rhodamine B Agar media when exposed to UV light with a wavelength of 350 nm (Telussa 2013). The formation of the orange complex is based on the change of the acid to the cation form and the formation of a complex with uranyl fatty acid ions which are inversely proportional to the fatty acid long chain to produce an orange glow excitation on exposure to UV light with a wavelength of 350 nm (Carissimi et al. 2007).

Based on the results of lipolytic bacteria screening, there were 11 isolates that had lipolytic activity, consisting of 6 isolates from cultivated *M. albus* and 5 isolates from wild *M. albus*. The isolates were BB.1.33.5.A; BB.1.32.5.A; BL.2.38.F; BB.2.27.5.C; BB.3.31.A; BB.3.31.D; BB.3.31.F; BL.3.36.5.A; BL.3.36.5.D; BL.3.36.5.J; BL.3.34.5.A. The results showed that several bacterial isolates with lipolytic activity were found in the digestive tract of wild and cultivated *M. albus*. This result is in accordance with the statement of Bairagi et al. (2012) that lipolytic bacteria can be isolated from the digestive tract of fish. According to Hungate (1996), lipolytic bacteria in the digestive tract play a role in the hydrolysis of fats into glycerol and fatty acids.

Morphological characteristics of lipolytic bacteria in the digestive tract of *M. albus*

The observed morphological characters of 11 bacterial isolates with lipolytic activity included macroscopic and microscopic characters. Macroscopic characters included colony shape, edge, color, and elevation. At the same time, microscopic characters included gram, cell shape, and cell size. The macroscopic morphological characteristics of lipolytic bacterial colonies are listed in Table 3, while the microscopic morphological characteristics of lipolytic bacterial colonies are shown in Table 4 and Figure 2.

Based on microscopic morphological characters, isolate BB.1.33.5.A (1) and isolate BB.3.31.D (6) had round cells (coccus) with purple cells indicating gram-positive. Isolate BB.2.27.5.C (4) was also gram-positive but had the form of rod cells (bacillus). Isolate BB.2.38.F (3) and isolate BL.3.36.5.J (10) had a round cell shape (coccus) with red cells indicating gram-negative. While the other isolates, namely, BB.1.32.5.A (2); BB.3.31.A (5); BB.3.31.F (7); BL.3.36.5.A (8); BL.3.36.5.D (9); and BL.3.34.5.A (11) had the same microscopic morphology, namely the shape of rod cells (bacillus) with red cell color indicating gram-negative. All lipolytic bacteria isolates had cell sizes ranging from 0.8-1.25 µm.

Hemolysis test on lipolytic bacterial isolate

Isolates of lipolytic bacteria were tested for hemolysis using BA (Blood Agar) media to determine the pathogenic potential. After incubation for 1x24 hours, the results of the hemolysis test were obtained, as shown in Table 5 and Figure 3.

Table 3. Macroscopic morphology of lipolytic bacterial colonies on NA media

No.	Isolation code	Shape	Edge	Color	Elevation
1	BB.1.33.5.A	*Circulair*	*Entire*	Beige	*Raised*
2	BB.1.32.5.A	*Irregular*	*Undulate*	Yellow	*Raised*
3	BL.2.38.F	*Circulair*	*Entire*	Beige	*Convex*
4	BB.2.27.5.C	*Filamentous*	*Lobate*	White	*Convex*
5	BB.3.31.A	*Circulair*	*Entire*	White	*Convex*
6	BB.3.31.D	*Circulair*	*Entire*	Beige	*Raised*
7	BB.3.31.F	*Circulair*	*Entire*	White	*Convex*
8	BL.3.36.5.A	*Circulair*	*Entire*	White	*Convex*
9	BL.3.36.5.D	*Circulair*	*Entire*	White	*Convex*
10	BL.3.36.5.J	*Circulair*	*Entire*	Beige	*Convex*
11	BL.3.34.5.A	*Circulair*	*Entire*	White	*Convex*

Table 4. Microscopic morphology of lipolytic bacterial cells

	Isolate code	Gram	Cell shape	Size (± µm)
1	BB.1.33.5.A	Positive	round (*coccus*)	0.83
2	BB.1.32.5.A	Negative	rod (*bacillus*)	1.2
3	BL.2.38.F	Negative	round (*coccus*)	1.25
4	BB.2.27.5.C	Positive	rod (*bacillus*)	1
5	BB.3.31.A	Negative	rod (*bacillus*)	0.8
6	BB.3.31.D	Positive	round(*coccus*)	0.8
7	BB.3.31.F	Negative	rod (*bacillus*)	0.83
8	BL.3.36.5.A	Negative	rod (*bacillus*)	1
9	BL.3.36.5.D	Negative	rod (*bacillus*)	1
10	BL.3.36.5.J	Negative	round (*coccus*)	1
11	BL.3.34.5.A	Negative	rod (*bacillus*)	1

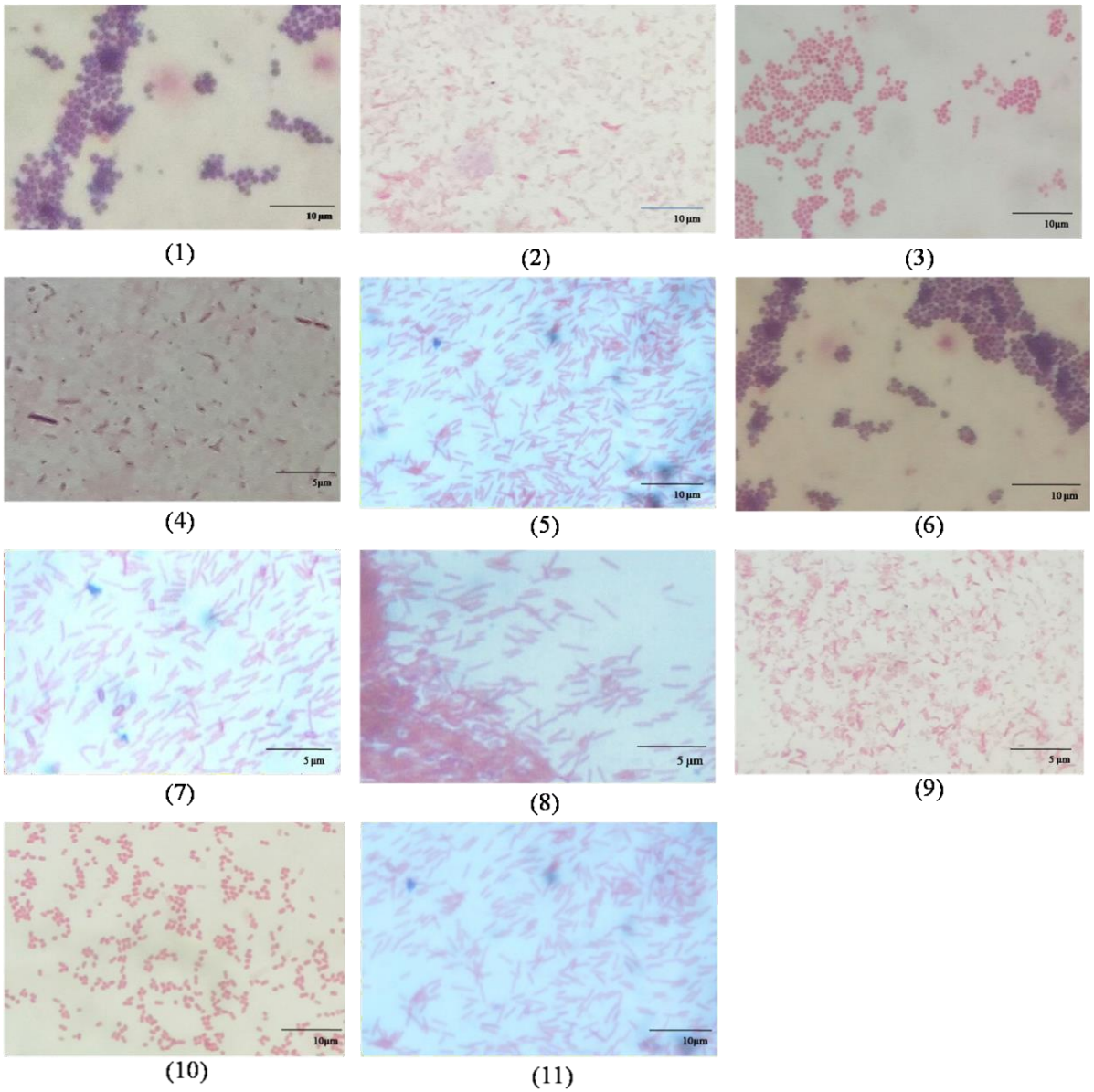

Figure 2. Macroscopic morphological character of lipolytic bacteria. Note: See Table 5 for isolate code

Table 5. Results of hemolysis test on lipolytic bacteria

No.	Isolation code	Clear zone	Hemolytic activity
1	BB.1.33.5.A	Absent	Negative
2	BB.1.32.5.A	Absent	Negative
3	BL.2.38.F	Present	Positive
4	BB.2.27.5.C	Absent	Negative
5	BB.3.31.A	Present	Positive
6	BB.3.31.D	Absent	Negative
7	BB.3.31.F	Present	Positive
8	BL.3.36.5.A	Present	Positive
9	BL.3.36.5.D	Present	Positive
10	BL.3.34.5.A	Absent	Negative
11	BL.3.36.5.J	Present	Positive

Isolate BB.1.33.5.A (1); Isolate BB.1.32.5.A (2); Isolate BL.2.27.5.C (4); Isolate BB.3.31.D (6); and Isolate BL.3.34,5.A (10) were bacteria with no hemolytic activity (gamma hemolysis (γ)) because they were colorless (clear) when they were planted in the media. They did not lyse the media, so no clear zone was formed around the colony. Meanwhile, the other 6 isolates, namely Isolate BL.2.38.F (3); Isolate BB.3.31.A (5); Isolate BB.3.31.F (7); Isolate BL.3.36.5.A (8); Isolate BL.3.36.5.D (9); Isolate BL.3.36.5.J (11) were bacteria with hemolysis activity (beta hemolysis (β)) because they were cloudy when they were planted in the media and lysed the media so that a clear zone was formed around the colony. So, when viewed from the hemolytic activity of 11 lipolytic bacteria isolates, only 5 isolates could be used as candidates for industrial-scale applications. These 5 isolates consisted of 2 from the digestive tract of wild *M. albus* and 3 from the digestive tract of cultivated *M. albus*. However, it is still necessary to carry out other pathogenicity tests on these 5 isolates.

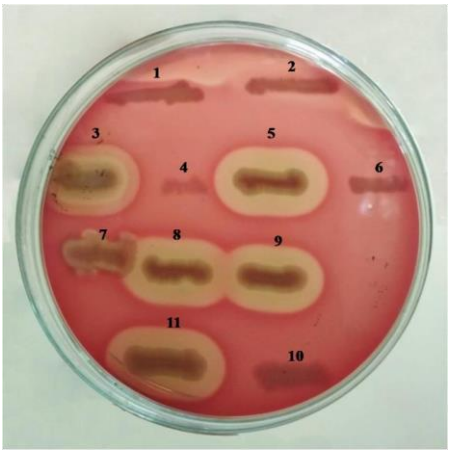

Figure 3. Hemolysis test on lipolytic bacteria. Isolate BL.2.38.F (3); Isolate BB.3.31.A (5); Isolate BB.3.31.F (7); Isolate BL.3.36.5.A (8); Isolate BL.3.36.5.D (9); and Isolate BL.3.36.5.J (11) had hemolytic activity (beta hemolysis (β)), which was indicated by the presence of a clear zone around isolate

The color change from blood red to dark color (transparent colorless) indicated growing bacterial colonies. This situation indicated incubation. The formation of the hemolysis zone was caused by bacteria secreting active glycolipid compounds in a hydrophilic substrate (Maneerat et al. 2007). In addition, some bacteria produce cytosolic, which can dissolve red blood cells. In this test, BA (Blood Agar) media was used to help the growth of microorganisms that were difficult to culture and to distinguish groups of microorganisms that did or did not lyse red blood cells (Sari et al. 2015).

Lipolytic bacterial species based on sequence analysis of the 16S rRNA encoding gene

Molecular identification of lipolytic bacterial isolates was based on the 16S rRNA encoding gene sequence (Pangastuti 2006). The lipolytic bacterial genome DNA samples were amplified using PCR (Polymerase Chain Reaction) with primers of 63F and 1387R. The primer pair 63F and 1387R can amplify the 16S rRNA coding gene sequence with a size of about 1,300 base pairs (Marchesi et al. 1998). In this study, the obtained PCR products were 11 isolates of lipolytic bacteria with almost equal size, consisting of about 1,300 base pairs (Figure 4). All 16S rRNA gene amplicons were sequenced to produce a sequence of nucleotide bases for the 16S rRNA encoding gene (Appendix 1). The results are processed using the BLASTN program and then synchronized with the database in the Gene Bank so that the percentage of similarity is obtained.

The obtained identity value determines the level of isolates similarity with the database on the Gene Bank. Hall (2001) stated that the higher identity value indicated higher similarity with the reference sequence in the Gene Bank. According to Janda and Abbot (2007), a bacterial species is said to be the same if it has a homology of more than or equal to 97%. Based on BLASTN analysis, 10 isolates of lipolytic bacteria had a similar percentage of 98% - 100% with the database in the Gene Bank, while 1 other isolate (BL.2.38.F) had a percentage similarity of 96% with the database in the Gene Bank (Table 6).

Staphylococcus is a group of gram-positive bacteria, and most of them are facultative anaerobic bacteria. This genus can be isolated from water, air, sand, soil, and various animal products such as meat, milk, and cheese. Some species are opportunistic pathogenic bacteria for humans and/or animals (Whitman 2009). In a research conducted by Tanasupawat et al. (1991) in the digestive tract of fish, there are *Staphylococcus* species that have lipolytic activity using *Tween 80* media, namely *Staphylococcus gallinarum*. Kurniasih et al. (2014) stated that *Staphylococcus* has lipolytic activity and is a candidate for probiotic bacteria in catfish feed because it can increase digestibility. Isolate BB.1.33.5.A and isolate BB.3.31.D were identified as *Staphylococcus saprophyticus*. This species is a gram-positive bacterium that belongs to the facultative anaerobic bacteria of the genus Staphylococcus. This bacterium grows optimally at a temperature of 28-35^{0}C. According to Sakinc et al. (2007), *S. saprophyticus* shows optimum lipolytic activity at a temperature of 30^{0}C and pH 6 but can lose its activity at a pH of 4.8-5. Isolate BB.1.33.5.A and Isolate BB.3.31.D showed non-pathogenic in the hemolysis test.

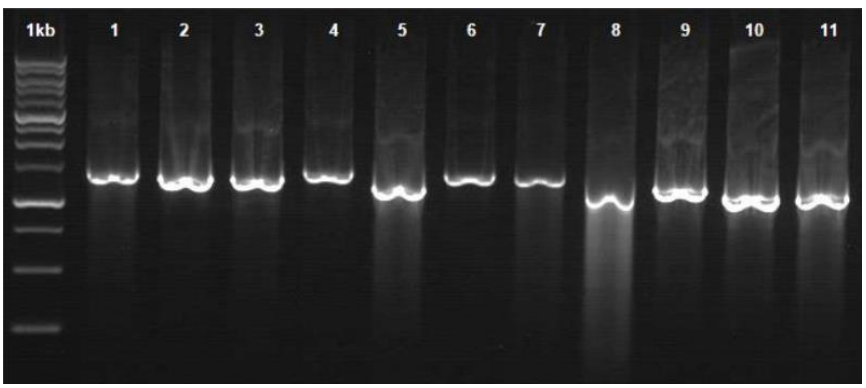

Figure 4. Electrophorogram of lipolytic bacteria PCR products. Note: M: Marker, 1: Isolate BB.1.33,5.A; 2: Isolate BB.1.32,5.A; 3: Isolate BL.2.38.F, 4: Isolate BB.2.27,5.C; 5: Isolate BB.3.31.A; 6: Isolate BB.3.31.D; 7: Isolate BB.3.31.F; 8: Isolate BL.3.36,5.A; 9: Isolate BL.3.36,5.D; 10: BL.3.34,5.A; 11: Isolate BL.3.36,5.J (Documentation: Genetics Science 2018)

Table 6. BLASTN analysis of 16S rRNA encoding gene sequences for lipolytic bacteria in the digestive tract of *M. albus*

Isolate name	Identity	Coverage	Closest relatives
BB.1.33.5.A	98%	95%	*Staphylococcus saprophyticus* strain L29
BB.1.32.5.A	99%	100%	*Stenotrophomonas maltophilia*
BB.2.38.F	100%	100%	*Acinetobacter junii* strain F-27
BL.2.27.5.C	96%	98%	*Paenibacillus lactis* strain PF4J 1-2
BB.3.31.A	99%	100%	*Aeromonas dhakensis* strain WWi303
BB.3.31.D	99%	100%	*Staphylococcus saprophyticus* strain P0081Karwar
BB.3.31.F	99%	100%	*Aeromonas dhakensis* strain WWi303
BL.3.36.5.A	99%	100%	*Aeromonas dhakensis* strain WWi303
BL.3.36.5.D	98%	99%	*Aeromonas caviae* strain R25-6
BL.3.34.5.A	99%	100%	*Acinetobacter pittii* strain BJ6
BL.3.36.5.J	99%	100%	*Aeromonas dhakensis* strain WWi303

Stenotrophomonas is a group of gram-negative bacteria that are motile using two or more flagella and are opportunistic pathogenic bacteria. These bacteria grow optimally at a temperature of 35^0C and have high lipolytic activity. *Stenotrophomonas maltophilia* is a species that has high lipolytic activity because it contains esterase in the outer membrane of bacteria which acts as a substrate for growth (Garrity 2009). Hasan-Beikdashti et al. (2012) succeeded in optimizing lipase production from *S. maltophilia* isolated from soil and found optimal by adding peptone, yeast extract, olive oil, and FeSO4 to the media. Isolate BB.1.32.5.A, identified as *S. maltophilia*, showed non-pathogenicity in the hemolysis test.

Acinetobacter is a genus of gram-negative bacteria and a common cause of human infections such as endocarditis, meningitis, and bacteremia. Species belonging to this genus are widely found in soil, water, and dry environments (Yakut et al. 2016). *Acinetobacter* can also be isolated from spoiled food, waste material, and contaminated soil (Musa and Adebayo-Tayo 2012). Based on Joseph et al. (2007), *Acinetobacter* is one of several lipase-producing microorganisms capable of producing lipase at a low temperature of around 5^0C. Musa and Adebayo-Tayo (2012) stated that *Acinetobacter* had the highest lipase activity during 7 hours of incubation compared to other bacteria such as *Arthrobacter* sp., *Brevibacterium* sp., *Staphylococcus* sp., *Yersinia* sp., *Lactobacillus* sp., *Citrobacter* sp., *Streptococcus* sp., *Acidomonas* sp., *Acetobacterium* sp., *Bacillus* sp., *Serratia marcescens*, and *Aeromonas hydrophila*. *Acinetobacter* ranks first as the bacteria that produces the highest lipase, with an average lipase production of 1.25 U/mL-8.65 U/mL. In this study, there were 2 species of the genus *Acinetobacter*, namely *Acinetobacter junii* and *Acinetobacter pittii*. The *A. junii* is an aerobic, gram-negative, opportunistic bacterial species with a size ranging from 0.9-1.6 m and grows optimally at a temperature of 15^0-35^0C (Garrity 2009). Isolate BL.2.38.F is a species of *A. junii*, but in the hemolysis test, this bacterium was positive for pathogens with the formation of a clear zone around the bacterial colony, so isolate BL.2.38.F was not possible to be developed for lipase production. The *A. pittii* is a gram-negative bacterium that infects humans (Atrouni et al. 2016). Isolate BB.3.34.5.A, identified as *A. pittii*, showed non-pathogenicity in the hemolysis test.

Paenibacillus lactis is a gram-positive bacterium with rod-shaped cells. These bacteria are pathogenic to insects and can be found in humus-rich soils because these bacteria play a role in the process of extracellular carbohydrate secretion and enzyme secretion. In addition, *P. lactis* can be found in waters, as shown by the research of Rawat et al. in 2018. The *P. lactis* was isolated from the Alaknada and Bhagirathi Rivers. Isolate BB.2.27.5.C, identified as *P. lactis*, did not show any hemolytic activity in the hemolysis test.

Aeromonas is a gram-negative bacterium that can grow optimally at a temperature of 22^0C-37^0C. *Aeromonas* is generally found abundantly in waters, brackish water, chlorinated water, and wastewater. Several species in this genus cause disease in humans, amphibians, freshwater fish, saltwater fish, and invertebrates (Garrity 2009). In Thenmozhi et al. (2015), Aeromonas species were isolated from *C. carpio*, namely *Aeromonas salmonicida* and *A. hydrophila*, and showed lipolytic activity. The *A. salmonicida* showed lipolytic activity of 69.23%, and *A. hydrophila* showed lipolytic activity of 75%. Divakar et al. (2012) showed that *Aeromonas caviae* AU04 is a bacterium that produces extracellular lipase and is thermostable. Lipase secretion by *A. caviae* AU04 was optimum at 31^0C and pH 7.0. According to the hemolysis test, isolate Bl.3.36.5.D identified as *A. caviae* was a positive bacterium with lipolytic activity, so it was not possible to develop this isolate in lipase production. Isolate BB.3.31.A; Isolate BB.3.31.F; Isolate BB.3.36.5.A; Isolate BL.3.36.5.J were identified as *Aeromonas dhakensis*. It is a subspecies of *A. hydrophila* that can grow optimally at a temperature of 28^0C (Garrity 2009). The *A. dhakensis* is a pathogenic bacterium that complies with the hemolysis test on Isolate BB.3.31.A; Isolate BB.3.31.F; Isolate BB.3.36.5.A; Isolate BL.3.36.5.J that all isolates were pathogenic. This condition made it impossible for these isolates to be developed in lipase production.

The *S. saprophyticus* (Isolate BB.1.33.5.A and Isolate BB.3.31.D); *S. maltophilia* (Isolate BB.1.32.5.A); *A. pittii* (Isolate BB.3.34.5.A); *P. lactis* (Isolate BB.2.27.5.C) are some of the species obtained in this study which allow it to be developed to the next stage, but other pathogenicity tests still need to be carried out to ensure these species are safe for lipase production.

In conclusion, 63 isolates of bacteria were isolated from the digestive tract of *M. albus*, consisting of 33 isolates of bacteria from the digestive tract of wild *M. albus* and 30 isolates of bacteria from the digestive tract of cultivated *M. albus*. 11 isolates had a lipolytic activity with 5 isolates being non-hemolytic, namely BB.1.33.5.A; BB.1.32.5.A; BB.2.27.5.C; BB.3.31.D; BL.3.34,5.A and 6 isolates were hemolytic, namely BL.2.38.F; BB.3.31.A; BB.3.31.F; BL.3.36.5.A; BL.3.36.5.D; BL.3.36.5.J. Based on the sequence of the gene encoding 16S rRNA, isolate BB.1.33.5.A was identified as *S. saprophyticus* strain L29 by 98%; Isolate BB.1.32.5.A was identified as 99% of *S. maltophilia*; Isolate BL.2.38.F was identified as *A. junii* F-27 by 100%; Isolate BB.2.27.5.C was identified as *P. lactis* PF4J 1-2 by 96%; Isolate BB.3.31.A, Isolate BB.3.31.F, Isolate BL.3.36.5.A, Isolate BL.3.36.5.J were identified as 99% of *A. dhakensis* WWi303; Isolate BB.3.31.D was identified as *S. saprophyticus* P0081Karwar by 99%; Isolate BL.3.36.5.D was identified as *A. caviae* strain R25-6 by 98%, and Isolate BL.3.34.5.A was identified as 99% of *A. pittii* strain BJ6.

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Biodiesel production from freshwater microalgae of Ghana

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Abstract. *Ruth OA, Ameka GK, Annag T. 2020. Biodiesel production from freshwater microalgae of Ghana. Bioteknologi 17: 37-45.* Due to rising oil prices and concerns about global warming, biodiesel has gained prominence as an alternative energy source. Biodiesel produced from microalgae is a potential carbon–neutral and renewable alternative to petroleum fuels. This study aimed to investigate the viability of manufacturing biodiesel from microalgae harvested from freshwater bodies in Ghana as an environmentally sustainable alternative to fossil diesel. In the study, water samples with algal blooms were taken from the Weija reservoir, wastewater ponds in and around the main campus of the University of Ghana in Prampram, and a freshwater pond in Accra's Teshie neighborhood. Four (4) microalgae were isolated and identified based on their morphology and cultivation ease: *Oedogonium* sp., *Chroococcus* sp., *Spirogyra* sp., and *Closterium* sp. After being grown in a 2-liter reagent bottle Photobioreactor (improvised) utilizing sunlight as the energy source, these isolates were collected, filtered to remove excess water, and centrifuged to form pastes. Extracting the oil from the algae required drying and grinding the pastes into powder. And then, the oils were transesterified into biodiesel. In terms of percentage dry weight, *Oedogonium* sp. exhibited a considerable increase (55.8%) and *Closterium* sp. exhibited a decrease (40.1%), while *Chroococcus* sp. and Spirogyra sp. ranged between 50.2 and 50.0%, respectively. Significant volumes (20- 38%) of microalgal oil were successfully extracted with hexane and diethyl ether and converted into biodiesel. Results also suggested that these species may grow abundantly under ambient temperature, air, natural source of light, and suitable substrate. 94% of biodiesel was produced from *Closterium* sp., compared to 80% from *Chroococcus* sp. The highest biodiesel yield was 49% for *Oedogonium* sp., and the lowest was 33% for *Spirogyra* sp. This study isolated and identified four freshwater microalgae that are seldom employed in biodiesel manufacturing studies. Conclusion: *Oedogonium* sp., *Closterium* sp., *Spirogyra* sp., and *Chroococcus* sp. are viable species capable of producing a large amount of algal oil for biodiesel synthesis. Consequently, efforts must be made to create a more effective and economically viable technology for the large-scale growth of algae for biodiesel production in Ghana. In addition, a comprehensive economic study must be conducted on every component of the algae-to-biodiesel production process. It can minimize the cost of biodiesel production, hence making it a more economical alternative to fossil fuel in Ghana.

Keywords: Biodiesel, microalgae, Ghana, sustainable bioenergy

INTRODUCTION

An estimated 27% of the world's primary energy supply goes toward the transportation industry, making it one of the fastest-growing industries (Antoni et al. 2007). However, the industry's lifeblood, crude oil, is becoming unsustainable due to dwindling reserves in a few geographically-favored areas (Ciubota-Rosie et al. 2008). As a result, fossil fuels meet about 80% of the world's energy needs today (Schenk et al. 2008). However, the biggest drawback of utilizing petroleum fuels is the pollution they cause to the atmosphere, especially diesel fuel, which produces a lot of carbon dioxide during burning (a greenhouse gas). In addition to this emission, petroleum diesel is a significant contributor to other air pollutants, including nitrogen oxides (NOx), sulfur oxides (SOx), carbon monoxide (CO), particulate matter (PM), and volatile organic compounds (VOC) (Hallenbeck and Benemann 2002). As a result, there is a worldwide push to create reliable renewable energy.

As an additional note, the cost of oil has been on the increase around the world. In 2010, rising demand from a recovering economy drove up global oil prices due to insufficient supply. Moreover, as social and political upheaval spread throughout numerous Middle Eastern and African economies, prices continued to rise through the end of 2010 and into 2011 (IEO 2011). Thus, non-oil alternatives have gained importance (Neltner 2008), and biodiesel's popularity as an alternative fuel source has skyrocketed in recent years (Koonin 2006; Durrett et al. 2008; Basha and Jebaraj 2009; Demirbas 2009).

In addition, a significant amount of effort has been invested into finding viable biomass feedstock from which biodiesel and gas fuels can be produced for power plants (Singh and Gu 2010). Soybean, rapeseed, sunflower, and safflower oil are some of the most studied oil sources since they are also edible (Altin et al. 2001; Lang et al. 2001; Twidell and Weir 2006; Parawira 2010). Other types of biomass, such as energy crops (both edible and non-edible oilseeds), bio-wastes, wood, and some types of aquatic plants like algae, have been identified as potential sources of bio-oil (Duku et al. 2011; Amirta et al. 2016).

Due to depleting fossil fuel sources, the world's economy is in danger of experiencing an energy crisis. In addition, countries face challenges, such as environmental pollution and health issues due to carbon dioxide, pollutant emissions, and the accompanying economic consequences. Significant effects on aquatic life are also caused by the ocean dissolving around a third of the carbon dioxide created by the combustion of fossil fuels (Mata et al. 2010).

Biodiesel has been suggested as a solution to many of these issues. However, difficulties in sourcing the correct feedstock have slowed the growth of the biodiesel production business worldwide. Recent research shows that using biomass to create biodiesel is a promising way to help the planet (Hossain et al. 2008). Soybean, corn, sugarcane, canola, Jatropha, and other crops are viable biomass sources, but they are controversial due to global issues of food-fuel conflict and competition for land. Microalgae are being used more frequently in biodiesel production due to these issues. However, there is almost no data on the topic in Ghana. This study aims to identify viable algae species from Ghanaian freshwater bodies for use in biodiesel manufacturing.

MATERIALS AND METHODS

Collection of samples

The Weija reservoir, wastewater ponds in and around the University of Ghana campus in Prampram, and a freshwater pond in Teshie, Ghana, all provided water samples for isolating microalgae. Figure 1 is a map showing where the samples were taken. The research laboratory of the Botany Department at the University of Ghana in Legon-Accra, Ghana, was used for all of the experiments.

According to this research, the following procedures are required to convert algae into oil: Step 1: harvesting and identifying algae; Step 2: cultivating algae in a medium; Step 3: dewatering and drying the algae; Step 5: solvent extraction of oil from the algae; Step 6: transesterification into biodiesel (fatty acid methyl esters).

Media compositions and preparation

To isolate and cultivate the microalgae, the researchers employed Bold's basal medium, Sach's solution, and BG-11 as their medium formula. These combinations represent a versatile freshwater algal growth medium. In the formulas, NaNO3 is used as the nitrogen source, $K_2HPO_4.3H_2O$ is the phosphorus source, and metal mixes are used as the micronutrients. Components of Bold's basal medium and Sach's solution developed for isolating microalgal species are listed in Tables 1 and 2, respectively. In addition, Table 3 displays the BG-11 developed methodology for cyanobacteria culture (isolated in the study).

The seventh stock solution (Table 1) was made by dissolving EDTA and KOH in 1 liter of distilled water. The eighth stock solution (Table 1) was made by dissolving the same amount of $FeSO_4.7H_2O$ into sterile water. The $FeSO_4.7H_2O$ solution was adjusted to 1 liter by adding H_2SO_4, followed by distillate water.

Micronutrients (stock solution 10) were mixed into 800 mL of distilled water, one component at a time until fully dissolved between additions. The volume was then brought up to 1 liter (Table 1).

10 mL of each stock solution (1-6) was added to 940 milliliters of distilled water to make one liter of Bold's basal medium. The final volume was brought up to l liter by adding one (1) mL of stock solution (7-10). Before being kept for later use in the experiments, the medium was autoclaved at 121°C for 15 minutes.

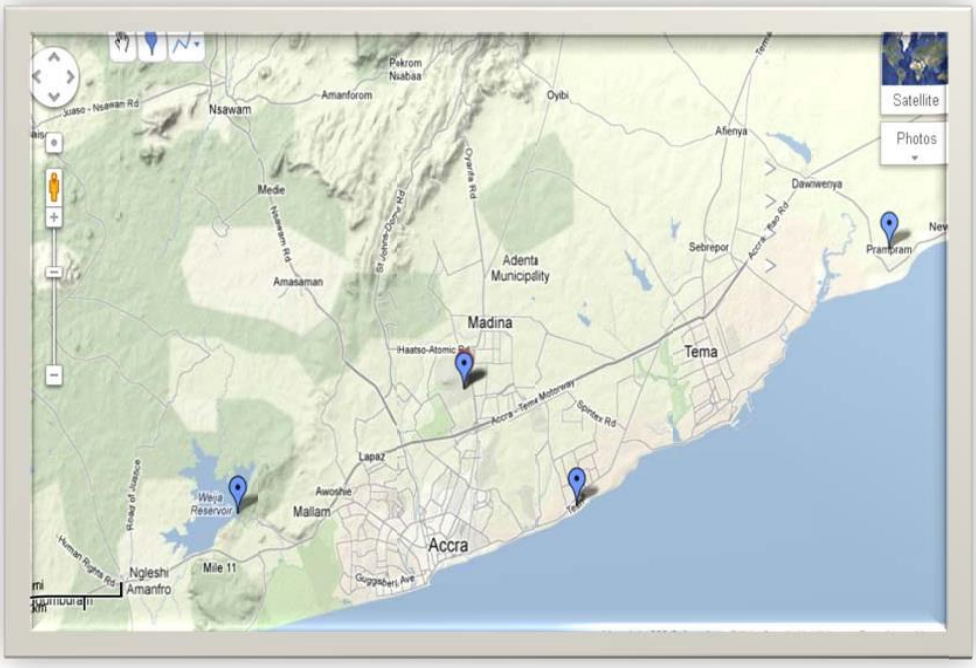

Figure 1. Map showing the location of collection site (source: Google Earth, retrieved April 10, 2013)

Table 1. Protocol for formulating Bold's Basal medium (BBM) (Kanz and Bold 1969)

Stock solutions	**per litre distilled water (dH_2O)**
1. $NaNO_3$	25.0 g
2. $CaCl_2.2H_2O$	2.5 g
3. $MgSO_4.7H_2O$	7.5 g
4. K_2HPO_4	7.5 g
5. KH_2PO_4	17.5 g
6. NaCl	2.5 g
7. EDTA (a)	50.0 g
KOH (b)	31.0g
8. $FeSO_4.7H_2O$ (c)	4.98 g
H_2SO_4 (d)	1.0 mL
9. H_3BO_3	11.42g
10. Micronutrients	**$g.L^{-1}$**
$ZnSO_4.7H_2O$	8.82
$MnCl_2.4H_2O$	1.44
MoO_3	0.71
$CuSO_4.5H_2O$	1.57
$Co(NO_3)_2.6H_2O$	0.49

Table 2. Protocol for the formulation of Sach's Solution (Full Strength)

Components	**Concentration in solution (g/L)**
$CaSO_4$	0.5
$Ca(PO_3)_2$	0.5
$MgSO_4$	0.5
NaCl	0.16
KNO_3	1.04
$FeCl_2$	Trace
Distilled water	2,000(ml)

Table 3. Protocol for the formulation of BG-11 (Stanier et al. 1971)

Stock solutions	**Per liter distilled water (dH_2O)**
1. $NaNO_3$	15.0 g
2. $K_2HPO_4.3H_2O$	4.0 g
3. $MgSO_4.7H_2O$	7.5 g
4. $CaCl_2.2H_2O$	3.6 g
5. Citric acid	0.6 g
6. Ferric ammonium citrate	0.6 g (Autoclave to dissolve)
7. EDTA	0.1 g
8. Na_2CO_3	2.0 g
9. **Trace metal mixture**	**$g.L^{-1}$**
H_3BO_3	2.86
$MnCl_2.4H_2O$	1.81
$ZnSO_4.7H_2O$	0.222
$Na_2MoO_4.2H_2O$	0.39
$CuSO_4.5H_2O$	0.079
$Co(NO_3)_2.6H2O$	0.0494

For the experiments, each component from Table 2 was dissolved individually in a measuring cylinder, then filled with distilled water until it reached a volume of 2 liters.

Dissolving the required mass of each component into one liter of distilled water yielded eight different stock solutions (1-8) (Table 3). Nonetheless, stock solution 9 was prepared by dissolving the specified mass of the trace metal mixture's constituents. Then, each component of the trace metal mixture was added individually to 800 mL of distilled water. It was guaranteed that each component thoroughly dissolved between additions and that the total volume reached 1 liter.

The stock solutions (1-9) were then used to prepare one liter of BG-11 medium for microalgae growth. First, an initial volume of 829 mL of pure water in a measuring cylinder was added to 100 mL of stock solution 1 (Table 3). Next, each stock solution (2-8) was added at a volume of ten (10) milliliters. Finally, 1 mL of stock solution 9 was added to raise the volume to 1 liter. Before autoclaving at 121°C for 15 minutes, the pH of the medium was adjusted to about 7.4 using 1M NaOH. The medium was preserved for use in future research.

Isolation and identification of microalgae

Microalgae were isolated and identified in the following ways: Ten (10) mL of water from each site was placed in a 250 mL conical flask with 200 mL of sterilized Bold's Basal Medium (BBM) (Kanz and Bold 1969). Sach's solution was used to do the identical operation, and the results were cultured for two weeks. Both cultures were kept at 29°C, illuminated by a 20-watt white fluorescent light, and aerated by a rotary shaker rotating at 150 revolutions per minute throughout a 12-hour day/night cycle. The flasks were inspected under an optical microscope for signs of algal growth after two (2) weeks of incubation (Leica DM 500). Next, isolated strains were grown in a BG-11 medium with 1% agar from 0.1 mL of water taken from a flask displaying growth. Sach's solution was used for the second round of testing. The algae were cultured as single colonies in BG-11 liquid medium and then re-cultured as non-axenic batch cultures. These were kept in subcultures for further testing. Three types of green microalgae and one kind of cyanobacteria were found and isolated for four different types of microalgae.

Culture of microalgae

Isolated green microalgae were cultivated in BG-11 medium for cyanobacteria, and 20 mL of the microalgal suspension was added to 200 mL of Bold's baseline medium (Table 1) (Stanier et al. 1971). A 500 mL conical flask was used to house the subcultures. The primary cultures were developed in a well-lit room with sunlight coming in via a window using homemade photobioreactors made from 2-liter reagent bottles. To facilitate gaseous exchange, plastic tubes were put into the openings of the makeshift photobioreactors (Figure 2). In this investigation, a continuous culture system was used.

Harvesting and drying of microalgae

Isolates of algae were grown in a petri dish for six weeks before being filtered and centrifuged every two weeks for harvesting. Cell suspensions of *Oedogonium* sp. and *Spirogyra* sp. were filtered before being air dried at room temperature (25°C) and then oven dried at 80°C. We also filtered and centrifuged at 500g for 10 minutes to remove *Chroococcus* sp. and *Closterium* sp. They were cooled to -70°C and freeze-dried for 12 hours.

Figure 2. Laboratory set up of some microalgal species (main cultures)

To begin the oil extraction process, all of the dried microalgae were ground into powder in an electronic mill.

Extraction of algal oil

For 24 hours at room temperature, twenty (20) g of each dried algal biomass was added to a 200 mL solvent mixture of 1:1 v/v hexane and diethyl ether. The mixture was agitated vigorously on a magnetic field stirrer (Figure 12). The extracted substance was filtered using a funnel and some filter paper to remove the biomass. To extract any remaining lipids, the biomass was washed three times using a combination of the solvents. After collecting the extracts (main and residual lipids), the solvents were evaporated using a rotary evaporator under a vacuum, resulting in the algal oil (Figure 12). Oil content in the biomass was calculated using the mass of extracted algal oil.

Transesterification of algal oil

Using a magnetic stirrer, one (1) gram of sodium metal was dissolved in 30 milliliters of methanol in a 500-milliliter conical flask. Hydrogen gas was released during the onsite production of sodium methoxide. Biodiesel (fatty acid methyl esters) and glycerol were obtained by washing algal oil with twenty (20) mL of diethyl ether and adding it to the sodium methoxide while stirring with a magnetic stirrer for twenty-four (24) hours.

Washing and drying of biodiesel produced

The transesterification by-products were collected in a separating funnel and rinsed three times with 25 mL of distilled water. The biodiesel was transferred from its original container into a volumetric flask after the aqueous layer had been collected in a conical flask and discarded. With the aid of anhydrous magnesium sulfate ($MgSO_4$), the biodiesel was dehydrated and filtered.

RESULTS AND DISCUSSION

Isolation and identification of microalgae

Using morphological analysis performed using a Leica DM 500 optical microscope, four (4) different types of microalgae were isolated and named for this study. *Chroococcus*, *Spirogyra*, *Oedogonium*, and *Closterium* were isolated (Figure 3-6). *Chroococcus* sp. is a cyanobacterium, while *Oedogonium* sp., *Spirogyra* sp., and *Closterium* sp. are all green algae. These species were chosen because they thrive in the designated growth medium combinations of Sach's solution and Bold's Basal Medium.

Productivity of microalgal species

Microalgae isolates cultured in photobioreactors were shown to have had tremendous cell multiplication, as depicted in Figures 7 and 8.

After harvesting microalgae, the total dry weight of microalgae was determined and used as the total biomass produced for cultures. Table 4 displays the total dry weight of biomass produced over six weeks.

The results show that *Oedogonium* sp. has the highest dry weight percentage (55.8%), followed by *Closterium* sp. at 40.1%, *Chroococcus* sp. at 50.2%, and *Spirogyra* sp. at 50%. (Table 4).

Figure 3. Micrograph of *Chroococcus* species

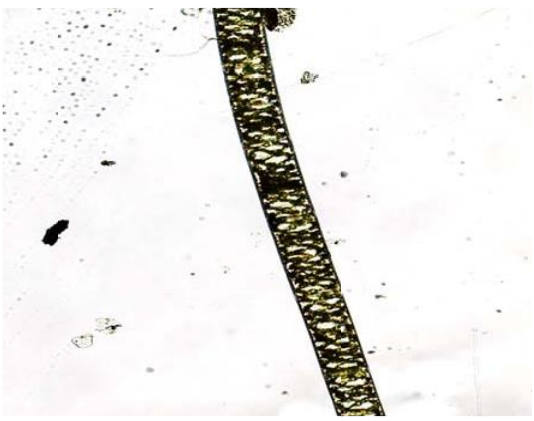

Figure 4. Micrograph of *Spirogyra* species

Figure 5. Micrograph of *Oedogonium* species

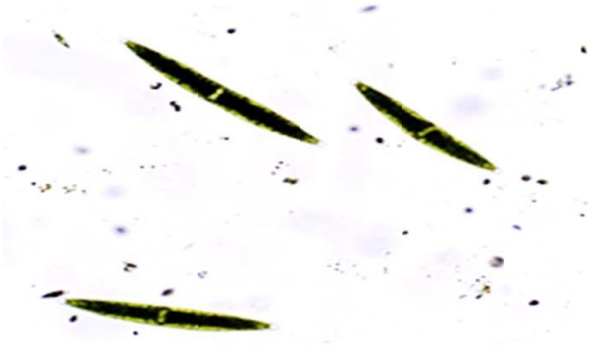

Figure 6. Micrograph of *Closterium* species

Figure 7. Photograph of growth observed after two weeks of culture

Figure 8. Photograph of growth observed after four weeks of culture

Table 4. Measurement of total wet weight, dry weight, and percentage dry weight

Microalgae species	Wet weight (g)			Total wet weight(g)	Dry weight (g)			Total dry weight(g)	Percentagedry weight (%)
	Week2	Week4	Week6		Week2	Week4	Week6		
Oedogonium sp.	21.6	21.6	21.8	65.0	12.0	12.1	12.2	36.3	55.8
Chroococcus sp.	20.0	20.1	20.4	61.5	10.0	10.4	10.5	30.9	50.2
Spirogyra sp.	16.7	16.8	17.3	50.8	8.1	8.4	8.9	25.4	50.0
Closterium sp.	16.7	16.9	16.9	50.5	6.5	6.8	7.0	20.3	40.1

Harvesting and drying of microalgae

The microalgae were collected every two weeks by filtration and centrifugation. Images of collected microalgae are shown in Figure 9. Photographs of dried microalgae are presented in Figure 10.

Algal oil production and transesterification

Biodiesel was created by transesterifying algal oil with sodium methoxide. Images of the transesterified algal oil are presented in Figure 11. This process involves the removal of organic solvents.

Algal oil was transesterified to create biodiesel, as seen in Figure 12. *Oedogonium* sp. was clear, *Spirogyra* sp. was brown, and *Closterium* sp. and *Chrooccocus* sp. were pale yellow as they produced the biodiesel collected in tubes.

The yield percentages of algal oil and biodiesel are displayed in Figure 13. According to the data, *Oedogonium* sp. produces the highest concentration of algal oil (38.1%). On the other hand, the oil yield from *Chroococcus* sp. and *Closterium* sp. was in the 20-25% range, while that of *Spirogyra* sp. was only around 21%.

The output of biodiesel was not proportionate to the yield of algal oil. Significant amounts of biodiesel oil (94%) were produced by the *Closterium* sp. strain, but the *Chroococcus* sp. strain only produced 80%. A 49% yield was achieved by cultivating *Oedogonium* sp., while *Spirogyra* sp. achieved the lowest biodiesel production at 33%.

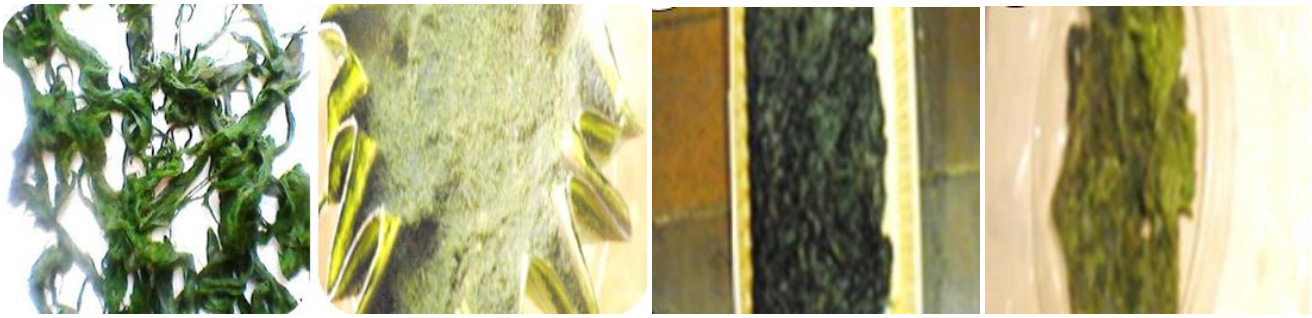

Figure 9. Photographs of harvested *Spirogyra* sp. (A) and *Chroococus* sp. (B), *Oedogonium* sp. (C), and *Closterium* sp. (D)

Figure 10. Photograph of dried *Oedogonium* species (A), *Chroococcus* species (B), *Spirogyra* species (C)

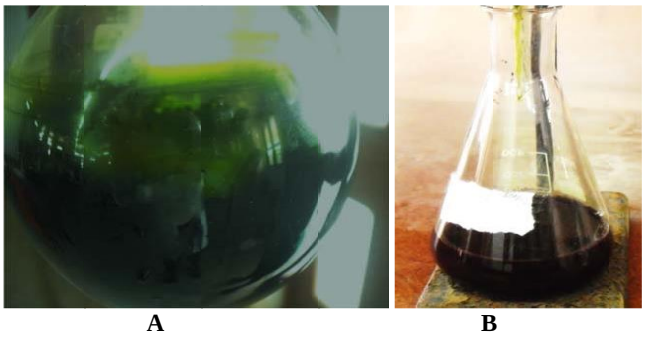

Figure 11. Photographs of algal oil and esterification (biodiesel and residue layers). (A) Algal oil, (B) Transesterification

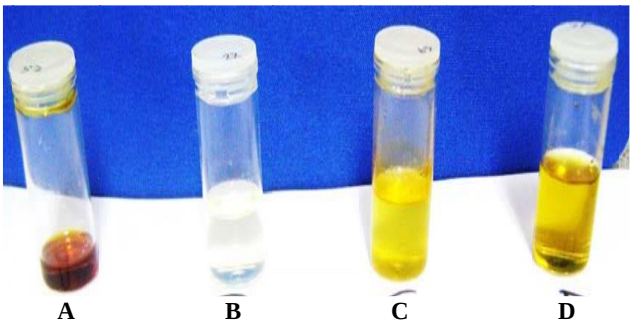

Figure 12. Photograph of Biodiesel Produced from Algal Oil. (A) *Spirogyra* sp. (B) *Oedogonium* sp. (C) *Chrooccocus* sp. (D) *Closterium* sp.

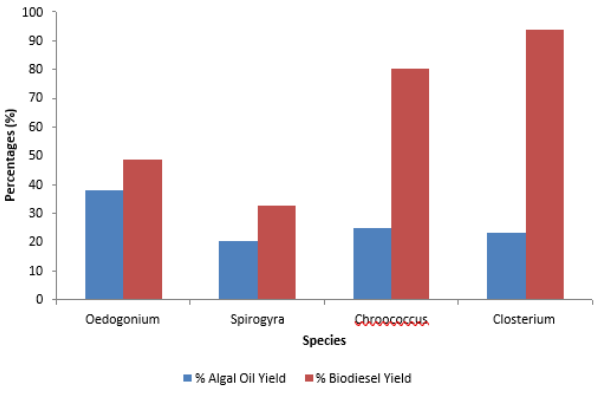

Figure 13. Percentage of oil and biodiesel yield

Discussion

Collection, isolation, and identification of microalgae

Microalgae are a heterogeneous group of organisms that can thrive in watery environments (Johnson and Wen 2009a). Though more than 50,000 species are thought to exist, only about 30,000 have been formally examined (Richmond 2004). This study gathered water samples from various freshwater sources, including wastewater ponds and reservoirs, to isolate microalgae. Three types of green algae and one type of cyanobacterium were found to have been isolated and identified from the samples (Figures 3-6). About 27 species of green algae, 20 species of cyanobacteria, and 8 species of diatoms were found in both treated and untreated wastewaters in a study by Chinnasamy et al. (2010), so these results are comparable.

Additionally, Reda et al. (2011) isolated 33 microalgal cultures, 18 from a reservoir and 15 from livestock effluent. Five green algae and three cyanobacteria were among the eight isolating organisms. Green algae and cyanobacteria were found in wastewater and reservoirs, indicating that these environments are a good place to collect microalgae isolates to grow into potential feedstock for biodiesel production.

Metzger and Largeau (2005) found that the morphology of algae might change with age and culture conditions, and it was true even within the same chemical race and strain. Because of their various shapes and sizes, algae can be challenging to study under a microscope. Because it is challenging to tell strains of the same genus apart under the microscope, the morphological examinations used in this work could only identify species to the genus level. It could lead to the misidentification of species. Hence DNA markers are preferable for determining which is which. In addition, if this restriction were lifted, more microalgae species could be discovered. However, some microalgae species were isolated and identified thanks to this study, which is a step in the right direction.

Two newly discovered species were found to have been isolated during the biodiesel production process. According to the results of this study, *Chroococcus* sp. and *Closterium* sp. both have promising applications as biodiesel feedstock. It is calculated using the percentage of algal oil and biodiesel they produce (Fig 4.1).

Productivity of microalgal species

Microalgae can rapidly multiply if provided with the right conditions and enough nutrients. Therefore, the growth dynamics of increasing algal populations should be considered for maximum productivity. In the first stage of algae cultivation, the algal cells adapt to the new medium conditions for a relatively short time. It's important to remember this when transferring cells to a new medium or reactor vessel, as it may be some time before the cells begin reproducing in their new environment. *Oedogonium* sp., *Chroococcus* sp., *Spirogyra* sp., and *Closterium* sp. all had dry weights of 12.1g, 10.0g, 8.1g, and 6.5g, respectively, in week 2 of this investigation (Table 4). Each species' dry weight rose about the same between weeks 4 and 6. It indicates that the growth of the diverse microalgal species was exponential during weeks 2, 4, and 6. Small gains in dry weight were seen across all species between weeks 2 and 4 and again between weeks 6 and 8. It is because new nutrients are added to the medium after each harvest, causing a domino effect of growth. Thus, the small rise in dry weights observed between weeks 4 and 6 can be attributed to the higher nutritional levels observed between those two periods (Table 4). This rapid expansion during the cultivation periods agrees with research by Posthuma (2009), who described the exponential phase of algal growth, during which the number of cells in a given volume increases constantly. Since this experiment was constrained in acquiring counting equipment for cell counts, a common way of determining algae growth rate, dry weights were measured as an indicator of microalgae productivity (Table 4). However, Zuka et al. (2012) have determined that the dry weights of collected algae are a good predictor of biomass yield. The production of dry biomass was reported in terms of g dry algae/L^{-day}.

In addition, the results of this work revealed cell proliferation (Figures 7 and 8), which resulted in a harvest every two weeks within the continuously functioning culture system. Harvesting the microalgae every fourteen (14) days is in line with findings by Zuka et al. (2012), who determined that after twelve (12) to fourteen (14) days in culture, algae reach their stationary phase and must be harvested before the lyses stage (death stage). Furthermore, Mulumba and Farag have corroborated this (2012).

The composition of the medium and its concentration have been discovered to affect microalgal production and the concentration of dry-weight cellular constituents like lipids throughout cultivation (Posthuma 2009). The results showed that the microalgae dry weight ranged from 55.8 to 40.1% (Table 4). It may be because Bold's Basal Medium and BG-11 media have larger quantities of critical nutrients, including Nitrogen ($NaNO_3$), Phosphorous, and Potassium (KH_2PO_4 and K_2HPO_4), than other media do (Tables 1 and 3). These nutrients account for a sizable fraction of the product's dry weight and are thus crucial (Basova 2005; Chisti 2007; Posthuma 2009). Vitamins are just one example of a chemical that has been shown to improve growth rates, but there are many others (Croft et al. 2006). The high production of microalgae in this study may be attributed to the fact that the formulated media contained not only the basic nutrients but also vitamins and other metals such as iron (needed for photosynthesis), magnesium, copper, and zinc (Tables 1 and 3). It is in line with the recommendations of Grobbelaar and Bornman (2004), who noted the importance of including nitrogen, phosphorus, iron, and even silicon in specially prepared media for algae culture.

It is also confirmed in Table 4 that the productivity of *Oedogonium* sp., *Chroococcus* sp., and *Spirogyra* sp. is above average, whereas the productivity of *Closterium* sp. is slightly below average. The results of this study demonstrate the potential of these species as feedstock for biodiesel production, although they are typically overlooked in biodiesel research.

Harvesting and drying of algal biomass

Microalgae were filtered and centrifuged for harvesting within six weeks of cultivation. They were carried out to divide the medium from the biomass. The efficiency or yield may vary depending on the harvesting technique employed. The solid content of the recovered microalgae may also be influenced by the efficiency of the harvesting procedure. Aside from that, the efficiency of the harvesting technique can be gauged by the rate of water extraction (Uduman et al. 2009; Xiang 2012).

High-speed centrifugation, which was used to recover biomass from *Chroococcus* sp. and *Closterium* sp., is a tried and tested method. It's a fast and energy-intensive process, yet it is the most commonly used (Molina-Grima et al. 2003). Due to their diminutive cell sizes, centrifugation served as the method of choice for harvesting these microalgae in this investigation.

The level of pressure drop needed to drive fluid through a filtration medium can be affected by centrifugal force, pressure, or gravity, among others (Shelef et al. 1984; Uduman et al. 2009; Xiang 2012). Media that are fine enough to retain the microalgae tend to bond, making frequent backwashing a necessary part of the filtration process. As a result, the production of microalgal concentrations slowed (Uduman et al. 2009). The microalgal species (*Spirogyra* sp. and *Oedogonium* sp.), which have relatively high cell sizes, were harvested using pressure-driven surface filters in this investigation. Pressure filtering methods are suitable for collecting microalgal species with large cell sizes but ineffective for recovering microalgal species with sizes approaching bacterial dimensions (in the range of micrometers), as was observed in a study of process options undertaken by Molina-Grima et al. (2003).

In this experiment, three different drying techniques were applied. Drying microalgae initially degenerates and weakens the cell wall and plasma membrane, decreasing the cell's ability to hold oil and improving the effectiveness of the extraction procedure.

Both Oedogonium sp. and Spirogyra sp. underwent two days of air drying at room temperature (29°C), followed by six hours in an oven at 80°C to remove any remaining moisture. Similarly, Dejoye et al. (2011) dried algae between 20°C and 70°C for 2 to 6 hours to decrease the remaining moisture. Similarly to Ziga et al. (2010) and Belarbi et al. (2000), *Chroococcus* sp. and *Closterium* sp. were freeze-dried to eliminate moisture content. They found that once microalgae are freeze-dried, intercellular metabolites like oils can be easily recovered using solvent extraction.

Extraction of algal oil and transesterification

Microalgal biomass was dried and then used to extract algal oil for biodiesel production in this study. Similar results were found by Johnson and Wen (2009b), who demonstrated that the biodiesel production from microalgae following transesterification was greater in dry biomass than in biodiesel yield following transesterification utilizing wet biomass. The studies conducted by Kumar et al. (2011) are likewise comparable to this one. They extracted lipids from dried microalgal biomass to make biodiesel. *Tolypothrix*, *Pithophora*, *Spirogyra*, *Hydrodictyon*, *Rhizoclonium*, and *Cladophora*, all of which were utilized to create biodiesel, had their dry weights determined after being dried in an oven or by air. Prior to extracting lipids for biodiesel synthesis, Hossain et al. (2008) also determined the percentage of dry weights of *Oedogonium* and *Spirogyra*.

Algal oil yield is proportional to biomass production for *Oedogonium* spp., *Chrooccocus* spp., and *Closterium* spp., as shown in Table 4 and Figure 13. *Spirogyra* sp. had significantly larger biomass than *Closterium* sp., but it produced significantly less algal oil. These results show that *Spirogyra* sp. may produce significantly less algal oil than the other three species. Figure 13 further shows that the fatty acids content of the algal oil was easily converted to biodiesel in the following order: *Closterium* sp. > *Chrooccocus* sp. > *Oedogonium* sp. > and *Spirogyra* sp. Research conducted by Hossain et al. (2008) indicates that *Oedogonium* sp. produced more biodiesel than *Spirogyra* sp. The result shown in Figure 13 was congruent with their findings.

However, Johnson and Wen (2009b) argue that lipid loss during the extraction stage of the two-stage transesterification process (i.e., extracting algal oil and then transesterifying it) is a possibility, so that direct transesterification (methylation) may result in greater crude biodiesel production.

The results of this study demonstrated that the isolated algae are suitable for cultivation for biodiesel production. The algae *Chrooccocus* sp., *Oedogonium* sp., and *Spirogyra* sp. produce the least biodiesel, whereas *Closterium* sp. produces the most.

Algal cells have oil, which is sealed inside the cell wall and plasma membrane. These formations impede the cell's natural capacity to ship oil out of the body. In addition, plasma membrane degeneration occurs during the drying of algal cells, reducing the cells' capacity to hold oil. Hexane, an organic solvent, can enter the dry algae sample's cell wall and dissolve the oil. After the hexane has been extracted from the algae sample, the oil is taken from the cell. This oil is extracted from algae by removing the hexane solvent (Browne et al. 2010).

In conclusion this thesis contributes to a better comprehension of the feasibility of utilizing algal biomass as a biodiesel feedstock. It is accomplished through efforts in all steps of the process, from cultivating microalgae to making biodiesel. Four freshwater microalgae, which are rarely employed in research projects for biodiesel production, were isolated and identified in this study; it is one of the primary findings of this work. Green algae (*Oedogonium*, *Closterium*, and *Spirogyra* species) and Cyanobacteria (*Chroococcus* species) comprise these microalgae. The results showed that these species might flourish in a room temperature environment (29°C), with sufficient air, natural light source, and an appropriate medium. It serves as "proof of concept" that, under the right conditions, even relatively obscure species like the aforementioned algae can contribute significantly to biomass for use in biodiesel manufacturing. After that, cultural endeavors on a grand scale will be possible.

According to the results of this research, filtering is an effective harvesting method for dehydrating cultured *Oedogonium* sp. and *Spirogyra* sp., while centrifugation is an effective harvesting method for cultured *Closterium* sp. and *Chroococcus* sp. Air drying and oven drying are appropriate for *Oedogonium* sp. and *Spirogyra* sp.. In contrast, freeze-drying is appropriate for *Closterium* sp. and *Chroococcus* sp. It can be deduced from this study that *Oedogonium* sp., *Closterium* sp., *Spirogyra* sp., and *Chroococcus* sp. are viable species that can supply a significant amount of algal oil for biodiesel synthesis. Biodiesel production is highest in *Closterium* sp., next in *Chrooccocus* sp., then in *Oedogonium* sp., and finally in *Spirogyra* sp. It means that, when implemented on a big scale, biodiesel from these microalgae can serve as a renewable energy source that can replace petroleum fuel.

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