EXTRACTION, ISOLATION AND CHARACTERIZATION OF COMPOUNDS FROM *Termitomyces letestui* AND *Ganoderma lucidum* AND THEIR EFFICACY AGAINST SOME PATHOGENIC MICRO-ORGANISMS

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A Thesis submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Chemistry of Masinde Muliro University of Science and Technology

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DECLARATION

This thesis is my original work prepared with no other than the indicated sources and support and has not been presented elsewhere for a degree or any other award.

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CERTIFICATION

The undersigned certify that they have read and hereby recommend for acceptance of Masinde Muliro University of Science and Technology a research thesis entitled "Extraction, Isolation and Characterization of Compounds from *Termitomyces letestui* and *Ganoderma lucidum* and their Efficacy against some Pathogenic Micro-organisms".

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DEDICATION

This thesis is dedicated to my uncle Wycliffe who supported me by all means throughout the Master of Science, Chemistry program.

ACKNOWLEDGEMENTS

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ABSTRACT

Studies on mushrooms and other fungi over along time have showed a number of physiological properties that provide health benefits. This is supported by the traditional belief that mushroom consumption provides remedies to various human infections. Bacteria and fungi have been developing resistance to the existing drugs compared to the rate at which new drugs are being discovered. The current study reports on the chemical investigation of the local *Termitomyces letestui* and *Ganoderma lucidum* from Kakamega, Kenya for their antibacterial and antifungal properties. Dried samples of the two mushroom species were extracted sequentially using *n*-hexane, ethylacetate and methanol. Separation of the extracts was done using Column Chromatography while purification was done using Thin Layer Chromatography. Chemical structural determination was done using Infra-Red (IR), 1D (^{1}H, ^{13}C-NMR) and 2D NMR spectroscopy and comparison with physical and spectroscopic data of previously isolated compounds. Antimicrobial studies of the crude extracts and isolates were carried out on six microbial strains (Bacteria: Methicillin Resistant S*taphylococcus aureus* (MRSA), *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa, Streptococcus pyogenes* and *Creptococcus neoformans* fungi). The extracts and pure compounds from the mushrooms were applied at a concentration of 100 μg/ml using well diffusion method. Zone of inhibition was analyzed statistically using bar graph and then compared with standard drugs ampicillin as antibacterial and nystatin as an antifungal. Standard procedures were used in the determination of phytochemicals from the crude extracts. The phytochemical qualitative data for the mushrooms revealed the following groups of compounds; steroids, triterpenoids, carbohydrates, phenolics (tannins), glycosides and flavonoids. Chromatograhic separation of ethylacetate extract of local *T. letestui* yielded an ergostane, ergosta-5,7,22-triene-3β-ol (22*Z*) (**69**). Similarly, the *n*-hexane extract of local strain of *G. lucidum* yielded a 14α hydroxyl derivative of **69**, ergosta-5,7,22-triene-3β,14α–diol (22*Z*) **(72)**. The crude extracts (hexane, ethylacetate and methanol) and the compound, ergosta-5,7,22-triene-3β,14α–diol (22*Z*) **(72)** from *Ganoderma lucidum* showed significant activity against Methicillin Resistant *Staphylococcus aureus* (MRSA) ($p > 0.022$) and *Streptococcus pyogenes* ($p < 0.05$). The most sensitive microbe was *Streptococcus pyogenes* (40.3±0.3 mm). These mushrooms can be a natural source of new drugs and nutrients for management of common infections which are threatening mankind. Their production and propagation should be encouraged.

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DEFINITION OF TERMS

Antimicrobial…….. agent that prevents growth of micro-organisms

Control……. experiment that separates the condition of one variable on a system by holding constant all variables other than the one under observation.

Pathogens…….. disease – causing micro-organisms such as bacteria.

Phytochemical……non-nutritive bioactive substance such as flavonoid or considered to have some medicinal value on human health.

Resistance……..ability of a pathogen to survive in the body even with administration of a drug against it.

Zone of Inhibition ……... a region of no growth of a micro-organism.

LIST OF ABBREVIATIONS

AIDS……..Acquired Immune Deficiency Syndrome

^{13}C- NMR……..Carbon-13 Nuclear Magnetic Resonance

COSY……Correlation Spectroscopy

DMAPP…….Dimethylallylpyrophosphate

DMSO……..Dimethylsulphoxide

EtOAc……Ethylacetate

fr. ……...fraction

GC-MS ……..Gas Chromatography Mass Spectrometry

^{1}H – NMR……...Proton Nuclear Magnetic Resonance

Hex…… Hexane

HIV…….Human Immunodeficiency Virus

HMBC…… Hetero-multibond Correlation Spectroscopy

HSQC……. Heteronuclear Single Quantum Coherence spectroscopy

IPP……...Isopentylpyrophosphate

IR…….Infra-red Spectroscopy

MHA……Mueller Hinton Agar

MeOH……..Methanol

MMUST……...Masinde Muliro University of Science and Technology

MRSA……...Methicillin esistance *Staphylococcus aureus*

PDA ……..Potato Dextrose agar

R_f……...Retention factor

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Mushrooms are hypogeneous or epigeneous macro-fungi with a fruiting body which can be observed and plucked (Shu-ting and Miles, 2004). They are classified into four categories: consumable such as *Agaricus bisporus*; curable like *Ganoderma lucidum* and toxic like for example *Amanita phalloides* (Shu-ting and Miles, 2004). Mushrooms produce phytochemicals and neutraceuticals (Shu-ting and Miles, 2004). In some developing countries, mushrooms are used to treat infectious diseases. Poor hygiene conditions brings about bacteria, fungi and other micro-organisms which cause diarrhea, dysentery and other deadly diseases resulting in high death rate (Soji and Oloketuyi., 2013).

Termitomyces letestui is an edible mushroom spread in many parts of the country (Wandati *et al*., 2013). *Termitomyces* species have also been used ethno-medicinally. For example, in Cameroon, *Termitomyces titanicus* has been used to treat stomach ache or pains (Kinge *et al*., 2017). About 19 species of *Termitomyces* have been used in treating gastrointestinal disorder, constipation, as a stimulant, wound alleviation and cure for measles and fever (Aryal and Budathoki 2016).

Scientific research has shown that *Termitomyces letestui* mushrooms contain neuritogenic cerebrosides which improve brain function. *Termitomyces letestui* also possesses antioxidant activities, scavenging activity and chelating effects on ferrous ions (Puttaraju *et al*., 2006). Most *Termitomyces* mushrooms contain phenolics and flavonoids (Puttaraju *et al*., 2006).

Termitomyces mushroom genus grows in symbiotic relationship with under or above the ground *termatorium*. The macro-fungus is found in most parts of Western and Coastal areas of Kenya (Wandati *et al*., 2013). It has been used as food for human beings from ancient times (Srivastava *et al*., 2012). They contain saccharides, albumen, amino acids and minerals (Elsayed *et al.,* 2014). Their truffles make them useful in traditional medicine in Africa and other places. Polysaccharides, terpenoids, phenolic compounds and other molecules have been isolated from *Termitomyces letestui* (Elsayed *et al*., 2014). *Termitomyces* genus spreads and grows naturally through transportion of spores by Termitidae (Isoptera) eusocial insects (termites) using plant materials through their stomach (Froslev *et al*., 2003). They also supply cellulose and xylanase which work synergistically with endogenous enzymes to concentrate nitrogen for the termites. Termites also appear on the genus *Basidiomycota* and *Agaricales* (Froslev *et al*., 2003).

Ganoderma lucidum, also known as "reishi" (Japan) and 'Ling Zhi" (China), is a fungus classified under Ganodermatacea family (polyporales) (Stamets, 2005). It is a large, dark mushroom with glossy or shiny exterior and woody texture (Wachtel-Galor *et al*., 2011). It grows in temperate and sub-tropical regions of North and South America, Europe and Asia. The species grows on its own or in small groups on the roots of *Acer*, *Quercus*, *Fraxinus*, *Celtis*, *Salix* or *Ulnus* (Gerhardt *et al*., 2000). It grows either in living hosts or saprophytic (dead hosts) bark of trees (Jong and Birmingham, 1992).

There are various species of *Ganoderma lucidum* worldwide due to different ecological conditions and adaptations (Staeyaert, 1980). In Asian parts, *Ganoderma lucidum* has been consumed traditionally to ease the mind, alleviate cough, asthma, prevents dizziness, insomnia, palpitation and shortness of breath (Wachtel-Galor *et al*., 2011). Ethnic medicine men use *Ganoderma lucidum* to improve the immune system (Zhu and

Lin, 2006). Recent innovation showed that *Ganoderma lucidum* methanol extract impaired breast cancer cells (Tiffany *et al*., 2018).

Ganoderma lucidum contains about 100 types of polysaccharides which possess immunomodulating activities (Boh *et al.,* 2007). Their triterpenoids possess anti-inflammatory, antitumorigenic and hypolipidemic activities (McKenna *et al.*, 2002). The mushroom has also been implicated in the treatment of various infectious diseases such as influenza (flu), swine flu, avian flu, asthma, liver and heart diseases (www.webmd.com accessed on 15/1/2019). These diseases are mainly caused by helminthes, protozoa, viruses, bacteria and fungi (www.webmd.com accessed on 15/1/2019). Scientists have shown creativity in identifying lead compounds against global infectious diseases (Turner, 2008). Antimicrobial secondary metabolites have been reported in many fungi bio-controls (Turner, 2008). *Ganoderma lucidum* is considered as a solution to many infectious diseases (Jong and Birmingham., 1992). In addition to about 400 active substances from *Ganoderma lucidum,* terpenoids have been the main phytochemical sources (Russell and Paterson 2006). Other compounds from *Ganoderma lucidum* are Ganoderic acids (Min *et al*., 2001), esters (Zhang *et al.,* 2008), alcohols (Min *et al.,* 1998) and lactones (Chen and Yu, 1991). Successful study has been done in other parts of the world but very little chemistry has been done on Kenyan *G. lucidum* species.

Kenyan *Ganoderma lucidum* is kidney shaped, wood textured with dark brown cap and no stalk. The mushroom is either sown or grows on bark of trees. Kenyan *Ganoderma lucidum* grows naturally in Kakamega forest which is a tropical rain forest. It is still unclear on the traditional uses of Kenyan *Ganoderma lucidum.*So far, there are little

reports on either chemical constituents or biological activity of the Kenyan *Ganoderma lucidum*, and therefore this research work sought to investigate the chemical and antimicrobial activity of this species.

1.2. Statement of the Problem

Although there has been advancement of modern medicine, microbes have become resistant to various drugs. For example, penicillin was discovered in 1929 by Fleming but in 1940`s, the emergence of new strains of bacteria began destroying the drug and negating its effects (Davies and Davies, 2010). Similarly, fungi have also developed resistance to anti-fungals. For instance, the fungi *Candida glabrata* has been found to be resistant against echnocandins drug while *Aspagillus fumigates* fungi has been found to be resistance against azole (Laxminarayan and Chaudhury, 2016). According to WHO, antimicrobial resistance is an urgent issue facing medical science (WHO report cited on 14/7/2020). Some compounds are discovered from plants or mushrooms but are not subjected to synergy. The discovered compounds can be used in combination with the existing convectional drugs and this could be a solution to the problem of antimicrobial resistance. Bioactive compounds have been isolated from many plants but only from a small number of mushrooms (Agrawal *et al*., 2017). Crude extracts from natural phenomena such as mushrooms or plants usually contain many phytochemicals. There is need to isolate drugs from natural sources such as mushrooms since they are less expensive, sometimes less toxic and easily available (Karimi *et al*., 2015). Kenyan *Ganoderma lucidum* and *Termitomyces letestui* may contain bio-active compounds. Phytochemical studies from these mushrooms could result into unreported compounds

that could be used as lead compounds for the management of pathogenic micro-organisms.

1.3 Objectives

1.3.1 Overall objective

To extract, isolate and characterize compounds from *Termitomyces letestui* and *Ganoderma lucidum* and determine their efficacy on some pathogenic micro-organisms.

1.3.2 Specific Objectives

i) To carry out phytochemical tests on local *Termitomyces letestui* and *Ganoderma lucidum*

ii) To determine and characterize compounds from local *Termitomyces letestui* and *Ganoderma lucidum*.

iii) To evaluate the antimicrobial potencies of crude and isolates from *Termitomyces letestui* and *Ganoderma lucidum* on some pathogenic microbes.

1.4 Hypothesis

i) The following phytochemicals are present in both *Termitomyces letestui* and *Ganoderma lucidum* mushrooms; phytosterols (steroids, and triterpenoids), polysaccharides and flavonoids.

ii) Kenyan *Termitomyces letestui* and *Ganoderma lucidum* possess bioactive compounds.

iii) Compounds from *Ganoderma lucidum* have activity against *MRSA (Methicillin Resistant Staphylococcus aureus)* and *Streptococcus pyogenes*.

1.5 Justification

The study was focused on the investigation of *Termitomyces letestui* and *Ganoderma lucidum* mushrooms for antibacterial and antifungal agents. Fungal and bacterial infections contribute greatly to human population mortality rate worldwide. This has been due to the increasing resistance of microbes to drugs worldwide (Odonkor and Addo, 2011; Weiderhold, 2017). Traditional uses of *Termitomyces letestui* mushrooms are not well known. However, in Cameroon, *Termitomyces titanicus* has been used in the treatment of stomach complications (Kinge *et al*., 2017). Local *Termitomyces letestui* mushroom needs to be investigated for its medicinal value. *Ganoderma lucidum* has been used ethno-medicinally as an immune booster especially in West African countries and Asia such as China and Japan due to the health benefits, useful bio-active compounds need to be isolated. A team of researchers found recently a drug from the American *Ganoderma lucidum* that impairs breast cancer stem cells (Tiffany *et al*., 2018), which proved that *Ganoderma lucidum* is a curative mushroom. Despite studies undertaken on *Ganoderma lucidum*, there have been little investigations on the antimicrobial activities of the Kenyan *G. lucidum* species. There has also been little knowledge on phytochemical, antibacterial and antifungal activities of local *Termitomyces letestui*. Thus the current study will provide scientific knowledge on the constituents of the two mushrooms and their antimicrobial activity. Their constituents or isolates could work as templates in the control of microbial agents.

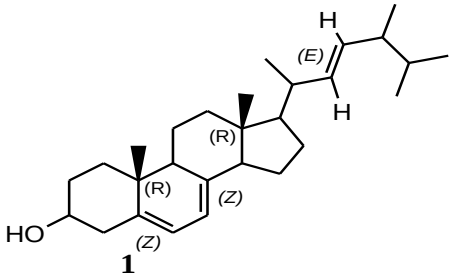
CHAPTER TWO

LITERATURE REVIEW

2.1 The general chemistry of *Termitomyces letestui* and *Ganoderma lucidum* with other mushrooms

Mushrooms produce useful bioactive metabolites and are also sources of useful drugs. The bioactive compounds produced either belong to sesquiterpenes, triterpenes, sterols, alkaloids or amino acids among which sterols are the most prevalent (Ulrike *et al*., 2005). One species of each can possess various bioactive compounds (Selima *et al*., 2012).

Investigations of Tanzanian edible mushroom species *Cantharellus symoensii* and *Cantharellus isabelinus* for their antibacterial, antifungal and cytotoxic constituents, yielded a sterol, ergosta-5,7,22-triene-3*β*-ol (22*E*) **(1)** that exhibited strong antifungal and antibacterial activities and was also toxic in brine shrimp test (Nkunya, 2002). Previous chemical investigations on *Pleurotus eryngii* revealed the presence of pleurolactone **(2)** that showed bioactivities such as antimicrobial, hepatoprotective, antioxidant, antitumor and immunomodulating functions (Liu *et al*., 2013). In previous studies dozens of human clinical studies have shown that oyster mushroom has no harmful effects and is used as food (Gilroy *et al*., 2000) and their derived compounds have been used to cure diseases such as typhoid and dysentery (Zhou, 2002).



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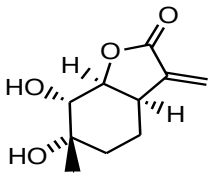

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Soji *et al*., (2013) researched on phytochemical and antimicrobial tests of *Lenzites betulina* and *Coriolopsis gallica* mushrooms. The study revealed the existence of tannins, phenolics, flavonoids, steroids and saponins in *Lenzites betulina* and *Coriolopsis gallica* mushrooms. Aqueous extracts of *Lenzites betulina* inhibited *E. coli* and *S. aureus* at 15 and 26 mm and its ethanolic extracts at 22.0 mm against *S. aureus*. Water soluble glucans from *Pleurotus tuber regium* and their sulphated derivatives inhibited the development of herpes simplex virus (Zhang *et al*., 2004). Alam *et al* (2011) stated that when rat is fed with hypercholesterol from *Pleurotus ferulae,* it lowered its serum cholesterol level.

Termitomyces mushrooms contain bioactive components for neuro-degenerative disorders, antioxidants, immunomodulators, antitumors and antimicrobials (Husieh and Ju, 2018). Although many studies have been done on other *Termitomyces spp,* not much has been done on *T. letestui* mushroom species. Preliminary studies have revealed that *Termitomyces* mushroom species possess substantial antimicrobial and antioxidant properties (Oyetayo, 2011). Research conducted by Mahamat (2018) showed that *T. letestui* water extract inhibited *S. aureus* and *E. coli*. The current information shows that the mushrooms tested were inhibitive against *Escherichia coli* and *Staphylococcus aureus*.

Ganoderma lucidum mushroom was used in customary medicine for curing human and animal illnesses (Shikongo *et al*., 2013). The mushroom has been proved to have broad pharmacological and antibacterial characteristics, fighting skin and wound infections (Shikongo *et al*., 2013). The Namibian *G. lucidum* showed that its benzene extract exhibited inhibitory zone (19 mm) against *E. coli* and *Neisseria meningitides* (Shikongo *et al*., 2013). This means that *G. lucidum* contains antimicrobial properties that can

inhibit various gram positive and negative bacteria. Most species contain triterpenoids, flavonoids, coumarins, quinones, carotenoids and amino acids (Shikongo *et al*., 2013). The same phytochemicals were shown to possess similar antimicrobial properties (Roberts, 2004). *Ganoderma* products are known to lack toxins and also have no side effects (Shu-ting and Miles, 2004).

2.2 The Genus *Termitomyces*

There are different varieties of *Termitomyces letestui* mushrooms in Kenya depending on their occurrence and are called by different local names. For example, *Termitomyces letestui* that occur in Western Kenya are called "obulando" in Luhya while the ones in Mt. Elgon are known as "mariondonik" in Sabaot language (Wandati *et al*., 2013). Other areas where *Termitomyces letestui* occur are Central Kenya where they call it "makunu ma mutitu" in Kikuyu and "oruka" according to Luos in Nyanza (Wandati *et al*., 2013). Since these varieties occur in different regions in Kenya, the regions have different ecological conditions. The species are adapted to their ecological conditions. For example *T. letestui* in Central Kenya (Mt. Kenya) is different from that of Western Kenya. *Termitomyces letestui* that occurs in Nyanza is the same as that of Western Kenya since the ecological conditions are almost similar (Wandati *et al*., 2013).

2.2.1 Classification of *Termitomyces* species

Kingdom…… Fungi

Division……..Basidiomycota

Class……...Agaricomycetes

Order……..Agaricales

Family……Lycophyllaceae

Genus…….*Temitomyces*

Species……*letestui*

Binomial Name……*Termitomyces letestui* (www.wikipedia.com accessed on 14/4/2019)

2.2.2 Characteristics of *Termitomyces letestui*

Most *Termitomyces* mushrooms species are edible. *Termitomyces titanicus* are the world`s biggest mushrooms and are found in West Africa and Zambia (Shikongo *et al*., 2013). The diameter of their cap is 1 m (3 ft). These fungi develop in "combs" which form termites excreta (Kirk *et al*., 2008). *Termitomyces* species are food for Macrotermitinae termites that enjoy symbiotic relationship same as that of *Atta* ants and *Attamyces* mushrooms (Kirk *et al*., 2008). However, spore transfer is fulfilled from mushrooms secretions which come from the termite mounds (Mueller *et al*., 2005). *Termitomyces* were first circumscribed in Uganda by Roger Heim in 1942 (www.wikipedia.com accessed on 14/4/2019). From 1965 to 1969, Arthur French did research in Uganda and worked with mushrooms and termites (French, 1993). Scientific information on mushroom species was present, but the information did not explain the symbiotic relationship between termites and the edible macro-fungi (French, 1993). French and Baganda women conducted research on the mushrooms and published (French, 1993). *Termitomyces* mushroom shows symbiotic relationship with termites (Herkonen *et al*., 2003). *Termitomyces* species grow in mounds of termites commonly known as *termatorium* (Srivastava *et al*., 2012). It has been noted that termites are most sensitive to their environment influenced by temperature, heat, humidity and carbon (IV) oxide concentration (Srivastava *et al*., 2012). *Termitomyces* fungus grows in the rainy season on the "brain like" comb of the termite insects (Srivastava *et al*., 2012). When heavy rains occur with thunderstorms, humidity increases while other environmental

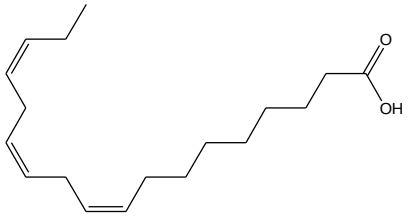
factors favorable for growth, development and differentiation of mycelium within the *termatorium* lead to the growth of *Termitomyces* mushroom (Srivasvata *et al*., 2012). This process of growth is similar to other types of wild mushrooms. In Namibia, the mushroom occurs only in tall termite mounds of central and northern Namibia with average rainfall of 350 mm or more. Their mounds reach 4 to 5 m high and their associated termite is the *Termes bellicosus* (*Macrotermes michaelseni*). In Zambia, *Termitomyces schimperi* is symbiotically related with *Odontotermes patruus* termites (Srivastava *et al*., 2012). In Kenya and East Africa, symbiotic relationship between *Termitomyces letestui* and their respective termite species has never been studied or no records are available. Symbiotic relationship of edible wild mushrooms with different termite species needs to be investigated so as to conserve the environment because this growth cycle is part of the natural ecosystem. *Termitomyces letestui* mushroom is shown in figure 1 below.

Figure 1: lKenyan *Termitomyces letestui* from MMUST compound

2.3 Compounds previously isolated from *Termitomyces letestui*

Not much has been done concerning isolation of compounds from *Termitomyces letestui* in many parts of the world. Neuritogenic cerebrosides were isolated from the

consumable Chinese mushroom *Jizong Termitomyces albuminosus* (Berk.) Heim (Qi *et al*., 2000). Information on phytochemistry or antimicrobial evidence of *Termitomyces letestui* from Western Kenya is unknown hence the motivation to carry out this study. GC-MS of lipid component of Tanzanian *T. letestui* indicated the existence of linolenic ($C_{18:2}$) **(3)**, stearic ($C_{18:0}$) **(4)**, oleic ($C_{18:1}$) **(5)**, palmitic ($C_{16:0}$) **(6)**, pentadecanoic ($C_{15:0}$) **(7)** and myristic ($C_{14:0}$) **(8)** acids (Baraza *et al*., 2007). The compounds, 5,8-peroxyergosterol **(9)** and α, α 1,1`- trehalose **(10)** have been reported from *Termitomyces letestui* (Baraza *et al*., 2007).



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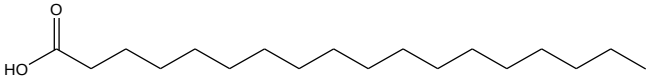

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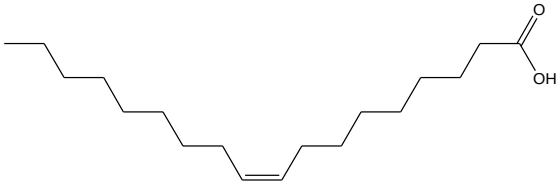

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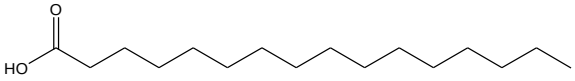

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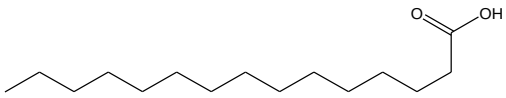

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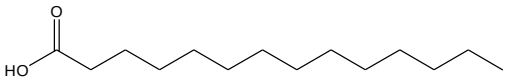

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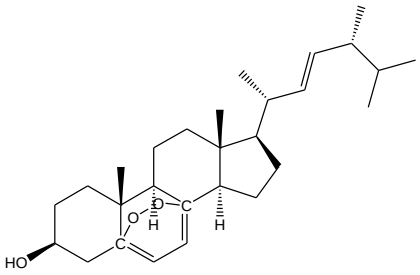

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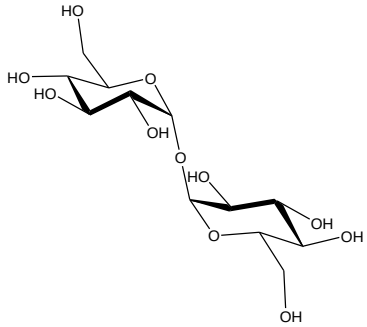

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2.4 The Genus *Ganoderma*

Different varieties of *Ganoderma lucidum* species are favoured in different geographical regions. For instance, the black *G. lucidum* thrives best in South China whereas the red *G. lucidum* does well in Japan (Chandana *et al*., 2008). *Ganoderma lucidum* germinates in warm and wet sub-tropical regions. Optimal temperature range for *Ganoderma lucidum* is 25 – 30 °C. This mushroom grows at pH range of 5 (Chandana *et al*., 2008). During fructification, good ventilation is necessary while during primordial initiation and fruiting light is required (Chen, 1999). *Ganoderma lucidum* fruity features occur in four colours: red, yellow, black and white. Initial colour of the fruits is white, turns into yellow and finally into reddish brown shade that is good looking and distinguishing. The red variety of *G. lucidum* is used for nutraceutical purposes and is cultivated as cash crop in China, Taiwan, Japan, Korea and North America (Shu-ting and Miles, 2004). *Ganoderma lucidum* is an annual fungus. Its fruiting body is rigid and can stay for a longer time. *Ganoderma* is cultivated by the following three methods: i) in woody or log (ii) in pot or bottle and (iii) in tank (Shu-ting and Miles, 2004). Woody log method has limitations because it requires a certain variety of tree logs which have to be seasoned and prepared (Shu-ting and Miles, 2018). Exotic *G. lucidum* is cultivated using cereal

materials, timber shavings (Wasser, 2005), cork residues (Riu *et al*., 1997) and hamada, glucose peptone and lilly media (Chandana *et al*., 2008).

Ganoderma mushrooms belong to Ganodermataceae family which consists of about eighty species of which *G. lucidum* is included. A few of these species are; *G. alba, G. annularis* and *G. artrum*. *Ganoderma* mushrooms are not edible due to their thick, corky, and rigid fruiting bodies (Jonathan 2002). The first classification of *G. lucidum* was done in 1881 by Karsten in the following way;

Kingdom……Fungi

Division……..Basidiomycota

Class……...Agaricomycetes

Order……..Polyporales

Family……Ganodermataceae

Genus……..*Ganoderma*

Species……*lucidum*

Name……..*Ganoderma lucidum* (www.wikipedia.com accessed on 15/4/2019).

All over the world, *Ganoderma* species have been regarded to be a source of cure (Wasser and Weis, 1999). This means that the medicinal value of local *Ganoderma lucidum* needs to be investigated.

2.4.1Botanical features of *Ganoderma lucidum*

From about 18th C, different taxonomic characters of *G. lucidum* have been classified in different geographical locations (www.wikipedia.com accessed on 15/4/2019). For example, Steyaert (1980) classified *Ganoderma* genus from almost every region of the

globe. Turner (2008) reported that the features of *Ganoderma* spores depend on the species. The upper side of sporophores depends on the species and is light to dark brown while the lower part is white. The laccate is shiny at the young stage and turns brown when spores are produced. According to (Wong, 2012), usually, different species of *Ganoderma* showed different features and pathogenecity. Basidiocarp is (7–12) × (11–19) × 1.5 cm, woody to corky, sub sessile to laterally stipitate, 2–3 cm in length (Bhosle *et al*., 2010). Upper Surface of the laccate is yellowish towards the edge and is also delicate and smooth. The edge is unpointed, rounded and brown to white depending with the ecological conditions while the pore is creamish to milky coffee. Each pore is 5 mm and is round (Bhosle *et al*., 2010). The tube is 2–9 mm and turns white or brown when brushed or when it ages. The cutis is thick walled and is of clavate form with diverticulate at the bottom, 35–42 × 6–8.5 μm. The hyphal is thin walled, developmental and 3.3 μm in diameter. The basidiospore is 8.3–10 × 6.6 μm, yellowish brown. The diameter varies with geographical location (Bhosle *et al*., 2010).

The South East African variety (Kenyan coast or Tanzania region) has the following features; spore index (1-1.5 μm), spore size (8) 9-12 (13 x 6-9 μm), curtis type is of claviform vera and is (63 – 85 x 10 μm). The tube layer is 15- 20 mm and the context is 15-20 mm. The stipe is eccentric, centric or lateral and is 10 cm in length (Bhosle *et al*., 2010).

The species that grows in China is reddish while the Kenyan variety is cream - brown as shown by photographs (Fig. 1 and 2 respectively). Both are kidney shaped, 5-10 cm in diameter with no stalk. Their underside (gills) is either white or brown. Their colours differ because they are found in different ecological zones and soil type. Local *G.*

lucidum grows in regions of acidic red soil at an altitude of 1500-1600 m temperatures between 11-27 ºC and over 2000 m rainfall (Okalo, 2014). Kenyan *G. lucidum* can easily be located at Kakamega forest on dead or live logs of trees (Okalo 2014). They can also be cultivated artificially (Kisiangani, 2014).

Figure 2: *Ganoderma lucidum* (Chinese variety); It has reddish, conked cap with fan-like stem.

Figure 3: The Kenyan Variety

2.4.2 Artificial cultivation of Kenyan *Ganoderma lucidum*

One third of each of the following substrates (maize cobs, dust bargas and saw dust) are mixed and placed in a polythene bag. Water is poured in a big tank to about 5 cm from the bottom and is heated. The mixed substrates in the bag are hanged slightly above the tank but should not touch the water level. Steam from the boiling water is supposed to

sterilize the substrates. Spawn (mushroom seed) is mixed with the substrates and placed in polythene bags. The mixture is transfered to a thatched house or cold room and put on shelves. Temperature is maintained at 20 °C or below. If thatched house is used, water is sprinkled on the floor and walls to maintain low temperatures. The mycelium usually grows after two weeks and then the holes are made in the polythene bags for easier growth of the mushrooms. *Ganoderma lucidum* matures after one month and then harvested (Kisiangani, 2014). The cultivated mushrooms have slightly different features to those that grow naturally in the forests. The cultivated ones are dark brown and smaller while natural ones are light brown and bigger. Comparison of artificial and local *G. lucidum* based on phytochemistry and antimicrobial tests is still unknown.

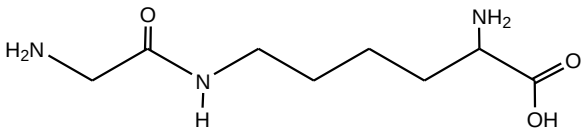
2.5 Ethnomedicinal Uses of *Ganoderma lucidum*

The Nigerian and Chinese traditional doctors use *Ganoderma lucidum* as immune booster (Jonathan, 2002). In Nigeria, the Yoruba medicinemen use *Ganoderma lucidum* in treating skin infections, high blood pressure and intestinal disorder (Akpaja *et al*., 2005). Akpaja *et al*. (2005) still notes as written proof that people consumption of native mushrooms in Nigeria are few in comparison to China and Japan where the macro-fungi have been exploited for over 4000 years in treating human ailments such as hepatitis, nephrosis, hyperpiepsis, rheumatism, nervousness, restlessness (sleeplessness), bronchospasm, respiratory disorder and abscess. Yun (1999) reports its use in China, Japan, Korea and Taiwan in treating hypercholesterolemia and boosts health and long life, reduces malignant and cardiovascular diseases (Chang and Buswell., 2003). Wasser (2005) wrote that traditional names of *G. lucidum* are not similar across civilizations. He adds that *G. lucidum* in Nigeria is used as a constituent in herbs and in magic or spriritualism.

2.6 Compounds from *Ganoderma lucidum* and their medicinal value

Ganoderma lucidum has antioxidative property, reduces inflammation and is also a disinfectant in human beings (Ogbe *et al*., 2008). Wasser (2002) wrote that this macro-fungus can enhance human protein level due to polysaccharides that are anti-tumour. Calcium in *Ganoderma lucidum* is important for bone and cartilage. The smooth appearance of the mushrom is due to tannins presence (Wasser, 2002). (Kubota *et al*., 1982) separated triterpene compounds, ganoderic acids A **(32)** and B **(28)** from *G. lucidum*.

Research on *Ganoderma lucidum* in Korea showed that the mushroom possesses anti-AIDS property (Kim *et al*., 1997). It is one of the known immune system enhancer and modulator and is safe for long term use. Currently AIDS is a challenge especially in Africa (Figlas and Curvetto, 2008). Water extract of *Ganoderma lucidum* has been known to display anti-HIV activities (Kim *et al.*, 1997). El-Mekkawy *et al*., (1998) obtained Ganoderiol F **(51)** and ganodermanontriol **(47)**. Ganoderic acid *β* **(31)**, ganodermanondiol **(50)**, ganodermanontriol **(47)**, ganolucidic acid A **(33)** and lucidumol B **(49)** displayed some activity against HIV (Min *et al*. 1998). Ubiquitin **(11)**, an anti-viral protein was separated and determined from oyster mushroom (Piraino and Brandt 1999). Many viral diseases have no cure. Water soluble glucans should also be investigated from the Kenyan *G. lucidum* and then tested against viruses.



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Polysaccharides bind to neutrophil or antihistamine proteins to cause the activation of phagocyte, CD4 cell, NK cells and other lymphocytes (Mueller *et al*., 2005).

Polysaccharides cause the growth of bacterial organism, therefore can antagonize the antibacterial activity of the active principles (Cushnie and Lamb, 2011). Polysaccharides, peptidoglycans and triterpenoids are the main components of *Ganoderma lucidum* (Boh *et al*., 2007; Zhou *et al*., 2007). In *G. lucidum*, lanosterol **(16)** biosynthesis is associated with the cyclization of squalene 2, 3-epoxide **(18)** (Scheme 2) (Haralampidis *et al*., 2002). *G. lucidum* contains many polysaccharides showing strong immunomodulating activity (Wasser, 2005). The immune-modulating effects involves blast transformation and activation of lymph cells, histiocyte and N.K cells which results in the formation of lymphokines such as interleukins, TNF alpha, an antiviral agent (Zhou *et al*., 2002). *G. lucidum* contains different kinds of triterpenes (Yue *et al*., 2010), which prevents histamine release, viral induction, cholesterol synthesis, exhibits anti-hepatotoxicity, anti-hypertensive, anti-inflammatory, necrobiosis, anti-oxidative, anti-cancer and anti-bacterial activities (Powell 2010). The enzyme tyrosinase in *G. lucidum* fruiting bodies indicated genoprotective effects (Shi *et al*., 2002). Jin *et al*., (2012) published a review on *G. lucidum* and its clinical studies on cancer patients. *Ganoderma lucidum* contains more than 400 antimicrobials which are made up of polysaccharides, triterpenes, sterols, nucleosides, fatty acids and proteins (McKenna *et al*.,, 2002; Boh, 2013).

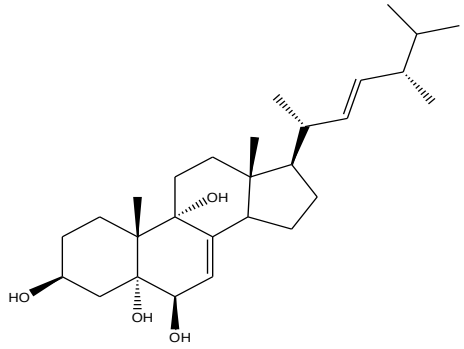
Five triterpenoids were separated from ether solvent of *G. lucidum* spores and identified as ganopsoreric acid A **(27)**, ganoderic acid B **(28)**, ganoderic acid C_1 **(29)**, ganoderic acid E **(30)** and ganodermanontriol **(47)** (Table 1). Ganopsoreric acid A **(27)** lowered Guanosine Triphosphate (GTP) in mice (Chen *et al*., 1993). Ganosporelactone A and B **(52, 53)** were obtained from lanostane skeleton through the construction of C_{18} and C_{23}

bond (Chen *et al*., 1991), ganoderic acid *β* **(31)**, lucidumol A **(48),** lucidumol B **(49)**, ganodermanontriol **(47)**, ganoderiol F **(51)**, ganoderic acid A **(32)** and structures (**28)**, (**29)** and (**47)** (Table 1). Of the compounds isolated, compounds **(43)**, **(60)**, **(62)** and **(46)** indicated anti-HIV-1 protease scheme with IC_{50} values of 20-90 µM **(43)** (Table 1). Seven oxygenated lanostane type triterpenes called ganoderic acid *γ* **(47)**, ganoderic acid *δ* **(48)**, ganoderic acid *ξ* **(37),** ganoderic acid *η* **(38)**, ganoderic acid *θ* **(39)**, ganoderic acid D **(43)**, ganoderic acid C_2 **(41)** were isolated from ganoderma spores (Table 1). A new oxygenated terpenoid, C_{27} lucidenic acid SP_1 **(42)** was separated. *G. lucidum* chloroform extract and eleven triterpenoids, namely ganoderic acid C_6 **(43)**, ganoderic acid G **(44)** and **(28)**, (**29)**, (**47)**, (**34)**, (**31)**, (**50)**, (**51)**, (**32)** and (**33)** (Min *et al*., 1998). Twelve compounds were investigated for anticomplementary activity whereby (**47)** and (**52)** had strong anticomplementary effect with IC_{50} values of 4.8, 17.2 and 41.7 µM. Zhang isolated two triterpenoids, methyl ganoderate A **(54)** and methyl ganoderate B **(55)** from *Ganoderma lucidum* spores (Zhang *et al*., 2008).

2.7 Characteristics of compounds isolated from other mushrooms and *Ganoderma lucidum*

One of the major components of phytochemical compounds in mushrooms is the terpenes. Terpenes such as (22*E*, 24*R*)-ergosta-7,22-diene-3*β*,5*α*,6*β*,9*α*-tetrol **(12)**, (22*E*,24*R*)-5*α*,8*α*-epidioxyergosta-6,22-diene-3β-ol-3-O-β-D-glucopyranose **(13)** and (22*E*, 24*R*)-5*α*,8*α*-epidioxyergosta-6,9,22-triene-3*β*-ol-3-O-*β*-D-glucopyranose **(14)** have been isolated from *Naematoloma fasciculare* (Yan *et al*., 2015). Trans-Ergosterol (ergosta-5,7,22-trien-3*β*-ol-22*E*) **(1)** is also terpene and has in the past been obtained from many mushrooms (Miguel *et al*., 2014; Wekesa, 2014; Nyigo *et al*., 2015; Tatiana

et al., 2017). Ergosterol peroxide (5,8-peroxyergosterol) **(3),** also a terpene has in the past been isolated from various mushrooms such as *Hericium erinaceum*, *Laetiporus sulfurous, Morchella esculenta, Boletus edulis* (king bolete), *Suillus bovines, Bacillus badius* fruiting bodies and *Agaricus sp. avensis*.aff. *arvensis* (Krzyczkowski *et al*., 2008; Baraza *et al*., 2007). Most of the compounds from mushrooms are classified as triterpenoids or steroids. The biosynthetic path way of triterpenes start with dimethylallylpyrophosphate (DMAPP) **(25)** and Isopentylpyrophosphate (IPP) **(26)** which are derived from pyruvic acid in fungal cells (Dupont *et al*., 2012). They undergo various cyclization processes through enzymatic reactions to form triterpenes (Scheme 1).



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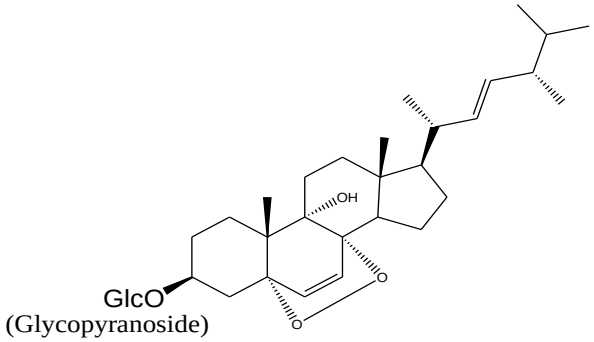

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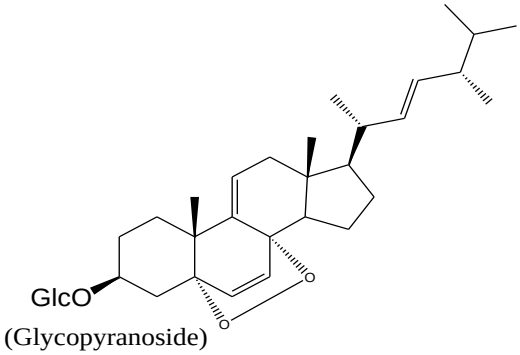

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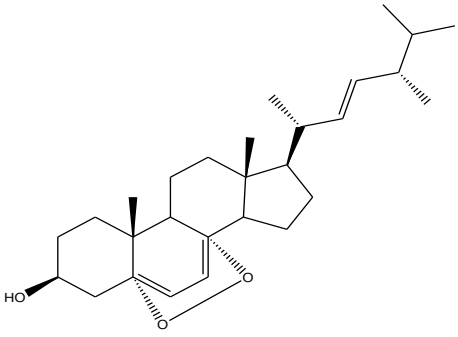

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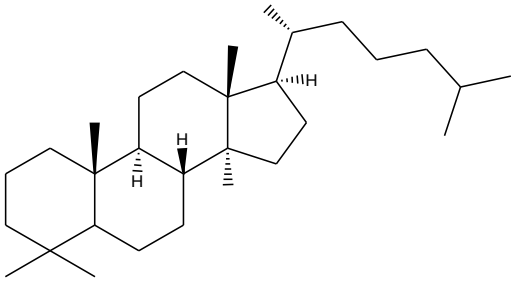

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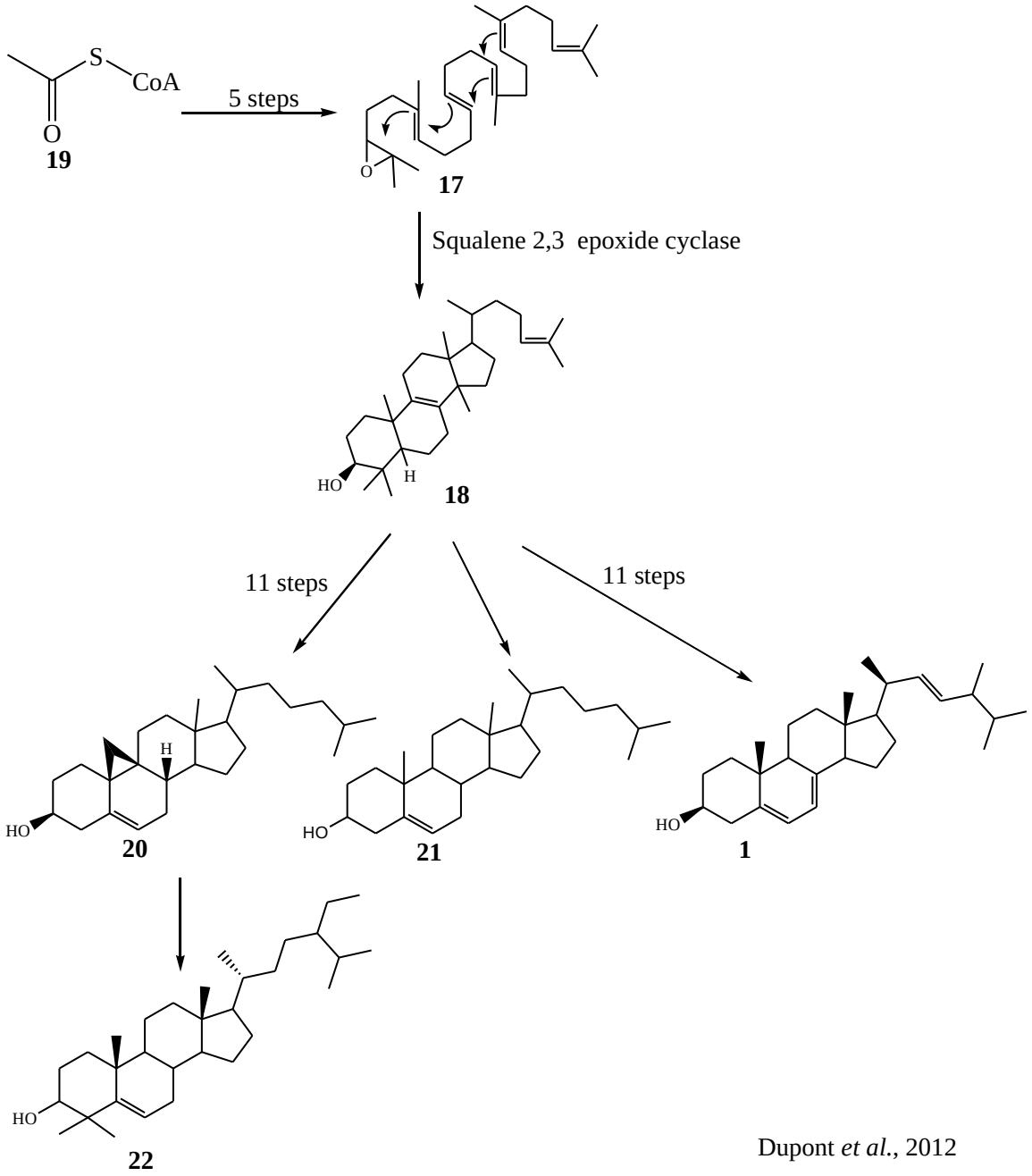
2.8 General biosynthetic pathway of compounds from Eukaryotes

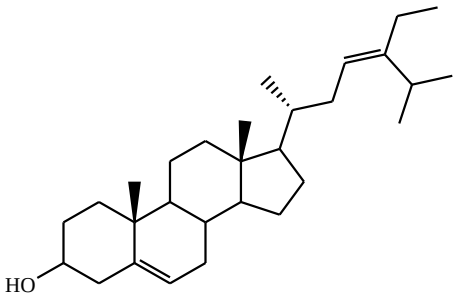
Eukaryotes contain sterols that organize the functions of the membrane. Cholesterol occurs in vertebrates; sitosterol **(22)**, stigmasterol **(23)**, campesterol **(24)** in plants and trans-ergosterol (Ergosta-5,7,22-triene-3β-ol (22*E*) **(1)** in macro-fungi. AcetylCoA **(19)** is the initial stage of the products (Dupont *et al*., 2012). Lanosterol **(18)** from squalene epoxide **(17)** produces cycloartenol **(20)** through various reactions which leads to the synthesis of sitosterol **(22)** in plants, cholesterol **(21)** in animals and Ergosta-5,7,22-triene-3β-ol (22*E*) **(1)** in fungi such as mushrooms.

Scheme 1: Showing end products from the biosynthetic pathway for the eukaryotes



Dupont *et al.*, 2012

Note: The compound, squalene appears as number **(16)** in scheme 8.



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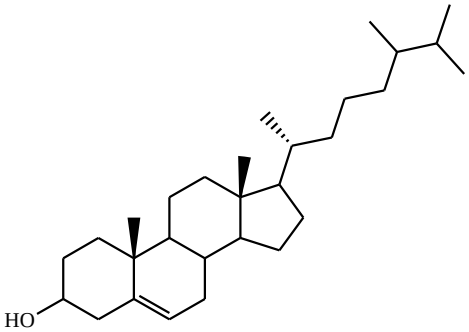

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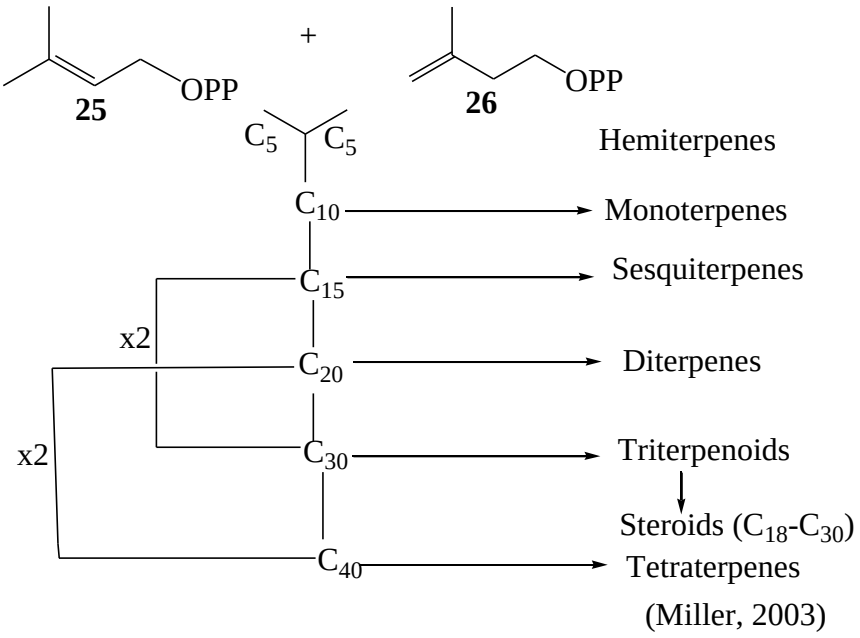
Scheme 2 below shows the general classification of terpenes. Most compounds from *Ganoderma lucidum* are triterpenes or steroids (C_{18-30}) (Table 3).

Scheme 2 : Flow diagram for the biosynthetic pathway for all terpenes



(Miller, 2003)

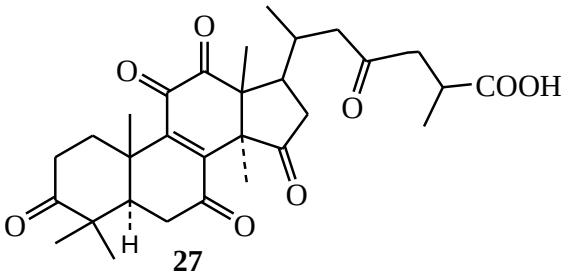
2.8.1 Triterpenoids present in *Ganoderma lucidum*

Table 1: List of *Ganoderma lucidum* terpenoids

(Min *et al*., 1998; Min *et al*., 2001; Zhang *et al*., 2008)

No.	Name	Molecular formula	Molecular weight
27	Ganopsoreric acid A	$C_{30}H_{38}O_8$	526
28	Ganoderic acid B	$C_{30}H_{44}O_7$	516
29	Ganoderic acid C_1	$C_{30}H_{42}O_7$	514
30	Ganoderic acid E	$C_{30}H_{40}O_7$	512
31	Ganoderic acid β	$C_{30}H_{44}O_6$	500
32	Ganoderic acid A	$C_{30}H_{44}O_7$	516
33	Ganolucidic acid A	$C_{30}H_{44}O_6$	500
34	Ganoderic acid γ	$C_{30}H_{44}O_7$	516
35	Ganoderic acid δ	$C_{30}H_{44}O_7$	516
36	Ganoderic acid ε	$C_{30}H_{44}O_7$	516
37	Ganoderic acid ξ	$C_{30}H_{42}O_7$	514
38	Ganoderic acid η	$C_{30}H_{40}O_6$	532
39	Ganoderic acid θ	$C_{30}H_{42}O_8$	530
40	Ganolucidic acid D	$C_{30}H_{44}O_6$	500
41	Ganoderic acid C_2	$C_{30}H_{46}O_7$	518
42	Lucidenic acid SP_1	$C_{27}H_{40}O_6$	460
43	Ganoderic acid C_6	$C_{30}H_{42}O_8$	530
44	Ganoderic acid G	$C_{30}H_{44}O_8$	532
45	Ganoderic acid D	$C_{30}H_{42}O_7$	514
46	Ganoderic acid H	$C_{32}H_{44}O_9$	572

47	Ganodermanontriol	$C_{30}H_{48}O_4$	472
48	Lucidumol A	$C_{30}H_{48}O_4$	472
49	Lucidumol B	$C_{30}H_{50}O_3$	458
50	Ganodermanondiol	$C_{30}H_{48}O_3$	456
51	Ganoderiol F	$C_{30}H_{46}O_3$	454
52	Ganosporelactone A	$C_{30}H_{40}O_7$	512
53	Ganosporelactone B	$C_{30}H_{42}O_7$	514
54	Methyl ganoderate A	$C_{31}H_{48}O_7$	530
55	Methyl ganoderate B	$C_{31}H_{48}O_7$	530



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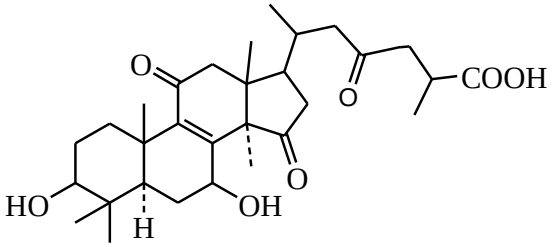

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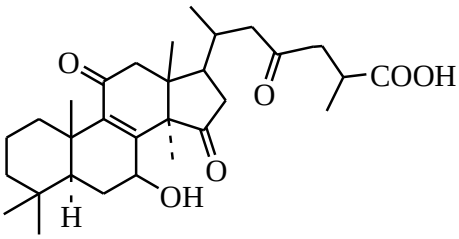

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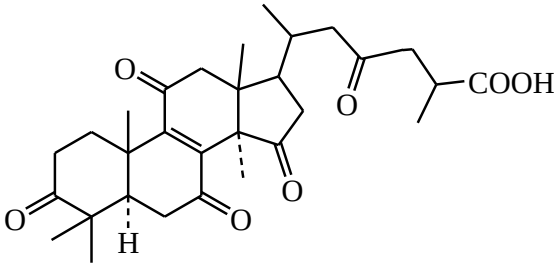

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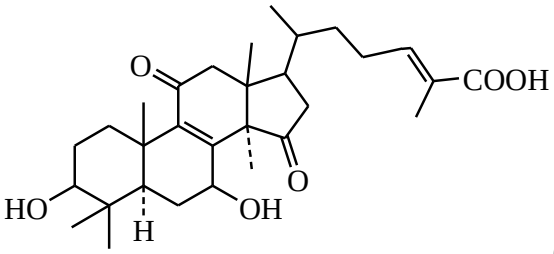

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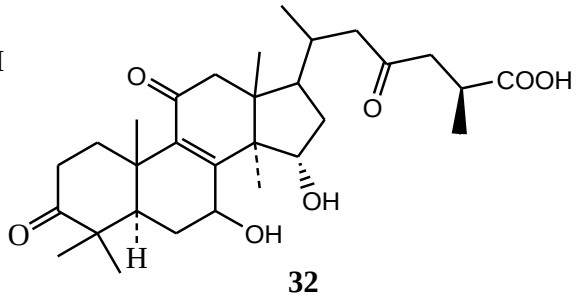

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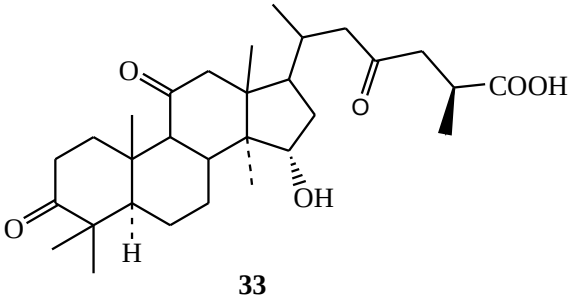

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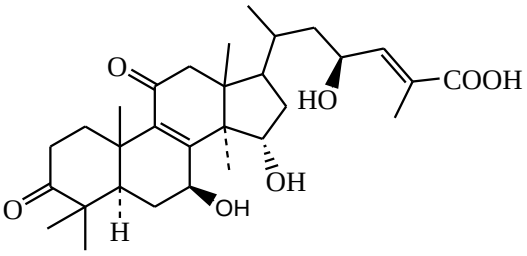

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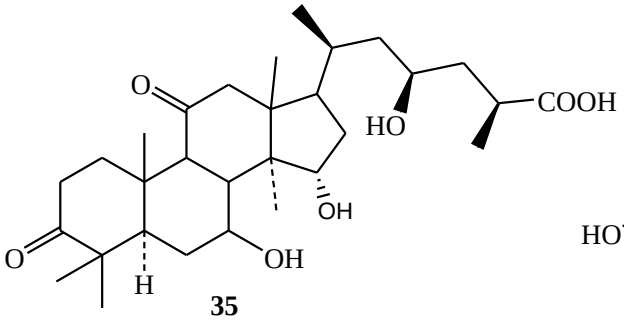

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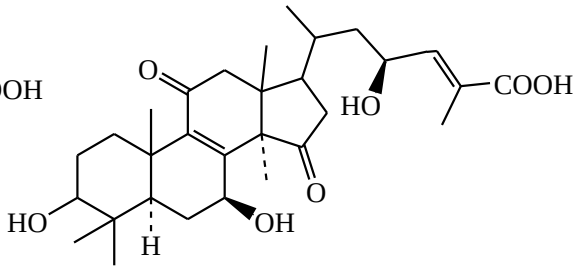

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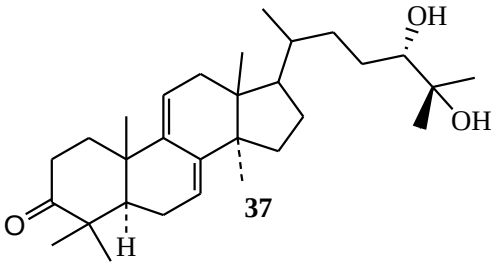

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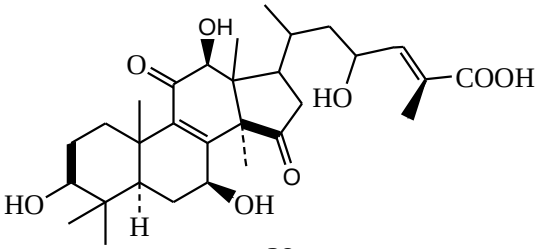

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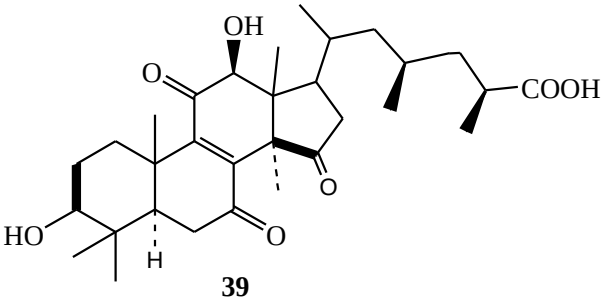

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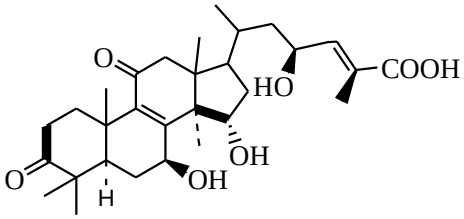

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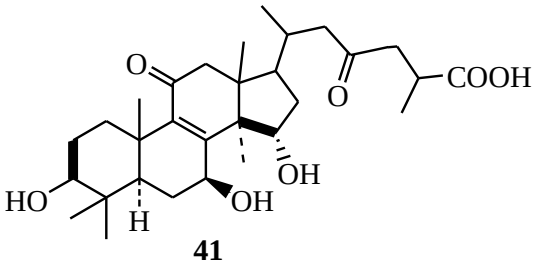

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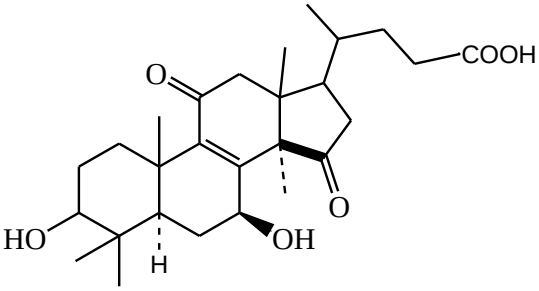

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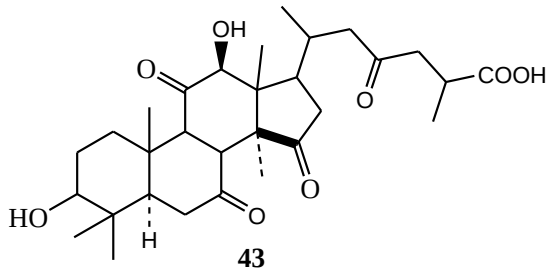

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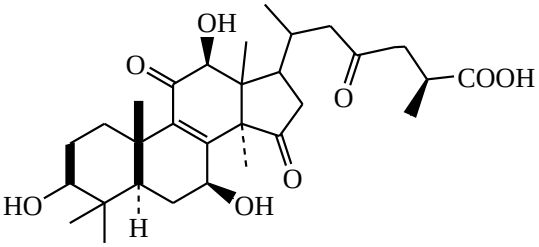

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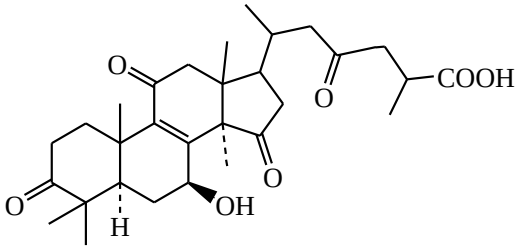

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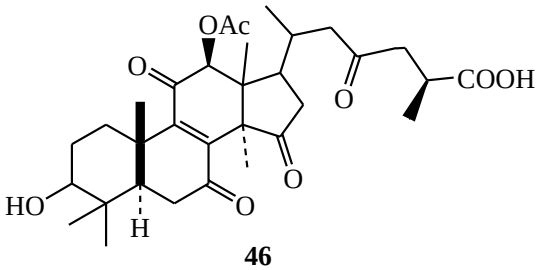

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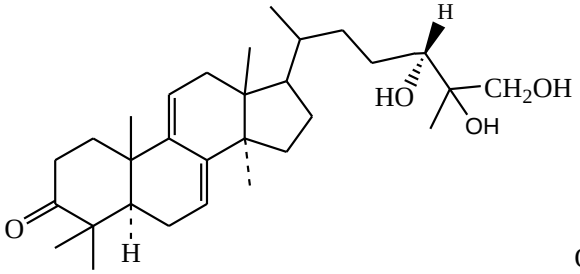

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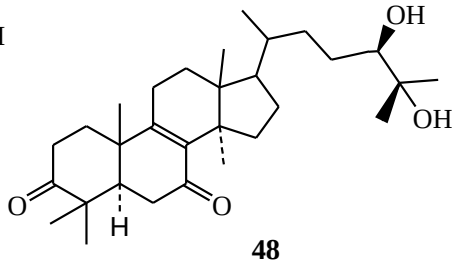

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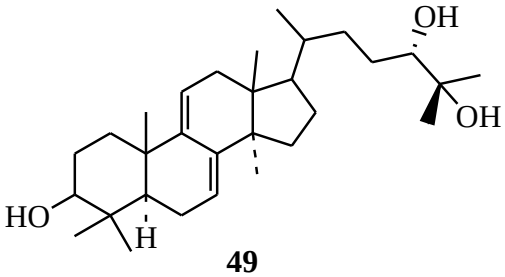

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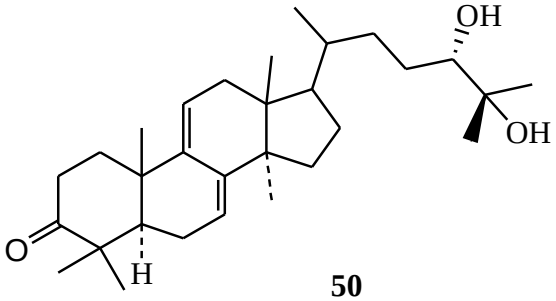

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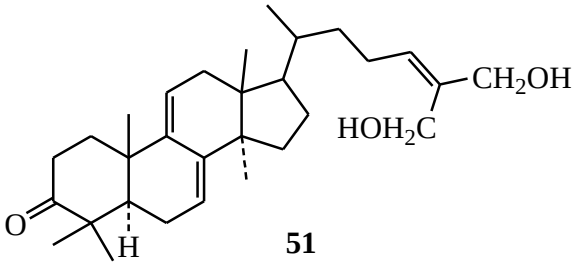

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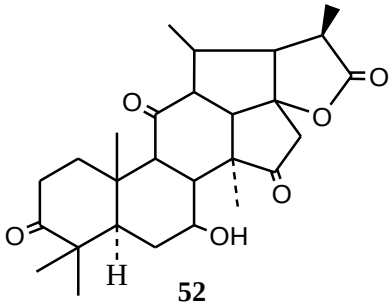

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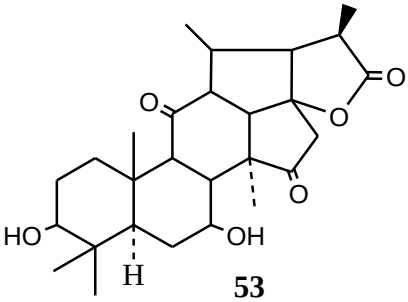

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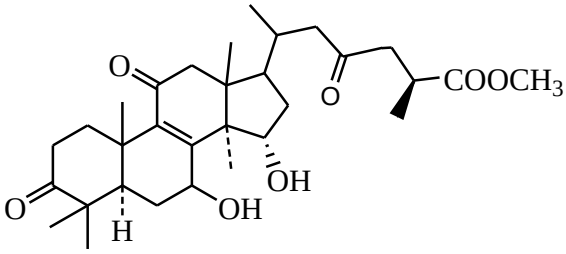

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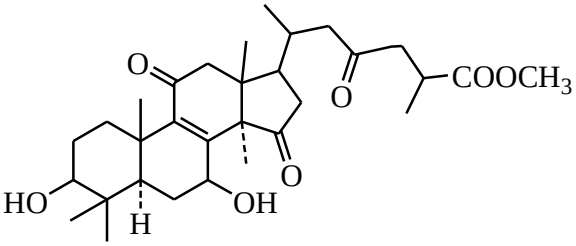

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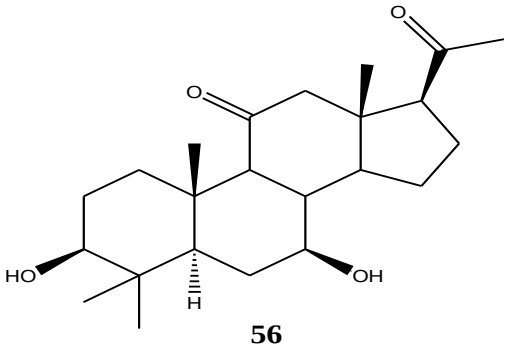
2.8.2 Volatile/essential oils - 2-8%

Terpenoids, triterpenes (>100 highly oxygenated lanostane-type triterpenoids), lucidone A **(56)**, vitamins, phenols and nucleotides (Naveen *et al*., 2018).



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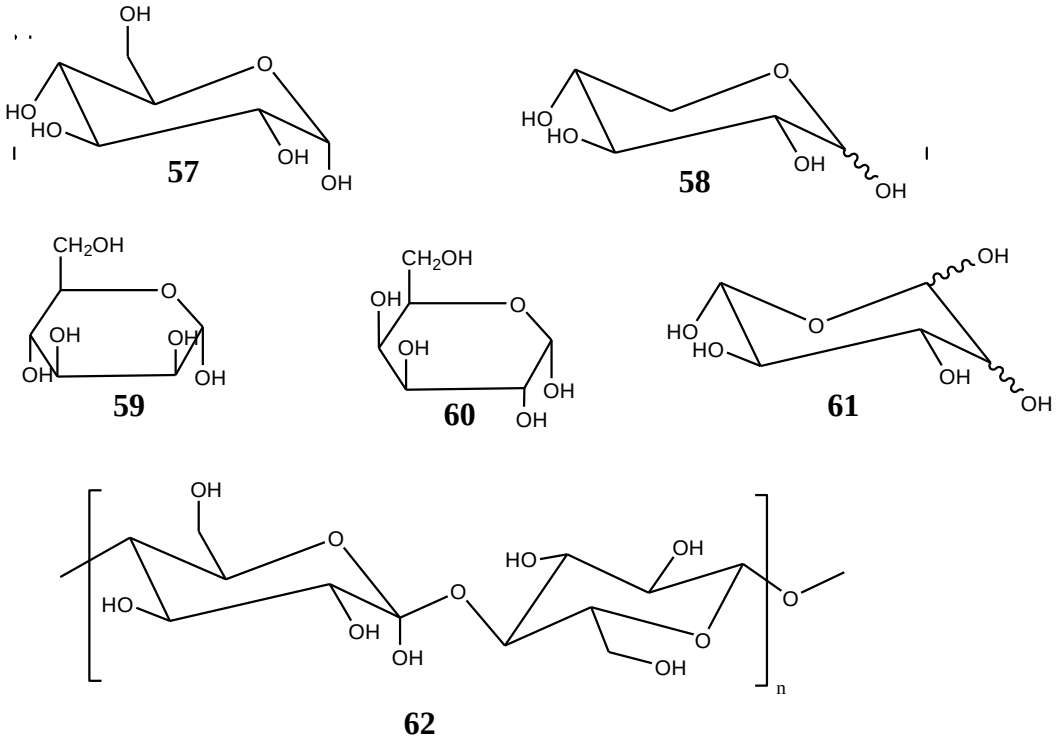
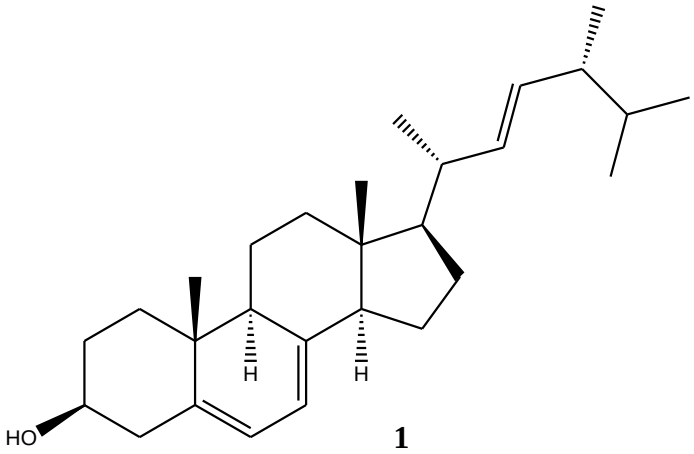
2.8.3 Carbohydrates – 26-28%-polysaccharides (P), heteropolymers- glucose **(57),** xylose **(58)**, mannose **(59)**, galactose **(60)**, fucose **(61)** and β-D- glucans particularly β-(1->3)- D- glucans **(62)** (Naveen *et al*., 2018).



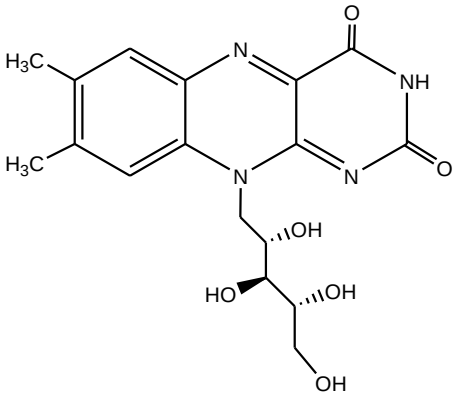
2.8.4 Sterols: Ergosta-5,7,22-triene-3*β*-ol (22*E*) **(1)** Trans Ergosterol



2.8.5 Amino acids: *Ganoderma* spores powder is made up of 13 kinds of amino acids: arginine, tryptophan, D-asparagic acid, glycine, L-Alanine, L-Threonine, L-Serine,

Glutamic acid, proline and small amount of methionine, leucine, tyrosine and phenylalanine (Naveen *et al*., 2018).

Riboflavin **(63)**, ascorbic acid **(64)**, alkaloids (lucidimine C and lucidimine D) **(65, 66)** and coumarins (coumarin and caffeic acid) **(67, 68)** (Naveen *et al*., 2018). Minerals such as calcium, magnesium, sodium, manganese, iron, zinc, copper and sulfur are also found in *G. lucidum*. fiber- (59%) while ash- (10%).



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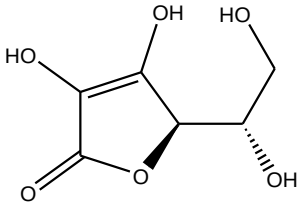

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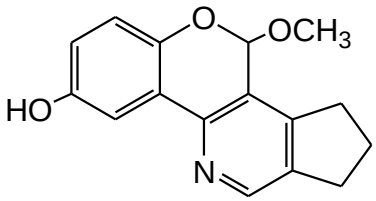

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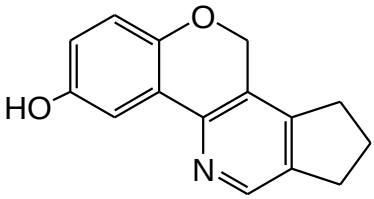

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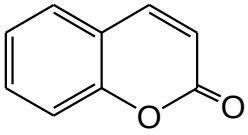

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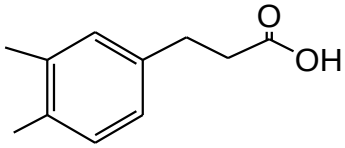

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2.9 *In vitro* analysis of *Ganoderma lucidum* extracts against various pathogens

Soji *et al*., (2013) carried out an experiment whereby the following pathogens showed resistance to both methanol and *n*-butanol extract of *Ganoderma lucidum*: *Streptococcus*

faecalis, Staphylococcus aureus, Creptococcus pyogenes, Bacillus subtilis, Salmonella typhi, Escherichia coli, Klebsiella pneumoeae, Pseudomonas aeruginosa and *Proteus mirabilis*. In this experiment, AmpicloxR was a positive control. However, *in vitro* analysis done on ethylacetate extract indicated different zones of inhibition on these bacteria. This means that the bioactive compounds against these micro-organisms were found in the ethylacetate extract of the *Ganoderma lucidum* mushroom (Soji *et al*., 2013). Wang and Tzi (2006) reported that *Ganoderma lucidum* exhibited antifungal activity. Previous investigations reported that lectin characterized from *Ganoderma lucidum* was inhibitive to growth of phytopathogens and dermatophytes (Vinay *et al.*, 2011).

2.10 Pathogenic Micro-organisms

A pathogenic organism is an organism that causes disease in its host. Common examples are bacteria like *Salmonella typhi* and *Listeria* and viruses such as *Cryptosporidium* (Hashimoto *et al*., 2016).

Escherichia coli*:* Gram-negative bacilli which cause diarrhoea, meningitis and urinary tract infections (Bhavsar and Krilov, 2015).

Streptococcus pyogenes*:* Gram-negative bacteria that causes rheumatic fever, glomerulonephritis, scarlet fever, meningitis, tonsilitis and skin infections (Todar, 2012).

Staphylococcus aureus: Are gram-positive bacteria that cause boils, styes, osteomyelitis, endocarditis, furunculosis and food poisoning (Foster, 1996).

Klebsiella pneumoniae*:* They cause cystis, pneumonitis, peritonitis, bloodstream infection, brain inflammation and pyogenic hepatic abscess (Yung-Fu, 2018). It is a gram-negative bacteria.

***Creptococcus neoformans*:** It is a fungus that causes harmless colonization of the airways and meningitis especially in people with immunity problems. The fungi causes deadly infection especially in people with HIV infection and is also a problem in corneal transplant, adrenal cortical steroid and mononuclear phagocyte system (Bilgrami and O`keefe 2014).

CHAPTER THREE

METHODOLOGY

3.1 General Information

The following solvents were used in extraction and isolation of compounds: *n*-hexane, ethylacetate and methanol. They were obtained from Scielab Chemicals Limited and distilled before use. Isolation techniques applied included: Thin Layer Chromatography (TLC) and Column chromatography (CC). TLC was run using silica gel 60 $PF_{254+366}$ pre-coated alumina plates (Merck, 0.25 mm thick) and silica gel packed analytical column (1.5 cm diameter and length 30 cm) was used in the purification process of the fractions obtained. Resulting spots were visualized using iodine tank. CC was conducted on Silica Gel (Loba Chemie, 60-120 cm mesh) or (CDH, 60-120 cm mesh).

3.2 Collection and Identification of *Ganoderma lucidum* and *Termitomyces letestui*

Ganoderma lucidum was sown and later harvested from a Kakamega farm in Western Kenya (1500-1600 m). The species was identified at the East African Herbarium - National Museums of Kenya where a specimen was kept and identification number EAHNMK 261 assigned to the mushroom while the number NMKEAH 510 was assigned to *Termitomyces letestui*.

3.3 Extraction of fruiting bodies of *Termitomyces letestui* and *Ganoderma lucidum*

3.3.1 Preliminary Extraction

Each species was separately washed, dried in air for one week, grinded into small particles using a blender and kept in suitable conditions to wait for investigation.

3.3.2 Solvent extraction and analysis of the mushrooms

About 1 kg of dried *Ganoderma lucidum* mushroom was soaked in about 3 L of hexane at room temperature for two days. The filtrate from the mixture was concentrated using a

rotar vapour. The resulting residue was taken through sequential extraction with ethylacetate (EtOAc) and lastly with methanol (MeOH) following the same procedure as hexane extract. The crude extracts were weighed and recorded (hexane yielded 6.2 g, ethyl acetate yielded 4.11 g while methanol yielded 10.03 g) (Appendix 3B, 3C and 3D). The same extraction procedure was repeated for local *Termitomyces letestui* mushroom. Ethylacetate extract yielded about 15 g, methanol extract yielded about 25 g (Appendix 2B and 2C) while *n*-hexane extract was about 1 g. Since *n*-hexane yield was very low, column chromatography could not be done. Even if it was done, any compound that would be separated would not be enough for structure determination and bio-activity tests.

3.3.3 Phytochemical screening of *Termitomyces letestui* and *Ganoderma lucidum*

Phytochemical analysis of crude extracts from *Termitomyces letestui* and *Ganoderma lucidum* were done inaccordance with the procedures by Harbourne (1998), Krishnaveni *et al*., (2016), Kavit *et al*., (2013), Wadood *et al*., (2013) and Yadav and Agarwala (2011). These analyses depended on colour changes or precipitate formation after adding particular reagents. Ethylacetate and methanol extracts of *Termitomyces letestui* were tested for phytochemical class of compounds. The same phytochemical procedures were repeated for *n*-hexane, ethyl acetate and methanol extracts of *Ganoderma lucidum*.

3.3.3.1 Test for Phytosterols (steroids/triterpenoids)

3.3.3.1.1 Salkowski test: About 0.5 g of ethylacetate crude extract of Kenyan *Termitomyces letestui* was kept in a test tube, a few drops of concentrated sulphuric (VI) acid added to the extract, then swirled. After settling, if the bottom layer changed red, steroids would be present. If the layer turned golden yellow triterpenoids would be

present in the extract. The method was re-run for ethylacetate and methanol extracts and also for *Ganoderma lucidum* crude extracts.

3.3.3.2 Test for saponins

Foam test: About 0.5 g of each extract was shaken with about 10 ml of water. When froth formed, it would be positive test for saponins in the extract.

3.3.3.3 Test for alkaloids: Some small volume of Mayer`s reagent was added to 1 ml of the crude material. White precipitate appearance was a positive indicator for the presence of alkaloids.

3.3.3.4 Test for carbohydrates

Fehlings tests: When Fehling`s A and B solution was mixed with the extract and warmed, if it produced brick red precipitate, reducing sugars would be present.

3.3.3.5 Test for tannins (phenolics): About 20 mL of distilled water was added to 0.5 g of powdered mushroom material, boiled in a water bath and filtered. About 3 drops of 0.1% $FeCl_3$ were added to the samples, filtered and noted for brown-green or a blue – black colouration. This colour change showed tannins presence.

3.3.3.6 Test for glycosides

3.3.3.6.1 Keller – Killiani test: About 10 ml of glacial acetic acid, 2.0 ml ferrous chloride, and 0.5 ml of concentrated sulphuric (VI) acid were added to each crude extract of the mushrooms. If present, acetic acid layer would show blue colour.

3.3.3.6.2 Ammonia test: Five drops 1% NH_3 were added to 0.5 g of hexane, ethyl acetate and MeOH extracts of the same weight in a test tube separately. The method was re-run for the extracts of EtOAc and MeOH of *Termitomyces letestui*.

3.3.3.7 Test for flavonoids

3.3.3.7.1 Ferric chloride test: Ethanolic extract of 100 g of dried mushroom was filtered and then reacted with freshly prepared 5% $FeCl_3$. If it gave black-green colour, it showed that flavonoids were present.

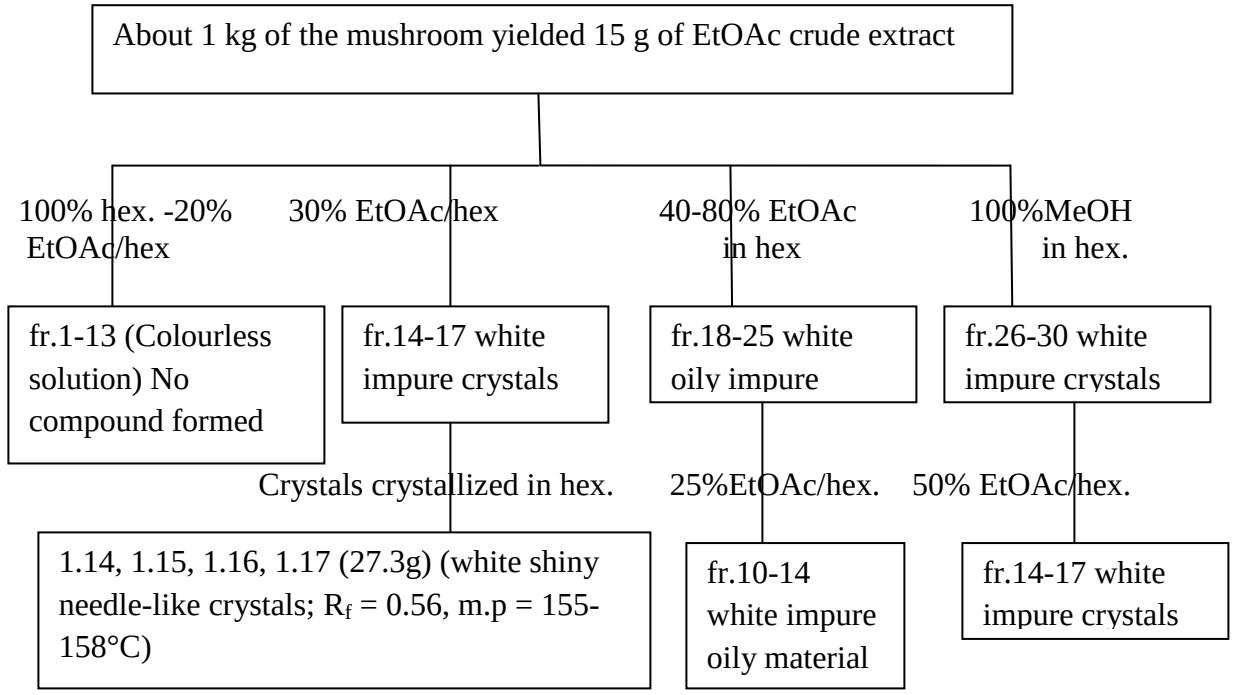
3.3.3.7.2 Lead acetate test: About 5 drops of 10% lead acetate were added to about 10 ml of ethanol extract. If it gave white precipitate, flavonoids would be present.

3.4 Isolation and Purification of *Termitomyces letestui* extracts

3.4.1 *Termitomyces letestui* Ethylacetate extract

Hexane/EtOAc solvent system was used in separating the extract while 100% MeOH was used in concluding the isolation process (Appendix 2B). Fractions (14 to 17) were collected at exactly 30% EtOAc in hexane as white crystals. They were recrystallised in hexane to obtain clear white needle-like crystals and coded as 1.14, 1.15, 1.16 and 1.17 with R_f = 0.56) (Appendix 2F). Any impurity present would dissolve in hexane and decanted so as to remain with white clear needle-like crystals (Appendix 2G). Their boiling points ranged between 155-158 °C. The samples were packed in sample bottles ready for identification using spectroscopic techniques and antimicrobial tests. No compound was obtained between fractions 1 to 13 at 100% hex.-20% EtOAc in hexane while fractions 18 to 25 resulted into white oily impure fraction at 40-80% EtOAC in hexane. The fractions were later combined and re-ran in a column to obtain fractions 10-14 as white impure oily fractions at 25% EtOAc in hexane. Fractions 26 to 30 were isolated as white impure crystals at 100% MeOH which were later combined and ran in an analytical column to produce fractions at 50% EtOAc in hexane as fractions 14 to 17. These results are indicated in scheme 3 below.

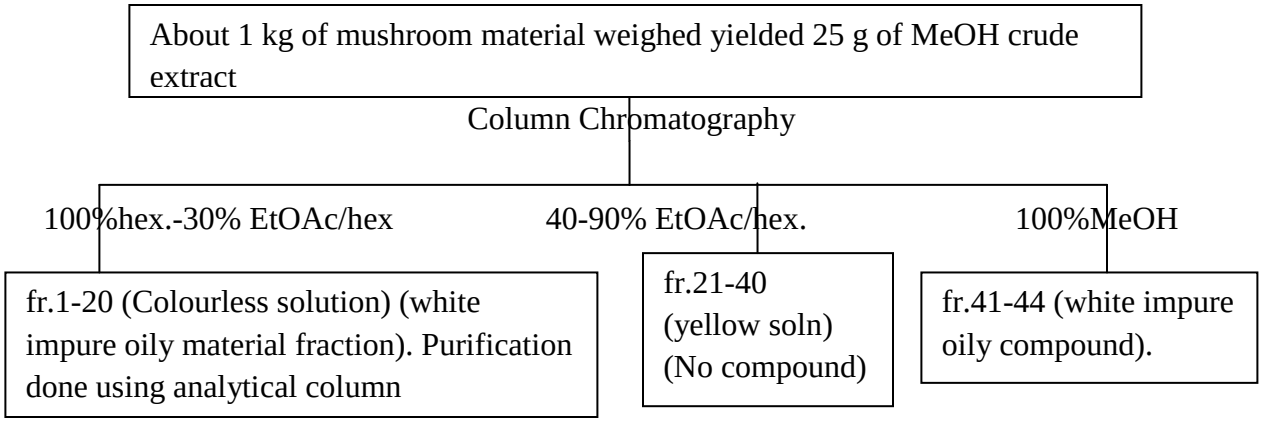
Scheme 3: Flow chart for isolation of compounds from EtOAC extract of *T. letestui*



3.4.2 Isolation and Purification of *Termitomyces letestui* methanol extract

Hexane/EtOAc was used as solvent system in separation. Fourty four fractions were collected (Scheme 4). Fractions 1 to 20 separated as white impure oily material at between 100% hexane to 30% EtOAc/hexane. Purification using an analytical column was unsuccessful. Fractions 21 to 40 separated as yellow solution but later on no compound was obtained. Fractions 41 to 44 were collected at 100% MeOH as white impure oily compound whereby purification did not result into a pure compound. Summary of the results is shown in Scheme 4.

Scheme 4: Flow chart for isolation of compounds from MeOH extract of *T. letestui*

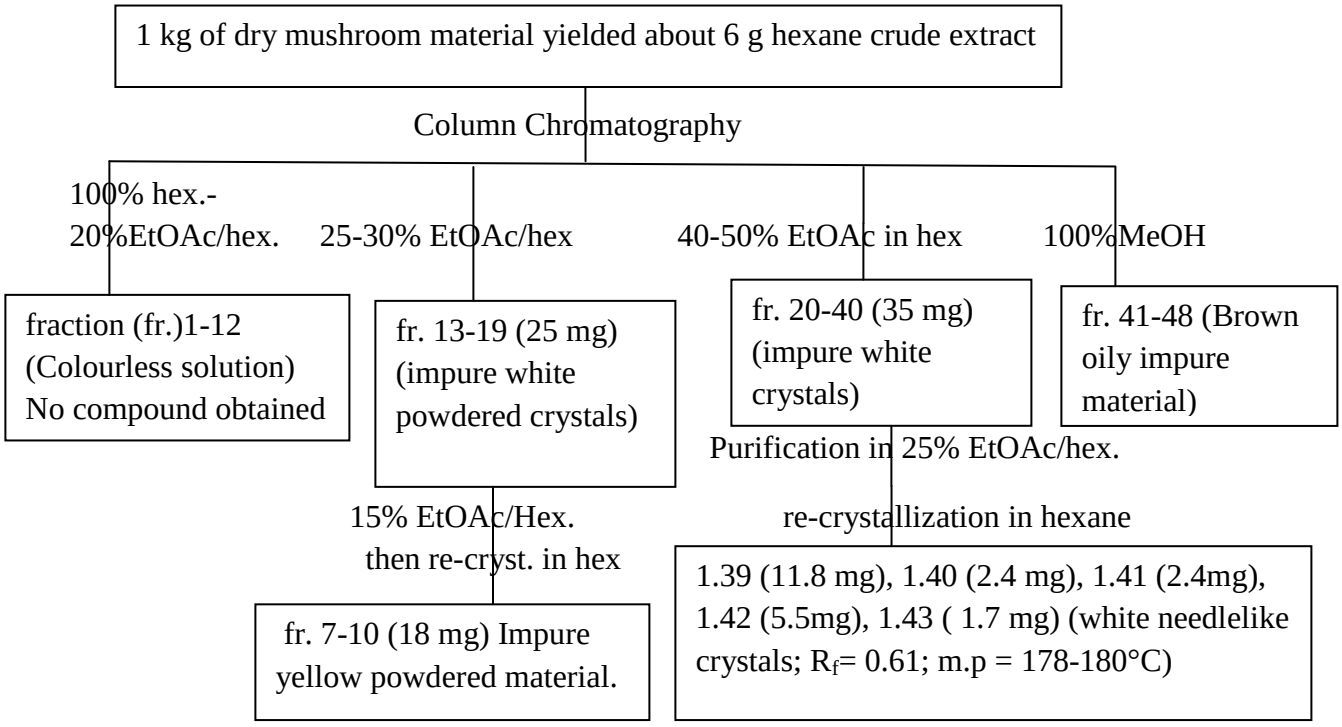


3.5 Isolation of *Ganoderma lucidum* crude extracts using Column chromatography

3.5.1 Isolation and Purification of *n*-hexane extract of *Ganoderma lucidum*

Hexane extract was isolated by use of *n*-hexane together with ethylacetate to increase polarity. Fourty eight fractions were obtained (Appendix 3E). Fractions 13 to 19 showed similar spots on TLC plate. In this case, the fractions (13 to 19) were combined and re-ran in an analytical column which later separated at 15% EtOA in hexane as fractions (7 to 10 as impure yellow powdered material). Further purification of (7 to 10) did not produce a pure compound. Fractions 21 to 40 separated at 40-50% EtOAc in hexane as impure white crystals. Since TLC for the fractions showed similar spots, the materials were combined and re-separated further using an analytical column. They were later separated at 25% EtOAc in hexane as fractions 39, 40, 41, 42 and 43 and later re-crystallized in *n*-hexane to obtain pure white needle-like crystals which were coded 1.39, 1.40, 1.41, 1.42 and 1.43 with R_f = 0.61, m.p = 178-180 °C (Appendix 3G). Fractions 41 to 48 were separated as brown oily impure material which remained impure after purification using an analytical column. Summary of these results is represented in scheme 5 below.

Scheme 5: Flow chart of compounds from *n*-hexane extract of *G. lucidum*

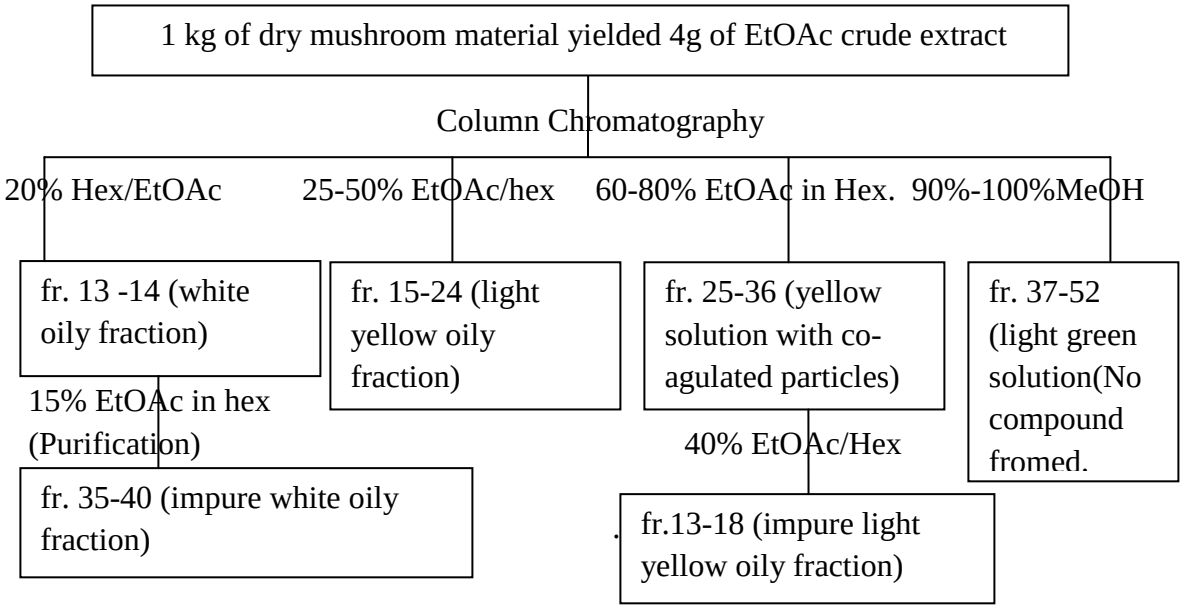


3.5.2 Isolation and Purification of ethylacetate extract of *G. lucidum* using Column Chromatography

About 4 g of ethylacetate extract was passed through the column using Hex./EtOAc solvent system. Fifty two fractions were obtained and combined accordingly (Scheme 6). Fractions 13 and 14 were observed as white oily fractions and were collected at 20% EtOAc in hexane. The fractions were combined and re-isolated using an analytical column and later separated as fractions 35 to 40 at 15% EtOAc in hexane. Further purification using an analytical column could not yield a pure compound (Appendix 3F). No compound was obtained between fractions 1 to 12 at 100% hexane to 10% EtOAc in hexane. Light yellow oily material moved down the column at 25-50% EtOAc in hexane (fractions 15 to 24). A pure compound could not be obtained even after further purification. Fractions 25 to 36 separated at 60-80% EtOAc in hexane as a yellow

solution with co-agulated particles. The same fractions (25 to 36) were later combined after TLC analysis and ran in an analytical column. The fractions separated at 40% EtOAc in hexane and were collected as impure fractions from 13 to 18 (impure light yellow oily fraction). Further purification of the fractions did not result into a pure compound. Between 90% EtOAc/hex. to 100% MeOH, light green solution was obtained (37 – 52). The light green mother liquor did not result into any compound. This information is summarized in scheme 6 below.

Scheme 6: Flow diagram for isolation of compounds from EtOAc of *G. lucidum*

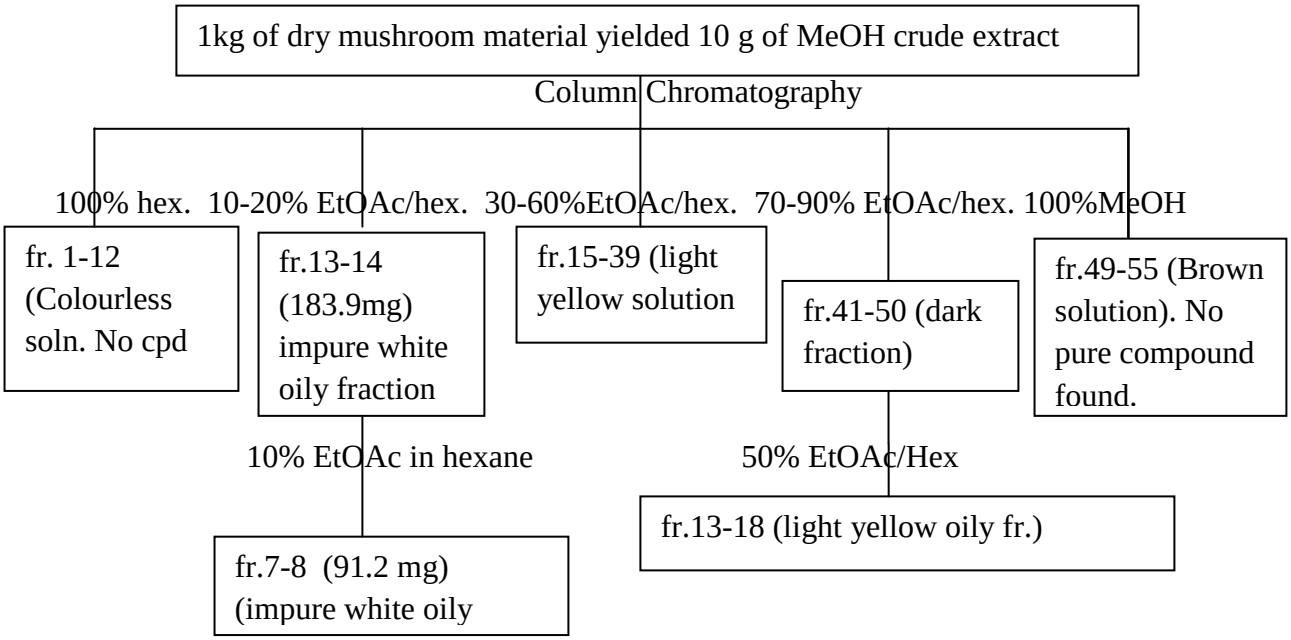


3.5.3 Isolation and Purification of methanol extract of *Ganoderma lucidum*

About 10 g of MeOH extract was placed in the column while Hex./EtOAc solvent system was used in running the column and concluded with 100 % MeOH. Polarity was increased at intervals of 10-20% EtOAc/hex. Impure fractions which weighed 183.9 mg were isolated at 10-20% EtOAc/hex. as fractions 13 and 14 and fractions 41 to 50 (dark fractions and weighed about 25 mg) at 90% EtOAc/hex. Fractions 13 and 14 were

combined, ran in the column and later on separated at 10% as fractions 7 and 8 (impure oily fractions). Fractions 41 – 50 were combined, ran in a column and separated at 50% EtOAc/hex as fractions 13 – 18 (light yellow oily fraction). Fractions 49-55 separated at 100% MeOH as brown solution which later did not result into a compound. This information is indicated in scheme 7 below.

Scheme 7: Flow chart for isolation of compounds from MeOH extract of *G. lucidum*



3.6 Biological Assays of the Local *Termitomyces letestui* and *Ganoderma lucidum*

3.6.1 Culture of test micro-organisms

The selected standard microbes [(*Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 13883), *Streptococcus pyogenes* (ATCC 12344) and Methicillin-Resistant *Staphylococcus aureus* (MRSA)] were cultured using Mueller Hinton Agar (MHA) then incubation applied at 37 °C for about 20 hrs. The microbes were then moved to Mueller Hinton Broth at 37 °C for 6 hours for stable and young culture for antimicrobial analysis. *Creptococcus neoformans* fungi were cultured in Potato Dextrose

Broth (PDB) while incubation was done at 28 °C for 5 days in preparation for the bio-activity.

3.6.2 Antibacterial tests

Mueller Hinton Agar media (MHA) was set based on the commercial directives given. Sterilization was done to the media using an autoclave set at 121 °C and 15 psi pressure for about 15 min. Wells were dug through the agar in the dishes using about 5 mm diameter cork borer (Balouiri *et al*., 2015). About 100 mg crude extracts of *Ganoderma lucidum* (hexane, EtOAc and MeOH) were dissolved in 1 ml of Dimethylsulphoxide (DMSO) and its isolated compound, *T. letestui* crude extracts (ethylacetate and methanol), its pure compound and control (ampicillin) were prepared. Concentrations of 100 µL of the prepared samples were placed into the wells using pipettes and 4 °C temperature set for 4 hours for each component to absolutely diffuse into the media. The experiment for each sample was done in triplicates. The microbes (*Escherichia coli*, *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Streptococcus pyogenes* and Methicillin-Resistant *Staphylococcus aureus,* (MRSA) were then uniformly spread on the plates while incubation was done at 37 °C for one day. Bacterial inhibition zones were observed and their diameters measured in millimeters in order to investigate their bio-activity. The measurements were used to assess the activity of the microbes by comparing the samples and standard antimicrobial (ampicillin).

3.6.3 Antifungal tests

Potato Dextrose Agar (PDA) was set according to the standard instructions stated. The antifungal control was nystatin. The concentration of 100 mg/mL in DMSO of crude extracts of *G. lucidum* and its isolated compound, *Termitomyces letestui* crude extracts,

its pure compound and control (10 mg/mL) in DMSO were prepared. The samples were run in triplicates. *Creptococcus neoformans* was placed into the plates by marking with a purified wire loop. They were allowed to stay undisturbed for about five hrs to be absorbed into the media at about 5 °C. The petri dishes were then incubated at 28 °C. They stayed there for one day and observations made.

3.7 Methods of Data Analysis

Antimicrobial activities of the crude extracts and pure compounds towards *Escherichia coli, Klebsiella pneumoniae, Methicillin Resistant Staphylococcus aureus (MRSA), Streptococcus pyogenes* and *Cryptococcus neoformans* were evaluated by applying the zones of inhibition in millimeters as median ± SEM (standard error of the median). Statistical significance was determined using the non-parametric statistical analysis. Significant differences in activity between the test samples and positive control was done by comparison of non parametric statistics for median by Kruskal – Walis test ($p \leq 0.05$). Samples with measures of $p < 0.05$ were found to be statistically significant (Weaver *et al*., 2017).

3.8 Physical and spectral data for the isolated compounds from the mushrooms

3.8.1 Physical and spectral data for Ergosta-5,7,22-triene-3*β*–ol (22*Z*) (69) isolated from *Termitomyces letestui*

Light yellow concentrated solution formed crystals which were recrystallised from *n*-hexane mother liquor solution to form colorless needle-like crystals isolated at 20% ethyl acetate/hexane. This compound appeared as a brown spot on TLC with R_f value = 0.56.

Ergosta-5,7,22-triene-3*β*–ol (22*Z*) **(69):** Colorless needle- like crystals, R_f value = 0.56, 19.4 mg, m.p: 140 – 145 °C. IR v max (cm^{-1}, KBr disc): 1400.2 cm^{-1}, 1650.0 cm^{-1}, 2956.7 cm^{-1},3168.8 cm^{-1}, 3300 cm^{-1}. ^{1}HNMR (500 MHz, CD_2Cl_2): 0.64, (3H, *s*), 0.79 (3H, *s*), 0.82 (3H, *d*, *J* = 10Hz), 0.84 (3H, *d*, *J* = 10 Hz), 0.92 (3H, *d*, *J* = 10 Hz), 1.05 (3H, *d*, *J* =10 Hz), 1.26 (1H, *m*), 1.31, 1.74 (2H, *m*), 1.50, 1.86 (2H, *m*), 1.61 (1H, *m*), 1.87 (1H, *m*), 1.90, 2.05 (1H, m), 2.06 (2 H, *t*, *dd*, (*J* = 5, 1 Hz), 2.44 (*ddd*, *J* = 15, 6, 2 Hz), 3.58 (1 H, *m*), 5.20 (1H, *dd*, *J* = 5, 10 Hz), 5.21 (1H, *dd*, *J* = 10, 5 Hz), 5.45 (1H, *dd*, *J* = 10, 5 Hz), 5.60 (1H, *dd*, *J* = 5, 10 Hz). ^{13}C NMR (500 MHz, CD_2Cl_2): 38.3 (C-1), 32.3 (C – 2), 70.5 (C – 3), 41.0 (C – 4), 140.3 (C – 5), 119.7 (C – 6), 116.5 (C – 7), 141.6 (C – 8), 46.5 (C – 9), 37.3 (C – 10), 21.3 C – 11), 39.3 (C – 12), 43.1(C – 13), 54.8 (C – 14), 23.3 (C – 15), 28.5 (C – 16), 55.7 (C – 17), 12.1 (C – 18), 17.7 (C – 19), 40.7 (C – 20), 21.1 (C – 21), 21.1 (C – 22) 135.9, 132.1 (C – 23), 43.0 (C – 24), 33.4 (C – 25), 19.7 (C – 26), 19.9 (C – 27), 16.3 (C – 28).

Infra-red signals for compound **69** were as follows: 3753.2 cm^{-1}(strong) for hydroxyl group (O-H peak), 3168.8 cm^{-1} (weak) for vinyl =C-H stretch, 2956.7 cm^{-1} (strong) for methyl (CH_3) stretch, 2800.4 cm^{-1}(weak) for methylene (-CH_2) stretch, 2659.7 cm^{-1} (weak) (methine, -C-H) *sp* C-H stretch, 1650.0 cm^{-1} (sharp) for cyclic double bond (-C=C-), 1242.1 cm^{-1} (strong) for C-O stretching vibration (Appendix 1A). These peak values were similar or approximately the same with those described by Pavia *et al*., (1996).

3.8.2 Physical and spectral data for Ergosta-5,7,22-triene-3*β*,14*α* –diol (22*Z*) (72) from *Ganoderma lucidum*

Separation of *n*-hexane extract using column chromatography from local *Ganoderma lucidum* followed by purification using analytical column led to isolation of colourless needle like crystals identified as ergosta-5,7,22-triene-3*β*,14*α*–diol (22*Z*) **(72)**.

Ergosta-5,7,22-triene-3*β*,14*α* –diol (22*Z*) **(72):** Colorless needle like crystals; R_f= 0.56 (20% EtOAc/*n*-hexane), yield, 19.4 mg, m.p: 178-180 °C. **IR** v max (cm^{-1}, KBr disc): 1226.6 cm^{-1},1334.6 cm^{-1},1650.0 cm^{-1}, 2657.7 cm^{-1}, 2869.9 cm^{-1}, 2954.7 cm^{-1}, 3176.5 cm^{-1}, 3300.0 cm^{-1}. **^{1}HNMR:** 1.84 (2H, *t*), 2.31 (2H, *m*), 3.59 (1H, *m*), 1.82, 2.28 (2H), 5.57 (1H, *d*, *J* = 2.5 Hz), 5.58 (1H, *d*, *J* =2.5 Hz), 1.58 (1H, *m*), 1.59 (2H, *m*), 1.91 (2H, *t*, *J* = 3.0 Hz), 3.49 (*s*), 1.50,1.83 (2H, *m*), 1.31, 1.745 (2H, *m*), 1.25 (1H, *m*), 0.63 (3H, *s*), 0.79 (3H, *s*), 1.87 (1H, *m*), 1.03 (3H, *d*, *J* = 4 Hz), 5.20 (1H, *dd*, *J* = 5.5, 2.5 Hz), 5.21 (1H, *dd*, *J* = 6.5, 5.0 Hz), 2.04 (1H, *m*), 1.45 (1H, *m*), 0.83 (3H, *d*, *J* = 6.5 Hz), 0.88 (3H, *d*, *J* = 6.0 Hz), 0.92 (3H, d, *J* = 4.5 Hz). **^{13}C NMR:** 38.4 (C-1), 32.0 (C-2),70.5 (C-3), 40.8 (C-4), 139.8 (C-5), 119.6 (C-6), 116.3 (C-7), 141.4 (C-8), 46.2 (C-9), 37.1 (C-10), 21.1 (C-11), 39.1 (C-12), 42.8 (C-13), 71.1 (C-14), 23.0 (C-15), 28.2 (C-16), 55.7 (C-17), 12.1 (C-18), 17.6 (C-19), 40.5 (C-20), 21.5 (C-21), 135.6 (C-22), 131.9 (C-23), 42.8 (C-24), 33.1 (C-25), 19.6 (C-26), 19.9 (C-27), 16.3 (C-28).

Infra-red signals for compound **72** were as follows: 3300cm^{-1}(strong) for hydroxyl group (O-H peak), 3176.5 cm^{-1} (weak) for vinyl =C-H stretch, 2954.7 cm^{-1} (strong) for methyl (CH_3) stretch, 2869.9 cm^{-1} (weak) for methylene (-CH_2) stretch, 2657.7 cm^{-1} (weak) -C-H (methine) sp C-H stretch, 1596.9 cm^{-1} (sharp) for cyclic double bond, (-C=C-), 1226.6 cm^{-1} (strong) for C-O stretching vibration (Appendix 1B). These peak values are similar or approximately the same with those described by Pavia *et al*., (1996).

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Phytochemical Analysis of Kenyan *Termitomyces letestui* and *Ganoderma lucidum*

4.1.1 Phytochemical screening of Local *Termitomyces letestui*

From Table 2, the presence of the following family/classes of compounds; steroids/triterpenoids, saponins, alkaloids, carbohydrates, phenolic compounds (tannins), flavonoids, phytosterols and polyoses were observed in *T. letestui* mushroom. However, it was observed that glycosides were absent in both extracts (Table 2). *Termitomyces letestui* mushroom is also found in Nyanza, Central Kenya, Mt. Elgon and Coastal region. The mushroom is called by different names by different communities in Kenya. Kikuyus in central Kenya call it "makunu ma mutitu", "mariondonik" (sabaot) in Mt. Elgon, "obulando" (luhya) in Western Kenya, "oruka" (luo) in Nyanza. Phytochemical studies done by Wandati *et al*., 2013 showed that "makunu ma mutitu", "mariondonik", "obulando" (luhya), "oruka" (luo) and "joga utuwe" (giriama) contained saponins, polyphenols and terpenoids. Alkaloids, tannins and anthraquinones were absent in all the five mushrooms.

Termitomyces mushrooms exihibit antioxidant, immune-modulation, antitumeric and antimicrobial properties (Huei-Mei *et al*., 2018). They are also used for treating neurodegenerative disorders (Huei-Mei *et al*., 2018).

Table 2: Phytochemical screening of *Termitomyces letestui*

		Extraction solvents	
No.	**Secondary metabolites**	**Ethylacetate**	**Methanol**
1.	Steroids	+	+
2.	Triterpenoids	+	+
3.	Saponins	+	+
4.	Alkaloids	+	+
5.	Cabohydrates	+	+
6.	Phenolics (tannins)	+	+
7.	Glycosides	-	-
8.	Flavonoids	+	+
9.	Polyoses	+	+

Key: Positive result (+), negative result (-)

4.1.2 Phytochemical screening of Local *Ganoderma luciderm*

Phytosterols (steroids and triterpenoids), carbohydrates, glycosides and flavonoids were revealed in local *Ganoderma lucidum* crude extracts. Phenolic compounds were observed in ethylacetate and methanol extracts but not in *n*-hexane extract. Saponins and alkaloids were absent in the local *Ganoderma lucidum*. The phytochemical analyses of *Ganoderma lucidum* are summarized in Table 3.

Table 3: Phytochemical screening of *Ganoderma lucidum*

		Extraction solvents		
No.	**Secondary metabolites**	***n*-Hexane**	**Ethyl acetate**	**Methanol**
1.	Steroids	+	+	+
2.	Triterpenoids	+	+	+
3.	Saponins	-	-	-
4.	Alkaloids	-	-	-
5.	Reducing sugar	+	+	+
6.	Phenolics (tannins)	-	+	+
7.	Glycosides	+	+	+
8.	Flavonoids	+	+	+

Key: Positive result (+), negative result (-)

Phytochemical tests of *Ganoderma lucidum* from different regions of the world have also been studied in the past. *Ganoderma lucidum* species from China indicates the presence of glycosides, carbohydrates (polysaccharides) and triterpenoids (Naveen *et al*.,

2018). Indian *Ganoderma lucidum* contains triterpenoids and polysaccharides (2014; Ranjith *et al*., 2015). Polysaccharides have been found to be important in enhancing the immune system while terpenes possess anti-inflammatory, anti-tumorigenic and hypolipidemic activity (Arora, 1986).

4.2 Compound isolated from *Termitomyces letestui* and *Ganoderma lucidum* mushrooms

4.2.1 Compound isolated from *Termitomyces letestui*

4.2.1.1 Ergosta-5,7,22-triene-3*β*-ol-(22*Z*) (69)

Column chromatography from ethylacetate extract of *Termitomyces letestui* followed by purification resulted in the isolation of colorless, needle-like crystals labelled as TLETA1.14 (R_f = 0.56, 20% EtOAc in Hexane). Multi view from ^{1}H-NMR data revealed that the four fractions were the same compound (Fig.4).

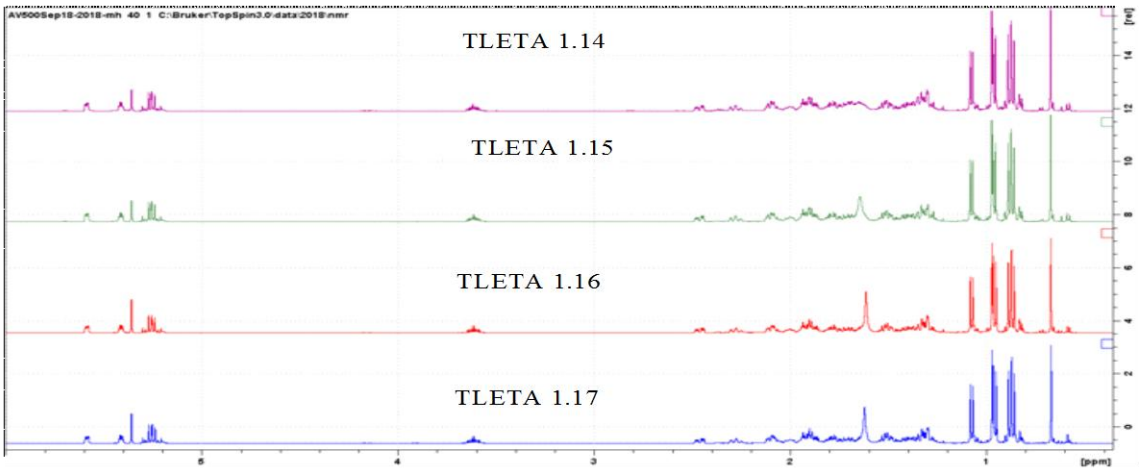


Figure 4: Multiview ^{1}H-NMR showing similarities in peaks

Compound **69** was analyzed using ^{1}H-NMR, ^{13}C-NMR, HMBC and COSY spectras. The spectral data revealed that the isolated compound was in agreement with the structure, ergosta-5,7,22-triene-3*β*-ol-(22*Z*) **(69)**. The data for the observed compound

indicated a *trans* isomer, ergosta-5,7,22-triene-3*β*-ol-(22*E*) (**1**) with R_f value of 0.56 and with m.p 140-145 °C (Wekesa, 2014). Reported (experimental data) melting points were in the range of 155-158 °C with R_f value being 0.56 (similar to the literature data).

Table 6 shows comparison for ^{1}H- and ^{13}C-NMR data for compound **(69)** isolated from local *Termitomyces letestui* (experimental data) and ergosta-5,7,22-triene-3β-ol (22*E*) **(1)** separated from *Basidiomycete pleurotus* and *Pleurotus ostreatus* mushrooms (Donadio *et al*., 2002; Ely *et al*., 2004; Wekesa, 2014). Most peak values for the isolated compound were consistent with the observed peaks from literature data (Tatiana *et al*., 2017) according to Table 4 below.

Table 4: Comparing ^{13}C and ^{1}H-NMR peaks with those from literature (Tatiana *et al*., 2017)

C/No.	^{13}C- NMR(CD_2Cl_2) (500 MHz) δ_C *Cis* **(69)**	^{1}H- NMR (CD_2Cl_2) (500 MHz) δ_H *Cis* **(69)**	^{13}C-NMR δ_C *Trans* **(1)**	^{1}H-NMR δ_H *Trans* **(1)**
1	38.3	1.86	38.3	1.83 (2H, *t*)
2	32.3	2.44 (*ddd*, *J* = 15, 6, 2 Hz)	32.1	2.32 (2H, *m*)
3	70.5	3.58 (1 H, *m*)	70.6	3.97 (1H, *m*)
4	41.0	1.90, 2.05 (2 H, *t*, *dd*, *m* (*J* = 5, 1Hz)	40.8	1.90, 2.07(2H, *t*, J=6.8Hz)
5	140.3		139.9	-
6	119.7	5.60 (1H, *dd*, *J* = 5, 10Hz)	119.7	6.24 (1H, *d*, *J*= 8.3Hz)
7	116.5	5.45 (1H, *dd*, *J* = 5, 10Hz)	116.4	6.50 (1H, *dd*, *J* = 8.3Hz)
8	141.6		141.5	-
9	46.5	1.61 (1H, *m*)	46.4	1.56 (1H, *m*)
10	37.3		37.1	-
11	21.3	1.50 (2H, *m*)	21.3	1.60 (2H, *m*)
12	39.3	1.90 (2H, *t*)	39.0	1.91 (2H, *t*, *J* = 6.8Hz)
13	43.1		42.8	-
14	54.8	1.85 (1H, *m*)	54.7	1.91 (1H, *m*)
15	23.3	1.50, 1.86 (2H, *m*)	23.1	1.50, 1.83 (2H, *m*)
16	28.5	1.31, 1.74 (2H, *m*)	28.4	1.30, 1.75 (2H, *m*)
17	55.7	1.26 (1H, *m*)	55.8	1.25 (1H, *m*)
18	12.1	0.79(3H, *s*)	12.2	0.79 (3H, *s*)
19	17.7	0.64 (3H, *s*)	17.7	0.63 (3H, *s*)
20	40.7	1.87 (1H, *m*)	40.5	1.87(1H, *m*)
21	21.1	1.05 (3H, *d*, *J* =10Hz)	21.2	1.04 (3H,*d*, *J*=6.5 Hz)
22	135.9	5.20 (1H, *dd*, *J* = 10, 5Hz)	135.5	5.13 (1H, *dd*, *J*= 15.6, 7.9Hz)
23	132.1	5.21 (1H, *dd*, *J* = 10, 5Hz)	132.1	5.21 (1H, dd, *J* = 15.6, 7.9Hz)
24	43.0	2.06 (1H, *m*)	42.9	2.05 (1H, *m*)
25	33.4	1.47 (1H, *m*)	33.2	1.45 (1H, *m*)
26	19.7	0.84 (3H, *d*, *J* = 10Hz)	19.8	0.82 (3H, *d*, *J* = 6.6Hz)
27	19.9	0.82 (3H, *d*, *J* = 10Hz)	20.1	0.89 (3H, *d*, *J* = 7.0 Hz)
28	16.3	0.92 (3H, *d*, *J* = 10Hz)	16.4	0.92 (3H, *d*, *J* = 7.0 Hz)

Note: Ergosterol obtained from literature was ergosta-5,7,22-triene-3*β*-ol (22*E*) (*trans*) **(1)** but local *Termitomyces letestui* indicated ergosta-5,7,22-triene-3*β*-ol (22*Z*) **(69)**. The

melting point for *trans*-ergosterol was 140-145 °C (Wekesa, 2014) while ergosta-5,7,22-triene-3*β*-ol (22*Z*) (*cis*-ergosterol) **(69)** was 155-158 °C. This meant that the two isomers had different physical properties.

4.2.1.1.1 Spectral analysis for Ergosta-5,7,22-triene-3*β*-ol (22*Z*) (69)

The isolated compound, ergosta-5,7,22-triene-3*β*-ol-(22*Z*) **(69)** was established using the following spectroscopic techniques: ^{1}H-NMR and ^{13}C-NMR. Both ^{1}H and ^{13}C-NMR results from spectral data were in agreement with ergosta-5,7,22-triene-3*β*-ol-(22*Z*) **(69)**. The ^{13}C-NMR spectra indicated 28 carbons which were; six methyl groups at (δ_C 12.1, 16.3, 17.7, 19.7, 19.9 and 21.1), seven methylenes (δ_C 21.3, 23.3, 28.5, 32.3, 38.3, 39.3, and 41.0), eleven methines (δ_C 33.4, 40.7, 43.0, 46.5, 54.8, 55.7, 70.5, 116.5, 119.7, 132.1, and 135.9) and four quaternary carbons (δ_C 37.3, 43.1, 140.3, and 141.6) (Fig. 6). Most carbon resonances obtained were close or similar to those obtained by (Wekesa, 2014; Nyigo *et al.* 2005; Miguel *et al*., 2015) for methyl and methine. The methine carbon (CH-3) at 70.5 ppm was shifted down field because of the deshielding of hydroxyl (-OH) attached (Fig. 7). The ^{13}C-NMR of **(69)** (Fig. 6) revealed six peaks because of sp^2 carbons at δ_C 141.6, 140.3, 135.9, 132.1, 119.7 and 116.5 which indicated three double bonds as consistent with values for ergosta-5,7,22-triene-3*β*-ol (22*E*) **(1)** (ergosterol) according to (Wekesa, 2014; Nyigo *et al.* 2005 and Miguel *et al*., 2015).

^{1}H-NMR spectrum for **(69)** indicated four olefinic protons (δ_H 5.60, 1H, *dd*, *J* = 10Hz, 5Hz, δ_H 5.45, 1 H, *dd*, *J* = 10Hz, 5Hz, δ_H 5.20, 1 H, *J* = 10 Hz, 5Hz, δ_H 5.22, 1 H, *J* = 10Hz, 5Hz) (Fig. 5 and 7). The two protons doublet of doublets at δ_H 5.20 and δ_H 5.22 were assigned to olefinic protons at C – 22 and C – 23, respectively, which usually appears in olefinic protons in sterols (Nyigo *et al*., 2005; Wekesa, 2014). The carbon 22 double bond for **69** was concluded to be *cis* from ^{1}H – ^{1}H coupling constant (*J* = 10 Hz)

between H-22 and H-23 (Pavia *et al*., 1996). According to ^{1}H-NMR spectrum obtained for **(69)**, the coupling constant was 10 Hz (Fig. 5). It was deduced from the information that the compound was ergosta-5,7,22-trien-3*β*-ol (22*Z*) **(69)**. Wekesa (2014) isolated ergosta-5,7,22-trien-3*β*-ol (22*E*) (*trans*-ergosterol) **(1)** from *Pleurotus spp* mushroom with coupling constant (*J* = 15.6 Hz). Olefinic protons at C-6 (^{1}H = 6.24) and C-7 (^{1}H = 6.50) in compound **(1)** are slightly higher than those of compound **(69)** which are between 5.45 and 5.60 ppm. This slight difference shows that the compounds are not the same. The ergosta-5,7,22-trien-3*β*-ol (22*E*) (*trans*-ergosterol) isomer is common in many mushroom species while *cis*-ergosterol isomer, ergosta-5,7,22-trien-3*β*-ol (22*Z*) **(69)** occurs rarely.

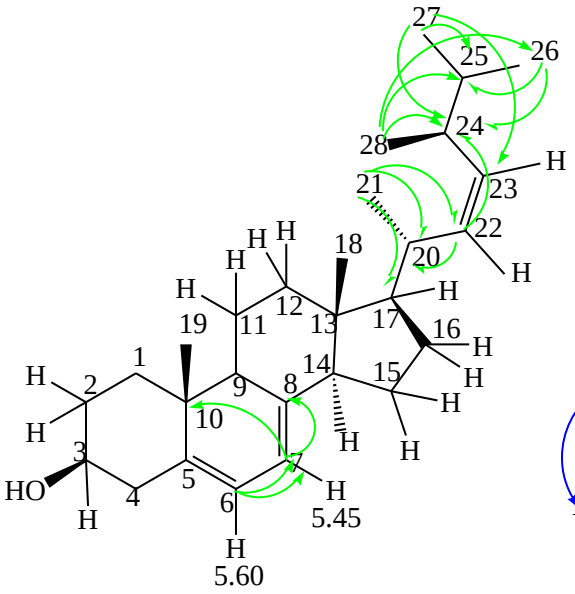
HMBC spectrum showed correlation peaks for methyl protons at C-26 (δ_H 0.84) with C-25 (δ_C 33.4) and C-24 (δ_C 43.0) (Fig. 9). Methyl protons at C-28 (δ_H 0.92) correlated with methine carbons at C-24 (δ_C 43.0), C-25 (δ_C 33.4) and C-26 (δ_C 19.7). Olefinic proton at C-22 (δ_H 5.20) correlated with methines at C-20 (δ_C 40.7) and C-24 (δ_C 43.0) (Fig. 9). A methyl proton at C-21 (δ_H 1.05) was coupled to methines at C-22 (δ_C 135.9), C-20 (δ_C 40.7) and C-17 (δ_C 55.7). The vinyl proton at C-7 (δ_H 5.45) coupled with methine carbons at C-6 (δ_C 119.7), C-8 (141.6) and C-10 (37.3) while that at C-6 (δ_H 5.6) correlated with methine carbon at C-7 (δ_C 116.5) (Fig. 9). The carbon chemical shift values were closely related with those of Miguel *et al*., (2015) and Baraza *et al*. (2005) for Ergosta-5,7,22-trien-3*β*-ol (22*E*) **(1)**.

The COSY ^{1}H-^{1}H spectrum revealed the coupling of H-2 and H-3; H-11 and H-12; H-14 and H-15; H-16 and H-17 (Fig. 8). There was also vicinal coupling of H-6 and H-7 and also H-22 and H-23; H-25 and H-26 (Fig. 8). Table 5 shows summary for the spectral data analysis for **(69)**.

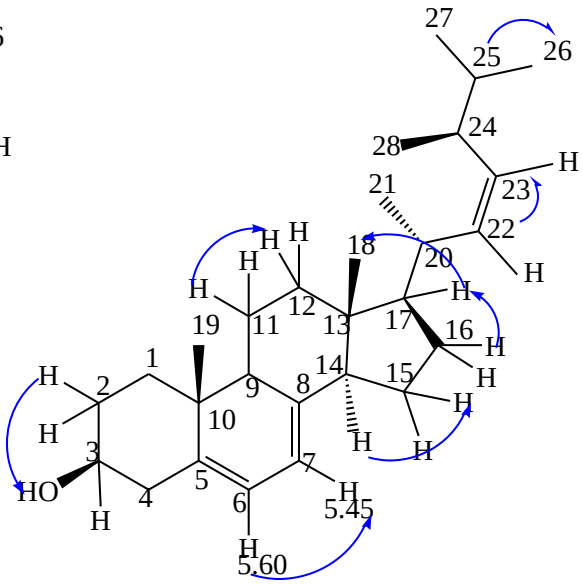
Table 5: ^{1}H and ^{13}C-NMR (500MHz, CD_2Cl_2) Data for ergosta-5,7,22-triene-3*β*-ol (22*Z*) (69)

C/No.	^{13}C NMR (CD_2Cl_2) (500 MHz)	^{1}H NMR (CD_2Cl_2) (500 MHz)	COSY	HMBC
1	38.3	1.86		
2	32.3	2.44 (*ddd*, *J* = 15, 6, 2 Hz)	H/C – 3	
3	70.5	3.58 (1 H, *m*)	H/C – 2	
4	41.0	1.90, 2.06 (2 H, *t*, *dd*,(*J* = 5, 1Hz)		
5	140.3			
6	119.7	5.60 (1H, *dd*, *J* = 10, 5 Hz)	H/C – 7	C – 7
7	116.5	5.45 (1H, *dd*, *J* = 10, 5 Hz)	H/C – 6	C – 6, C – 8, C – 10
8	141.6			
9	46.5	1.61 (1H, *m*)		
10	37.3			
11	21.3	1.50 (2H, *m*)	H/C – 12	
12	39.3	1.90 (2H, *t*)	H/C – 11	
13	43.1			
14	54.8	1.85 (1H, *m*)	H/C – 15	
15	23.3	1.50, 1.86 (2H, *m*)	H/C – 14	
16	28.5	1.31, 1.74 (2H, *m*)	H/C – 17	
17	55.7	1.26 (1H, *m*)		
18	12.1	0.79(3H, *s*)	H/C – 17	
19	17.7	0.64 (3H, *s*)		
20	40.7	1.87 (1H, *m*)		
21	21.1	1.05 (3H, *d*, *J* =10 Hz)		C – 22, C -20, C – 17
22	135.9	5.20 (1H, *dd*, *J* = 10, 5 Hz)	H/C – 23	C – 24, C – 20
23	132.1	5.21 (1H, *dd*, *J* = 10, 5 Hz)	H/C – 22	
24	43.0	2.06 (1H, *m*)		
25	33.4	1.47 (1H, *m*)	H/C – 26	
26	19.7	0.84 (3H, *d*, *J* = 10 Hz)		C-25, C-24
27	19.9	0.82 (3H, *d*, *J* = 10 Hz)		C- 24, C – 25,C - 23
28	16.3	0.92 (3H, *d*, *J* = 10 Hz)		C-25, C-24, C-26

The assignments were made from COSY, HSQC and HMBC spectra



69

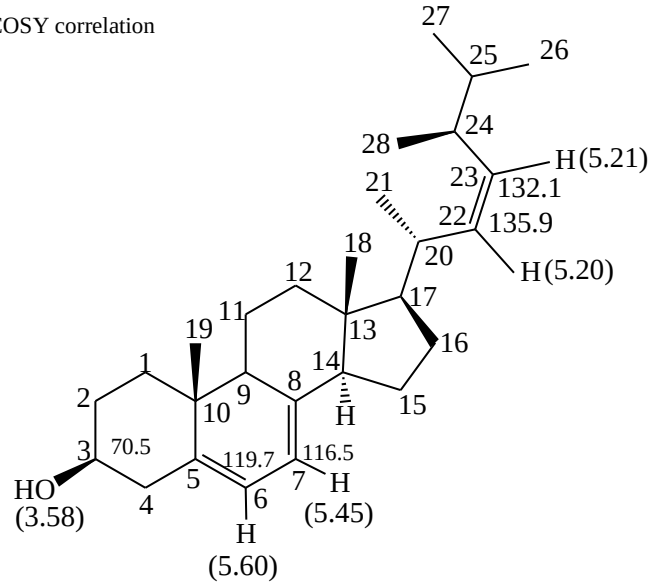


69

Key

HMBC correlation

COSY correlation



HSQC for the main peaks for the compound (69)

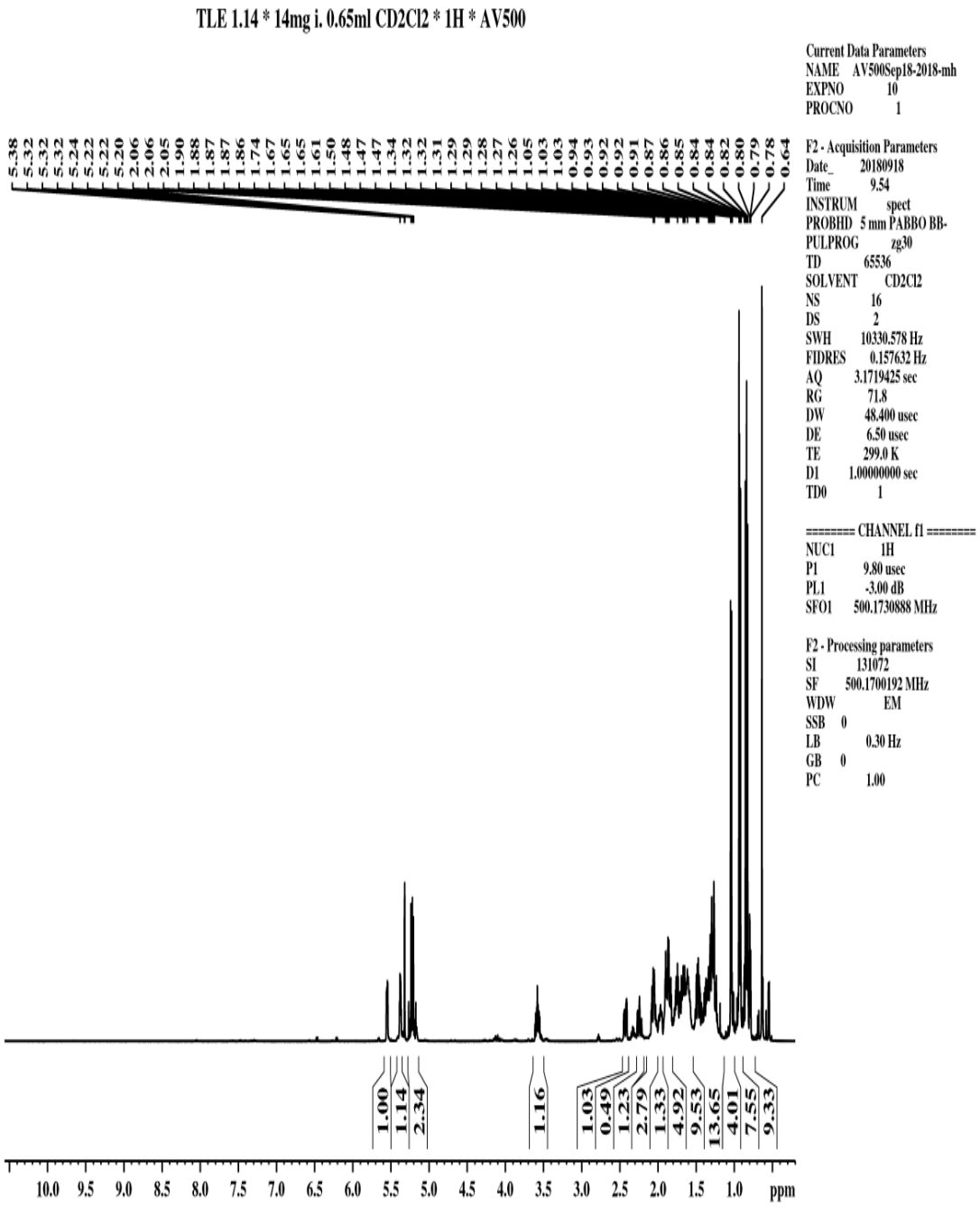


Figure 5: ^{1}H-NMR spectrum for Ergosta-5,7,22-triene-3β-ol (22*Z*) (69)

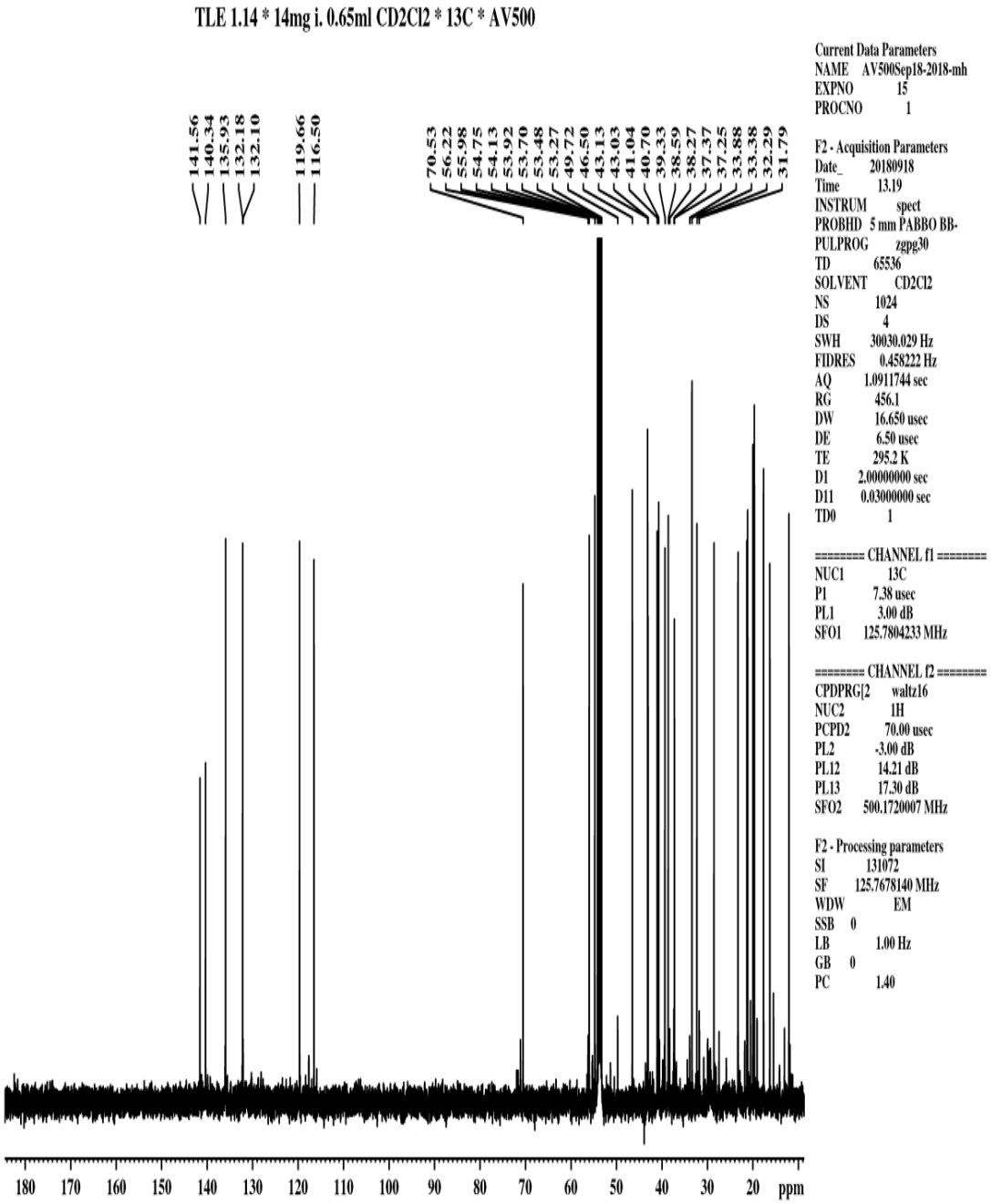


Figure 6: ^{13}C-NMR for Ergosta-5,7,22-triene-3*β*-ol (22*Z*) (69)

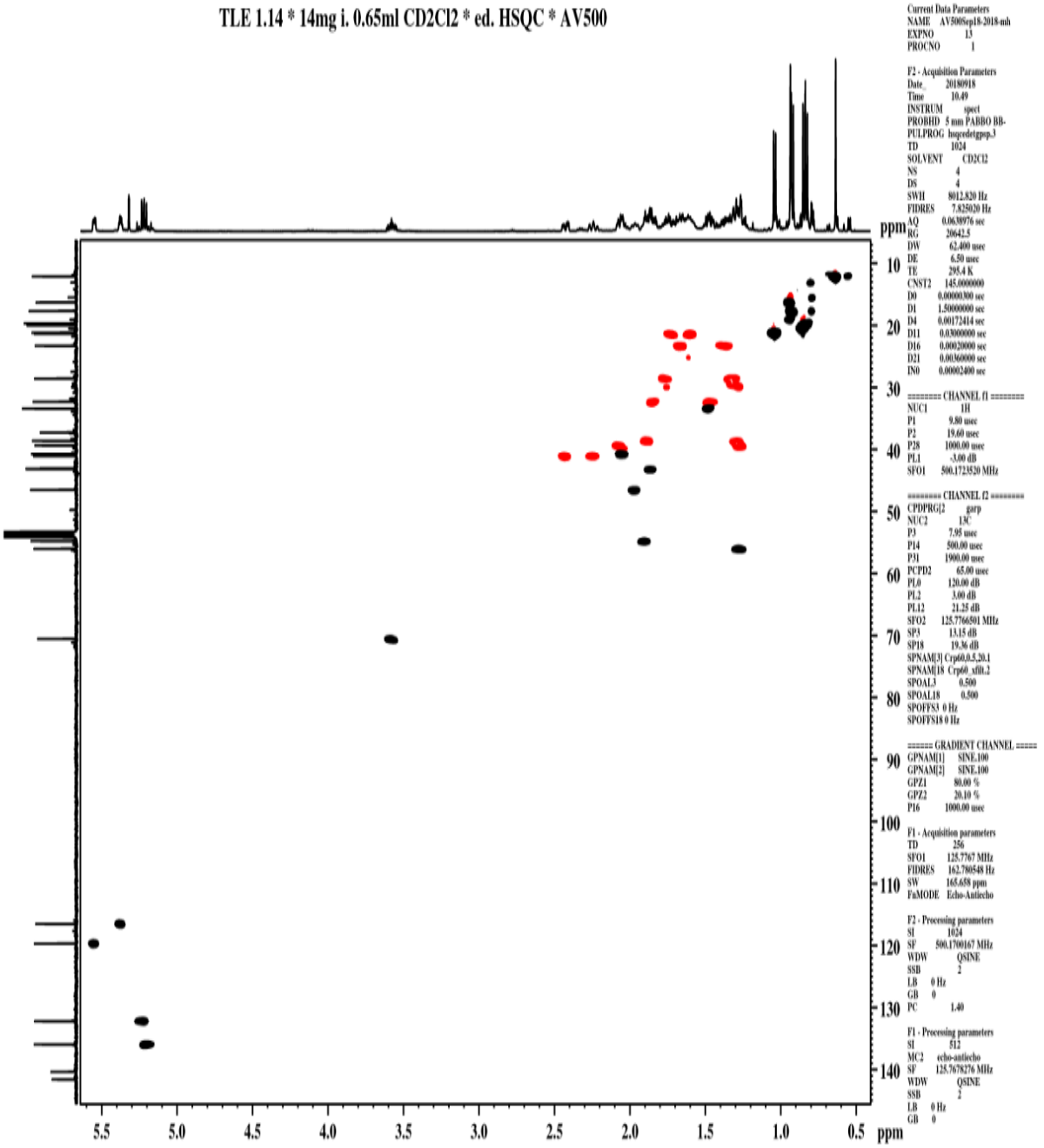


Figure 7: HSQC spectrum for Ergosta-5,7,22-triene-3*β*-ol (22*Z*) (69)

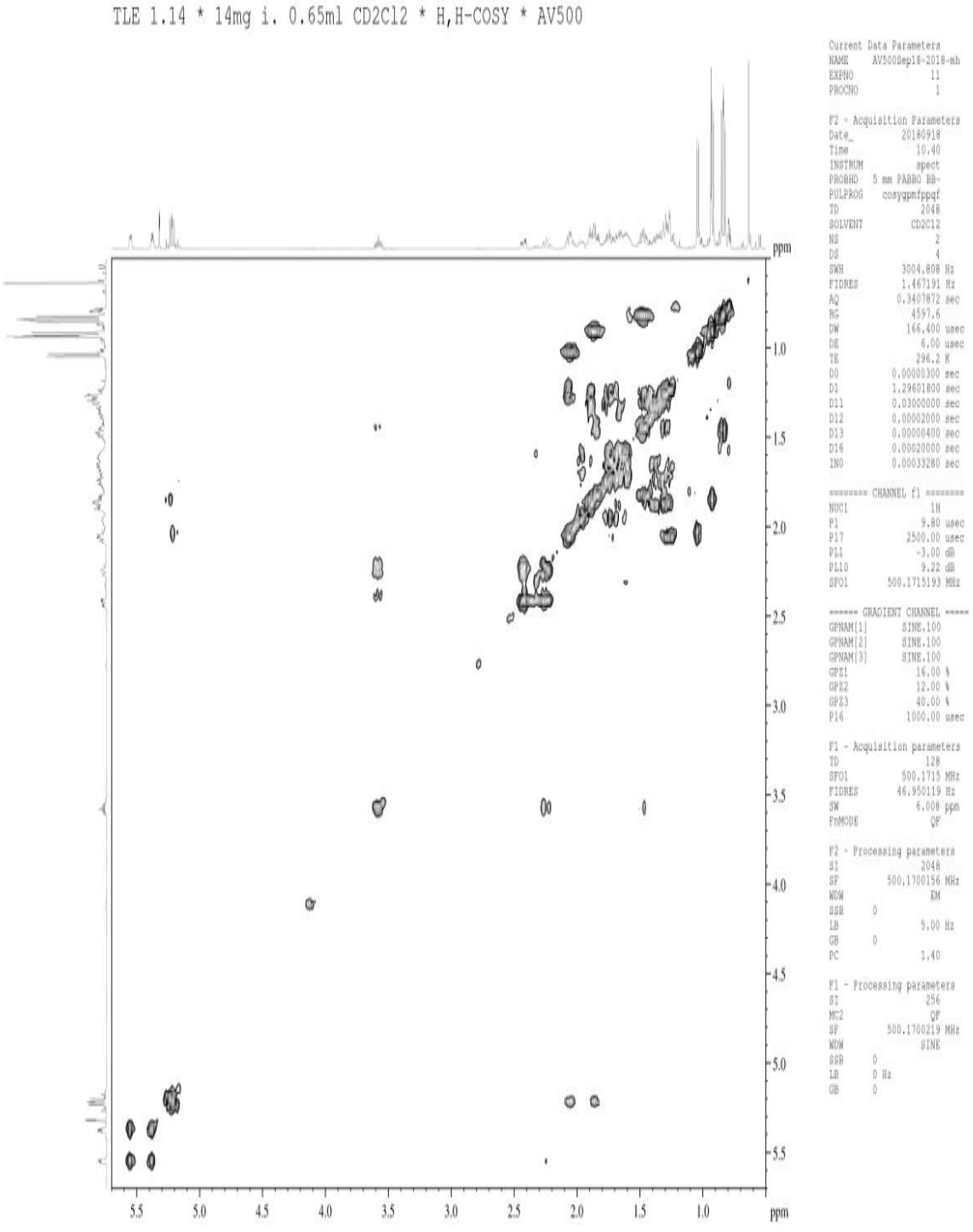


Figure 8: COSY spectrum of Ergosta-5,7,22-triene-3*β*-ol (22Z) (69)

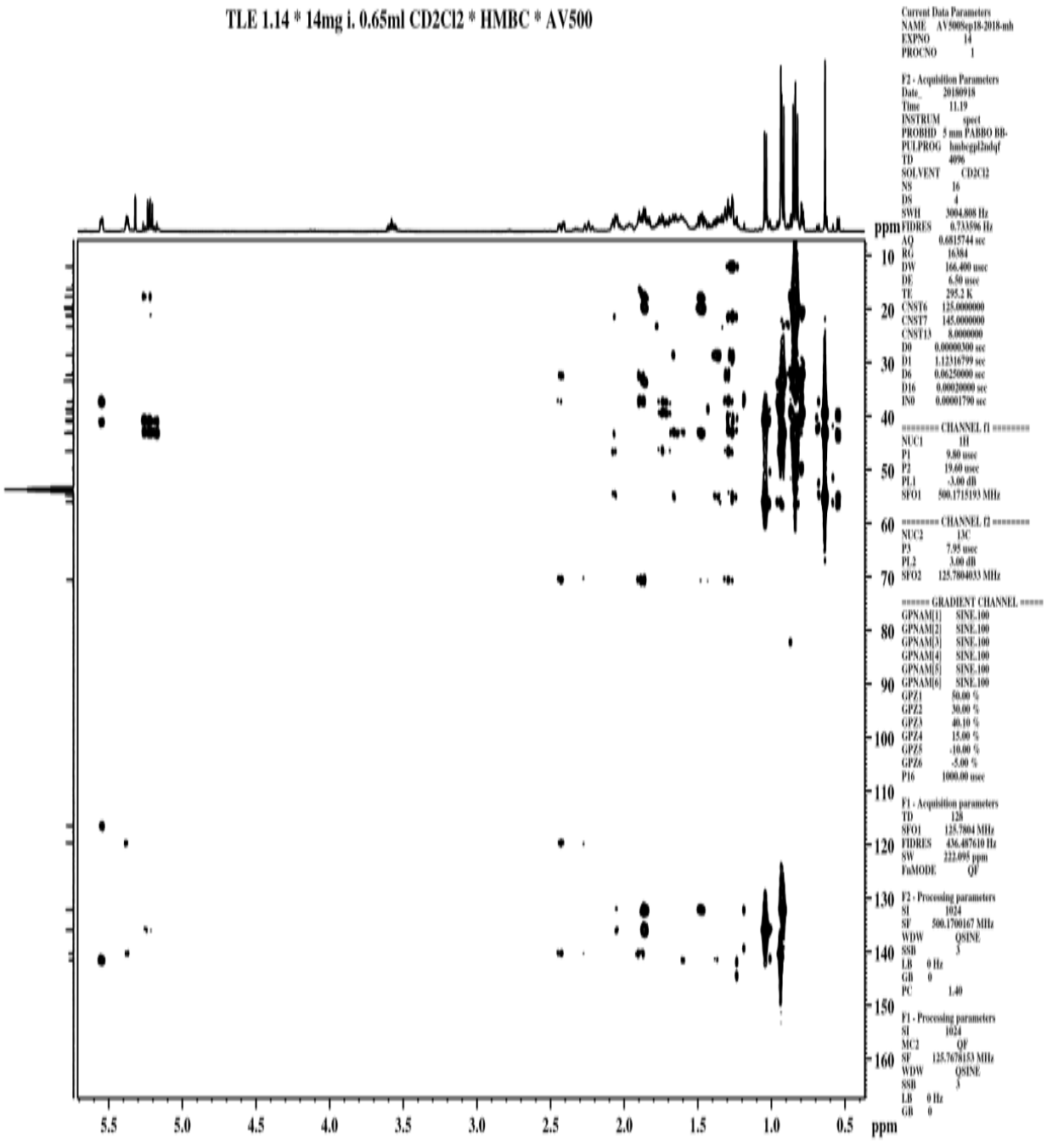
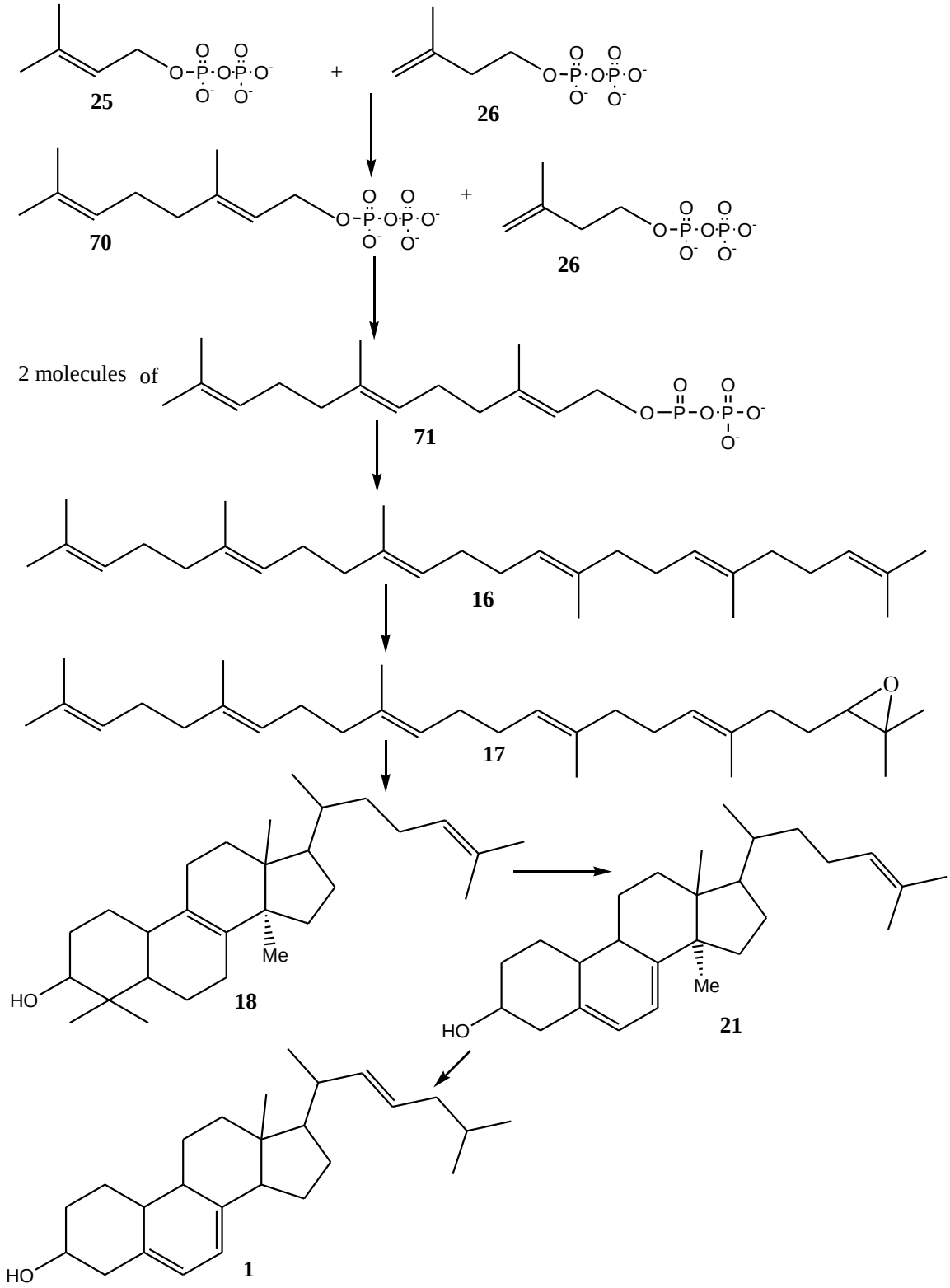


Figure 9: HMBC for Ergosta-5,7,22-triene-3*β*-ol (22*Z*) (69)

4.3 Biosynthetic pathway for Ergosta-5,7,22-triene-3*β*-ol-(22*Z*) (69)

Biosynthetically, ergosterols are derived from squalene which is an important precursor of cholesterol. Squalene, like other terpenoids is synthesized biologically from five carbon isoprene units. Overall coupling of isopentyl pyrophosphate (IPP) **(26)** with its isomer, dimethylally pyrophosphate (DMAPP) **(25)** forms geranyl pyrophosphate (GPP) **(70)**. GPP **(70)** then condenses with IPP **(26)** to produce a 15-carbon molecule, farnesyl pyrophosphate (FPP) **(71)**. Squalene synthase catalyst enables 2 molecules of FPP **(71)** to combine through a head fashion to give rise to a 30-carbon triterpene squalene **(16)**. Epoxidation of squalene catalysed by squalene epoxidase enables squalene epoxide **(17)** to be successively transformed into lanosterol **(18)**, 7-dehydrocholesterol **(21)** and ergosta-5,7,22-triene-3*β*-ol (22*E*) **(1)** (*trans*) **(1)** (Nyigo *et al.,* 2005; Dupont *et al*., 2012). The ergosterol synthesized in this way is *trans* (Nyigo *et al.,* 2005; Dupont *et al*., 2012).The biosynthetic pathway for ergosta-5,7,22-triene-3*β*-ol (22*Z*) (*cis*-ergosterol) isomer **(69)** is still unknown. The biosynthetic route for ergosta-5,7,22-triene-3*β*-ol (22*E*) (*trans*-ergosterol) **(1)** is shown in scheme 8 below.

Scheme 8: Biosynthetic pathway for Ergosta-5,7,22-triene-3*β*-ol (22*E*) (trans-**ergosterol) (Du pont *et al.*, 2012)**



4.4 Medicinal and Nutritional Value of Ergosta-5,7,22-triene-3*β*-ol (22*E*) (Ergosterol) and its derivatives

Ergosta-5,7,22-triene-3*β*-ol(22*E*) **(1)** stays on the cell membranes of fungi and maintains the cell membrane tugidity, same as cholesterol in mammals (Marek and Timmons., 2019). Polyene antimycotic agents together with ergosterol form a pore which allows electrolytes and small molecules to leak out of the cell (Marek and Timmons, 2019). Ergosterol and its derivatives have been noted for the biological processes and help in the survival of mushroom (Baraza *et al*., 2007). The compound also induces pyroptosis-mediated macrophage lysis (Koselyn *et al*., 2018). Ergosterol biosynthesis routes enhance multiple targets for antifungal development and also act as an immunoactive molecule for macro-fungi or mushrooms (Klemptner *et al*., 2014).

4.5 Compound isolated from *Ganoderma lucidum*

4.5.1 Ergosta-5,7,22-triene-3*β*, 14*α*-diol (22*Z*) (72)

Compound **(72)** was isolated as colourless needle-like crystals with R_f=0.61, 25% EtOAc in Hexane) and m.p of 178-180 °C. The ^{1}H and ^{13}C NMR and IR spectra together with physical properties of **72** were consistent with literature values for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*). The five fractions obtained from *n*-hexane extract of *Ganoderma lucidum* shows that the compound was the same (Fig.10).

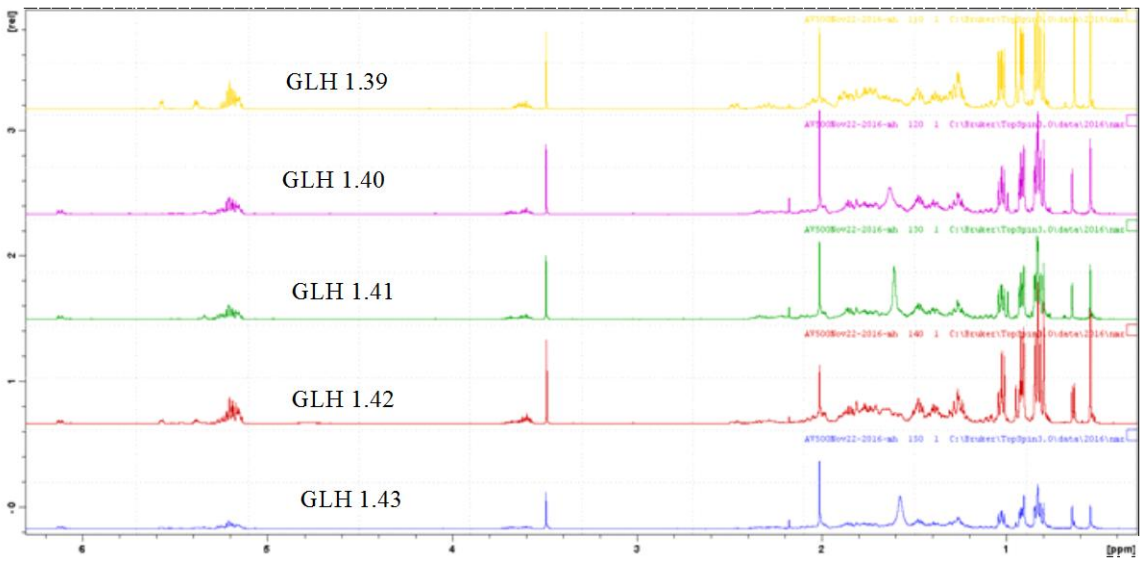


Figure 10: Multiview ^{1}H-NMR showing similarities in peaks for compound 72

Compound **(72)** was isolated as colourless needle-like crystals with R_f value of 0.61 (25% EtOAc in *n*-hexane) and m.p of 178-180 °C. The ^{1}H and ^{13}C-NMR and IR spectra data together with physical properties of **72** were in agreement with literature values for ergosta-5,7,22-triene-3*β*,14*α*-diol-(22*Z*) (Miguel *et al.*, 2015; Tatiana *et al.*, 2017; Wekesa, 2014; Baraza *et al.*, 2007). Reported melting points of the fractions were in the range of 178-180 °C.

The compound isolated was a sterol. Most of the peaks were consistent with the observed values in Tables 6 except for carbon (C-14) which had a hydroxyl group (-OH). The carbon at position 3 was also assigned hydroxyl group (-OH) consistent with in ergosta-5,7,22-triene-3*β*-ol-(22*Z*) **(69)** according to the observed data in Tables 6. The compound therefore had two hydroxyl groups (–OH).

4.5.1.1 Spectral analysis of Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*)

The isolated compound, ergosta-5,7,22-triene-3*β*,14*α*-diol(22*Z*) **(72)** was established using combination of spectral analysis of ^{1}H-NMR and ^{13}C-NMR and HMBC. Both ^{1}H-

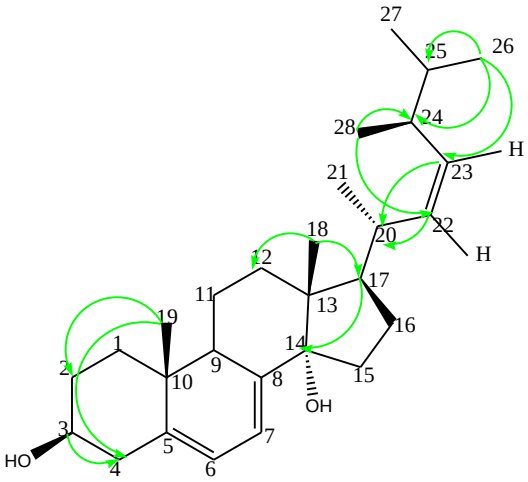
NMR and ^{13}C-NMR spectras showed peaks that were consistent with ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) **(72).**

Most ^{1}H-NMR and ^{13}C-NMR signals for compound **72** were same as those of compound **69** as shown by Table 6. Olefinic (-C=C-) and hydoxyl groups (-OH) were the main peaks in compound **72**. The olefinic carbons; C-5, C-6, C-7, C-8, C-22 and C-23 were similar to those of compound **69** (Table 6). Compound **72** isolated from hexane extract of *G. lucidum* had two hydroxyl groups at (C-3 and C-14). Their signals were (3.59, 1H, *m*) and [3.49, 1H (*s*)] (Fig. 12). The one with the peak 3.59 ppm was a multiplet at carbon-3 since it splitted its neighbours at carbon-2 and carbon-4 while the singlet appeared at C-14 (Fig.12). Previously, it was observed that compound **69** also had a hydroxyl (–OH) multiplet at carbon-3. Compound **72** also had a hydroxyl (-OH) group at C-14 (Table 6).

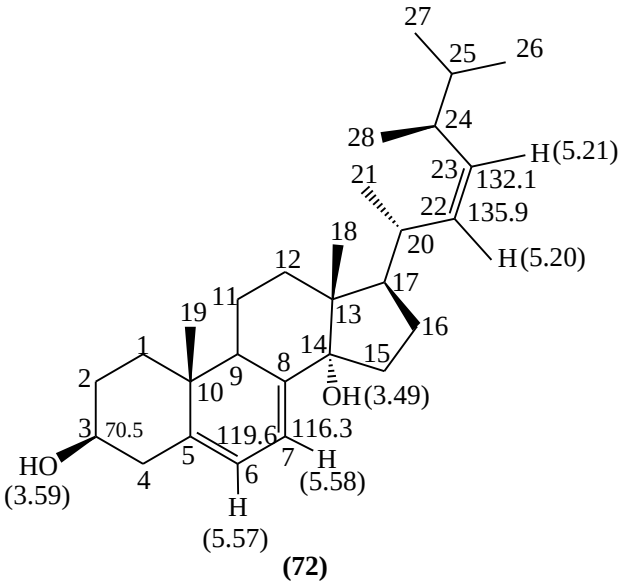
According to HMBC, there was correlation between C-14 and C-17. The signal at about 1.18 ppm was assigned to C-17 and it correlated with the quarternary carbon-14 with the value 71.1 ppm (Fig. 15 and 16). This showed that a hydroxyl (-OH) singlet appeared at C-14. Nai-Yun Ji *et al*., (2011) isolated a sterol from *Algicolous Gibberella zeae* strain and gave carbon-17 the peak value of 1.06 ppm for the compound (22*E*,24*R*)-5*α*,6*α*-epoxy-3*β*,8*β*,14*α*-trihydroxyergosta-22-en-7-one **(73)**. The value by Nai-Yun Ji *et al*., (2011) was comparable with the value 1.18 ppm at C-17 according to HMBC data (Fig. 15 and16). Combination of other spectroscopic techniques and literature sources (Miguel *et al*., 2015; Pavia *et al*., 1996) deduced the compound to be ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) **(72)**.

Table 6: ^{13}C and ^{1}H-NMR comparison data for compounds (72) and (69)

C/No.	^{13}C NMR (CD_2Cl_2) (500 MHz) **(72)**	^{1}H NMR (CD_2Cl_2) (500 MHz) **(72)**	^{13}C NMR (CD_2Cl_2) (500 MHz) **(69) (ref.)**	^{1}H NMR (CD_2Cl_2) (500 MHz) **(69) (ref.)**
1	38.4	1.84 (2 H, *t*)	38.3	1.86 (2H, *t*)
2	32.0	2.31 (2 H, *m*)	32.3	2.44 (2H,*m*)
3	70.5	3.59 (1 H, *m*)	70.5	3.58 (1H, *m*)
4	40.8	1.82, 2.28 (2 H, *d*, (*J* = 5.0, 1.0 Hz)	41.0	1.90, 2.06 (2H, *t*, *J* = 5.0, 1.0 Hz)
5	139.8		140.3	
6	119.6	5.57 (1H, *d*, *J* = 2.5 Hz)	119.7	5.60 (1H, *dd*, *J* = 10.0, 5.0 Hz)
7	116.3	5.58 (1H, *d*, *J* = 2.5 Hz)	116.5	5.45 (1H, *dd*, *J* = 10.0, 5.0 Hz)
8	141.4		141.6	
9	46.2	1.58 (1H, *m*)	46.5	1.61 (1H, *m*)
10	37.1		37.3	
11	21.1	1.59 (2H, *m*)	21.3	1.50 (2H, *m*)
12	39.1	1.91 (2H, *t*, *J* = 3.0 Hz)	39.3	1.90 (2H, *t*)
13	42.8		43.1	
14	71.1	3.49 (*s*)	54.8	1.85 (1H, *m*)
15	23.0	1.50, 1.83 (2H, *m*)	23.3	1.50,1.86 (2H, *m*)
16	28.2	1.31, 1.75 (2H, *m*)	28.5	1.31,1.74 (2H, *m*)
17	55.7	1.25 (1H,*m*)	55.7	1.26 (1H, *m*)
18	12.1	0.63 (3H, *s*)	12.1	0.64 (3H, *s*)
19	17.6	0.79 (3H, *s*)	17.7	0.79 (3H, *s*)
20	40.5	1.87 (1H, *m*)	40.7	1.87 (1H,*m*)
21	21.5	1.03 (3H, *d*, *J* = 4.0 Hz)	21.1	1.05 (3H, *d*, *J* = 10.0 Hz)
22	135.6	5.20 (1H, *dd*, *J* = 5.5, 2.5 Hz)	135.9	5.20 (1H, *dd*, *J* =10.0, 5.0 Hz)
23	131.9	5.21 (1H, *dd*, *J* = 6.5, 5.0 Hz)	132.1	5.21 (1H, *dd*, *J* = 10.0, 5.0 Hz)
24	42.8	2.04 (1H, *m*)	43.0	2.05 (1H, *m*)
25	33.1	1.45 (1H, *m*)	33.4	1.47 (1H, *m*)
26	19.6	0.83 (3H, *d*, *J* = 6.5Hz)	19.7	0.84 (3H, *d*, *J* = 10.0 Hz)
27	19.9	0.88 (3H, *d*, *J* = 6.0 Hz)	19.9	0.82 (3H, *d*, *J* = 10.0 Hz)
28	16.3	0.92 (3H, *d*, *J* = 4.5 Hz)	16.3	0.92 (3H, *d*, *J* = 10.0 Hz)



72

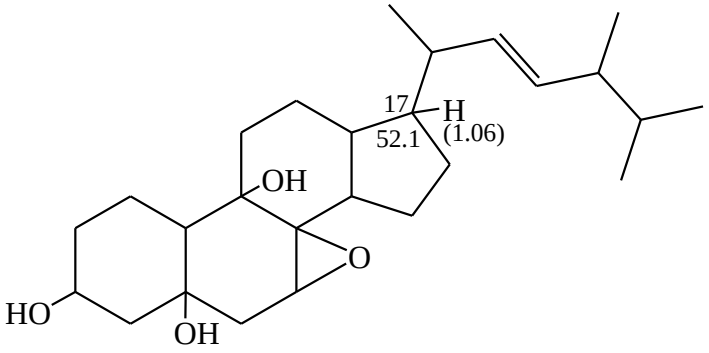


(72)

HSQC for the main peaks for the compound

Key

HMBC correlation



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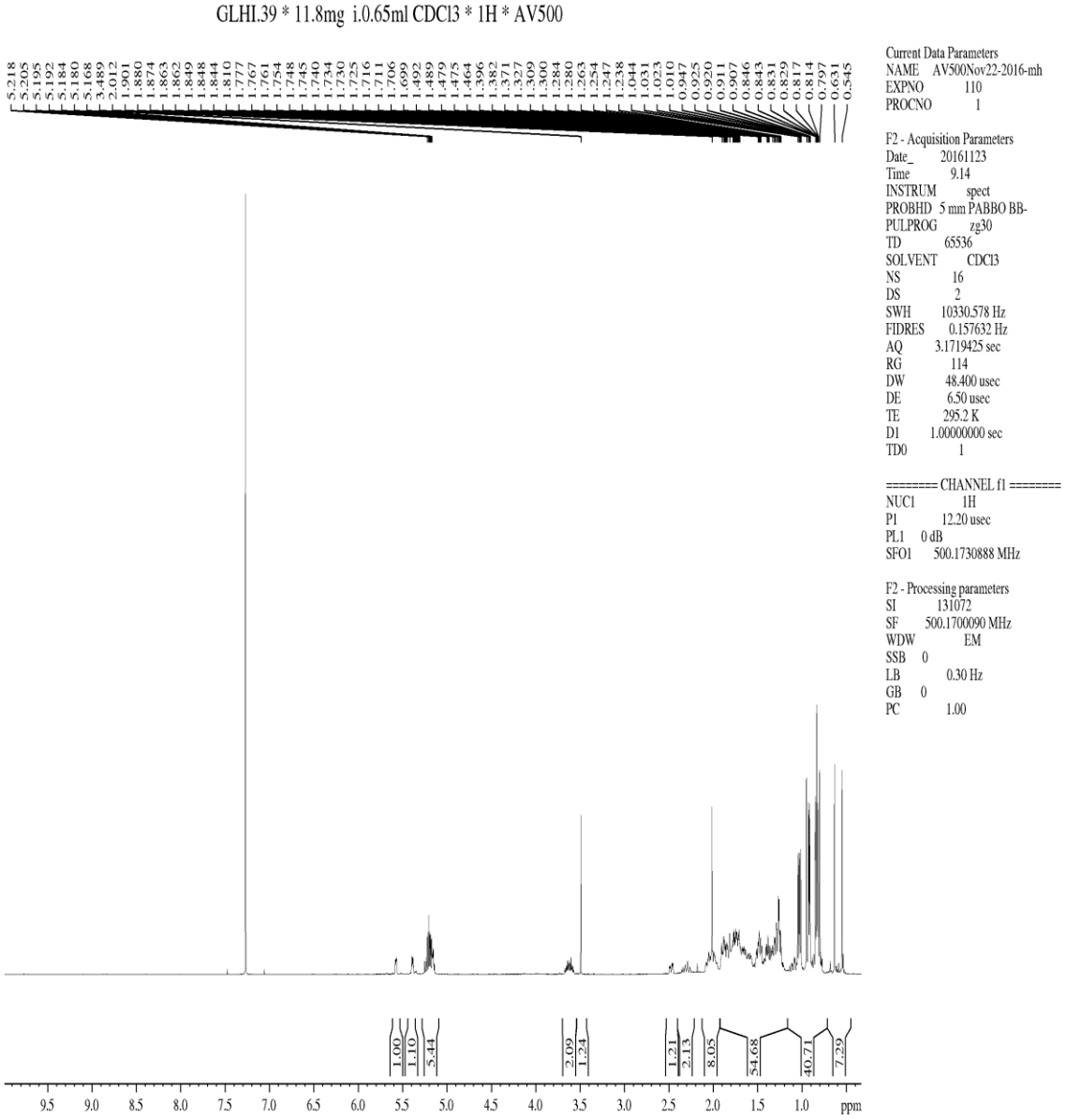

Figure 11: 1H-NMR for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) (72)

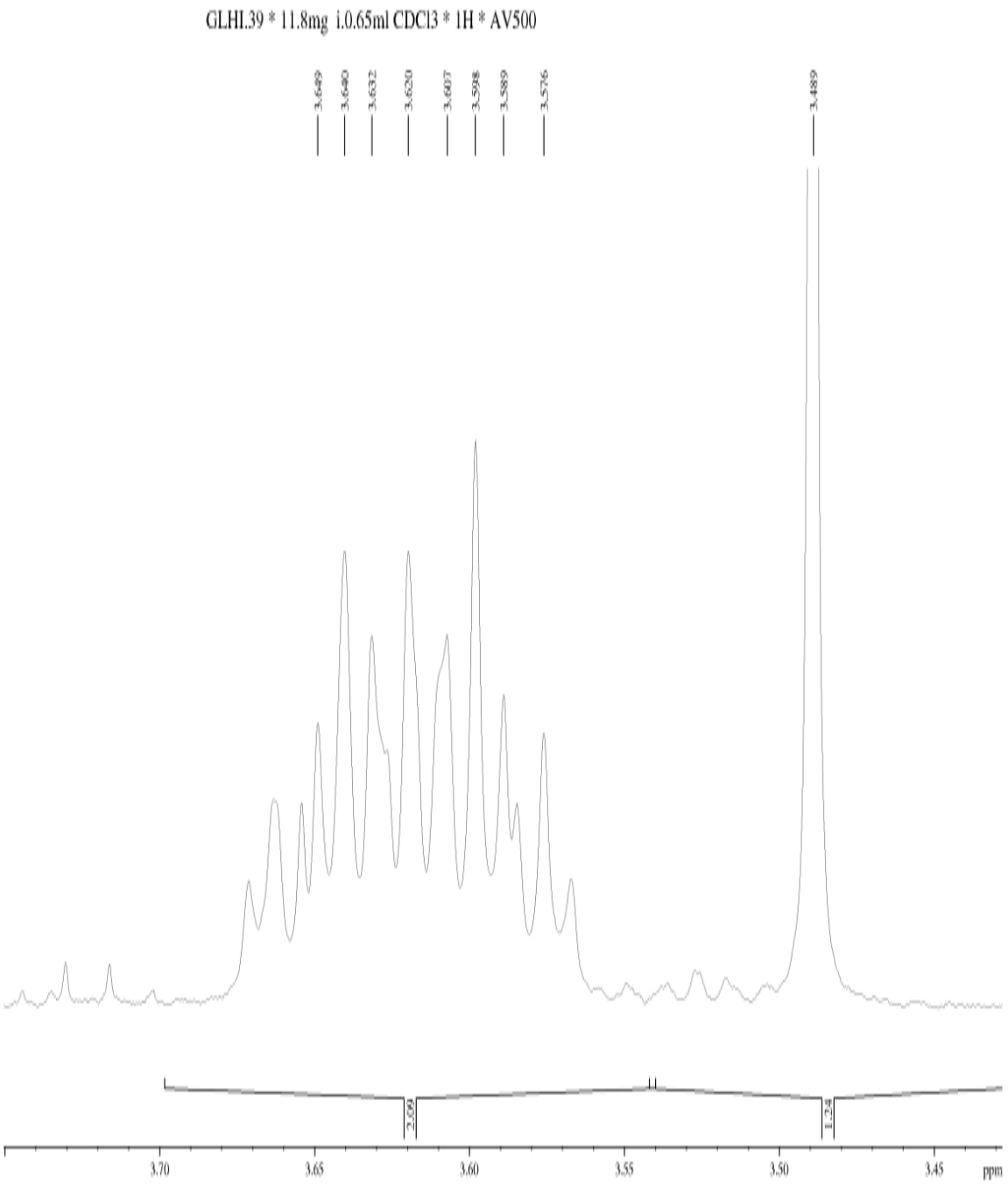

Figure 12: ^{1}H-NMR showing a multiplet and a singlet of hydroxyl groups (72)

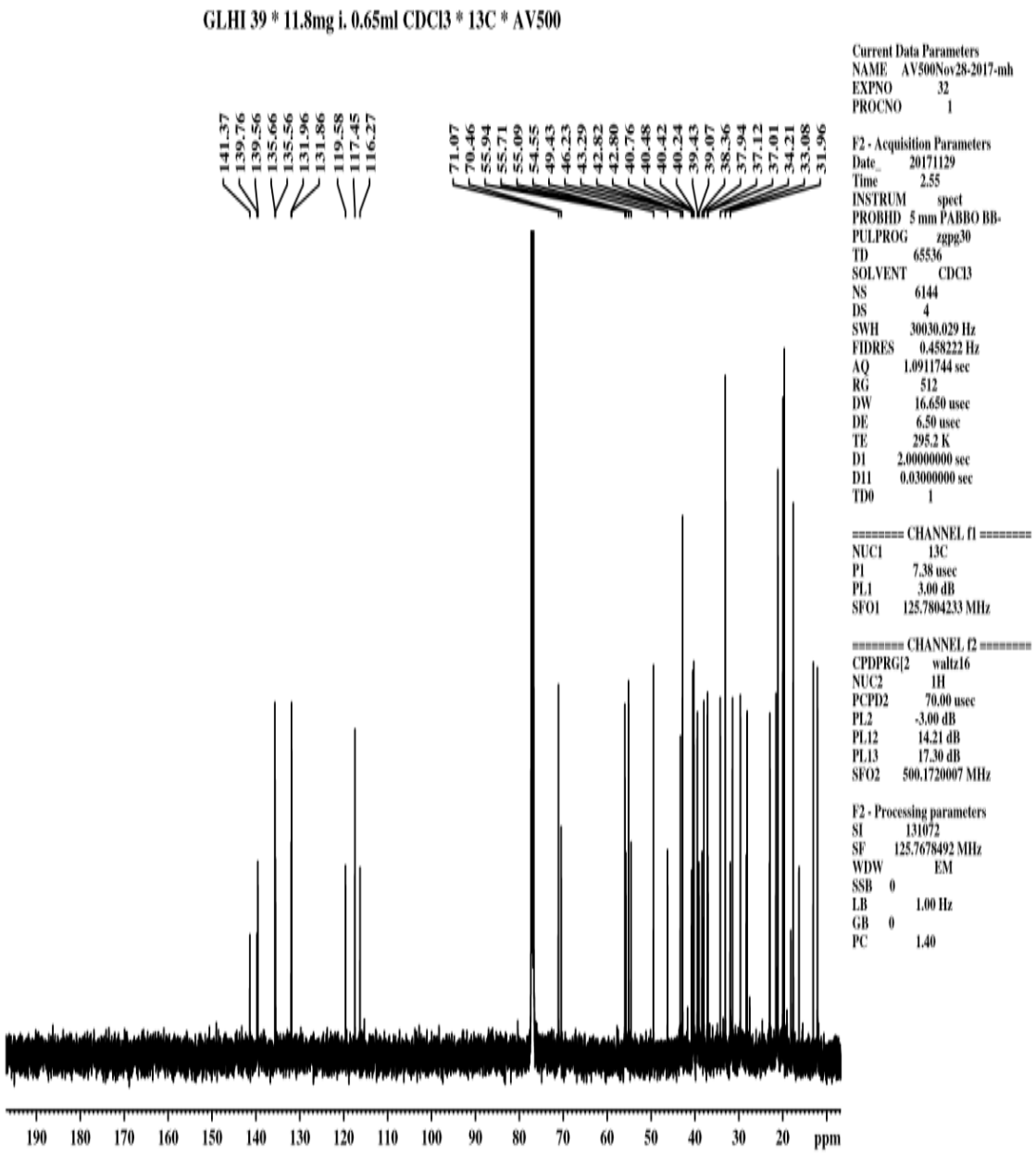

Figure 13: ^{13}C-NMR spectrum for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) (72)

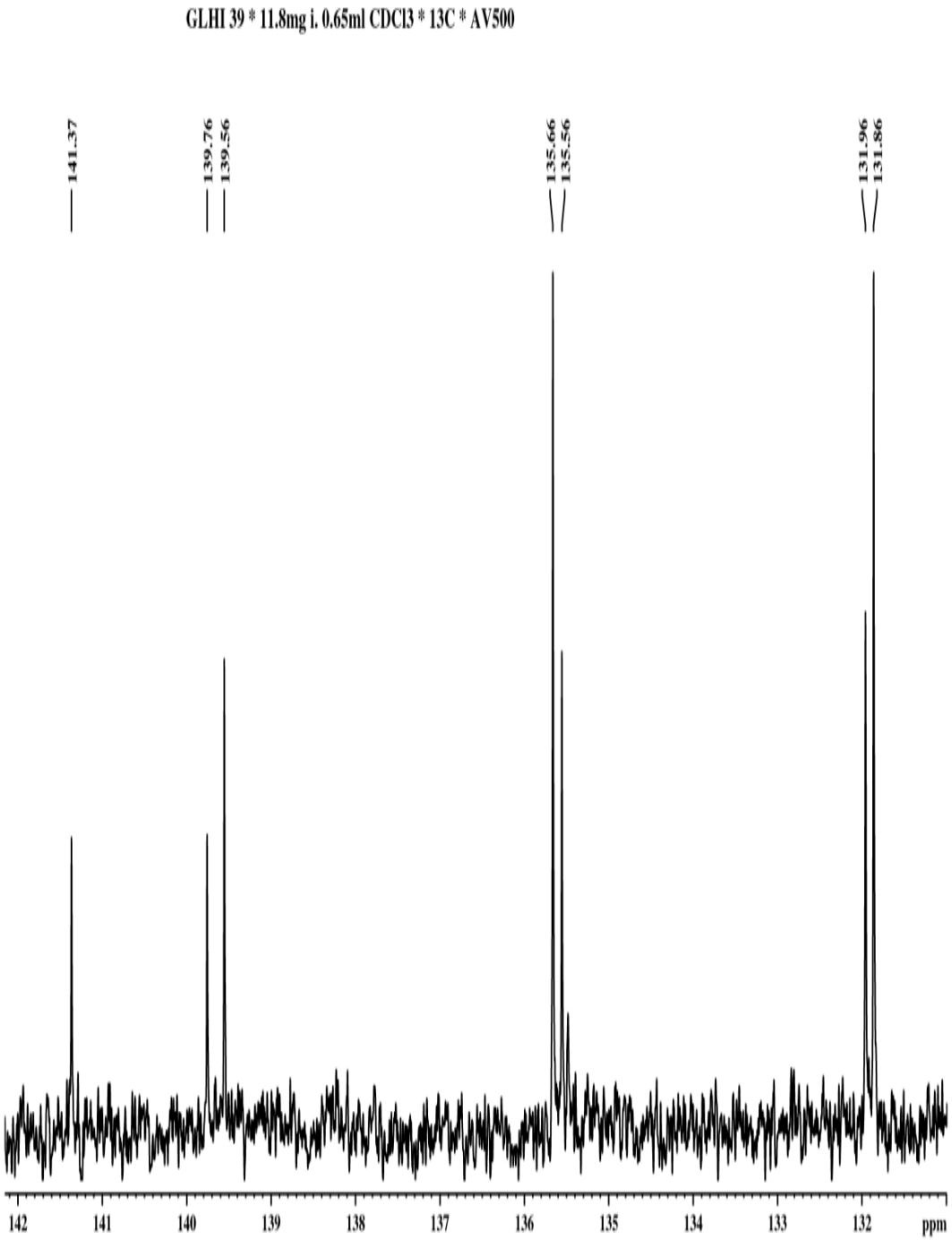

Figure 14: ^{13}C-NMR for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) (72)

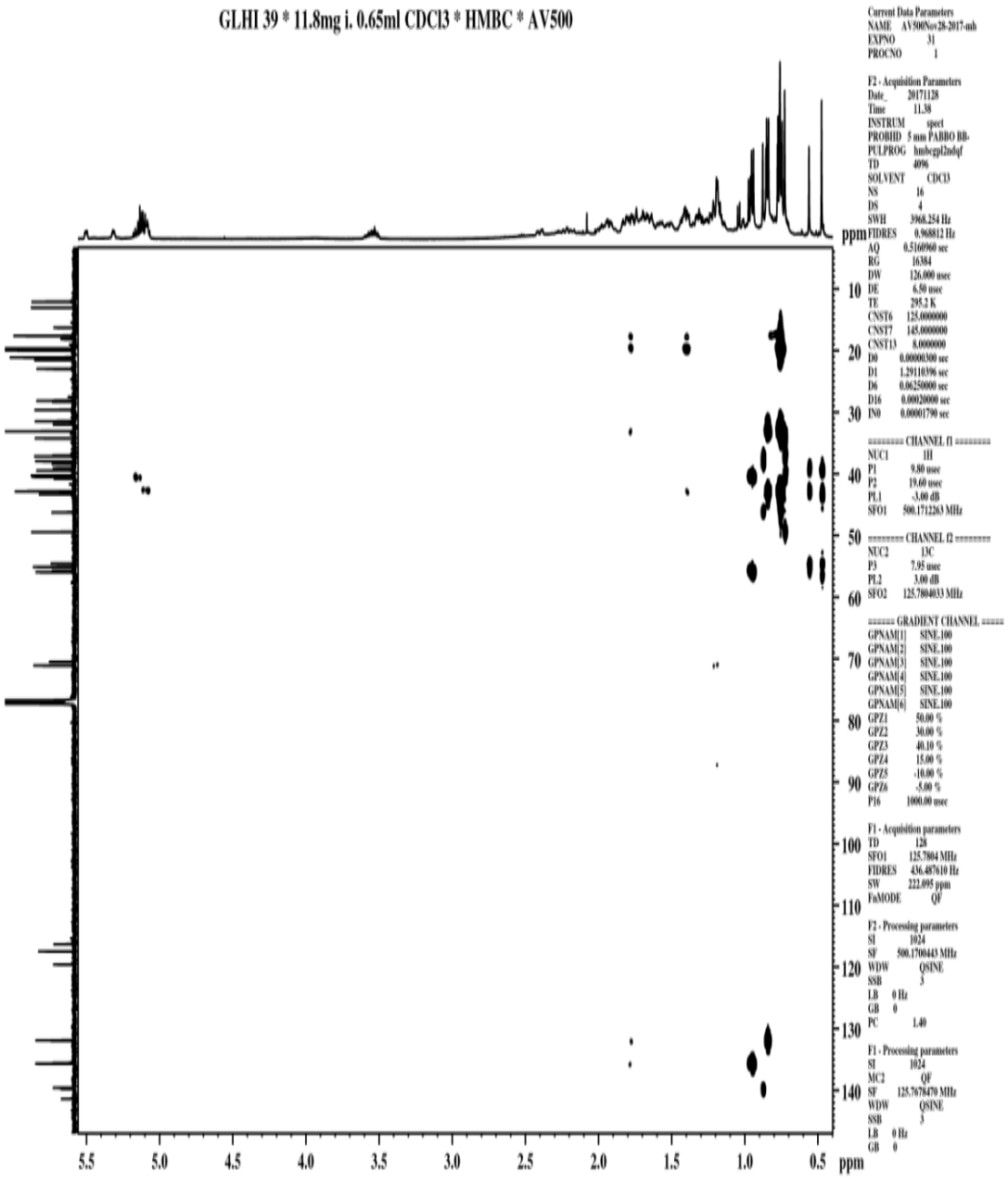

Figure 15: HMBC for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) (72)

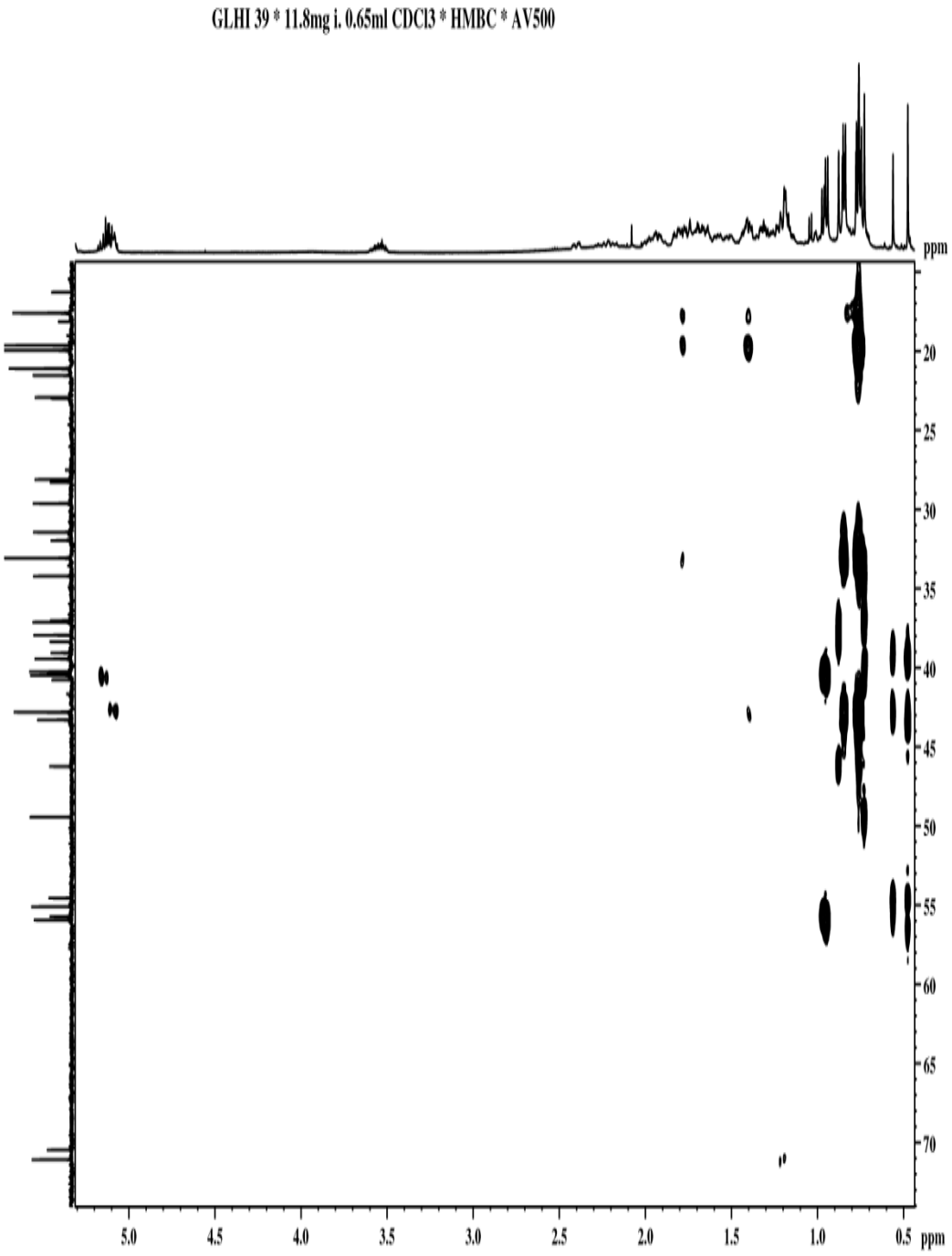

Figure 16: Extended HMBC for Ergosta-5,7,22-triene-3*β*,14*α*-diol (22*Z*) (72)

4.6 Biological Assays of Local *Ganoderma lucidum* and *Termitomyces letestui*

4.6.1 Biological Activities of Local *Termitomyces letestui*

The crude extracts (ethyl acetate and methanol), isolated compound ergosta-5,7,22-triene-3β-ol(22*Z*) **(69)** and controls were subjected to both antibacterial and antifungal activities at 10 μg/ml concentration. Both crude extracts (EtOAc and MeOH) and the isolated compound were inactive against the bacteria and fungi tested. In a separate study, ergosta-5,7,22-triene-3β-ol(22*E*) **(1)** (*trans*-ergosterol) isolated by (Wekesa, 2014) showed activity against *S. aureus* at 10 μg/ml with inhibition zone of 12.0 mm but was inactive against the gram negative *E. coli* bacteria. The differences in activity show that the two compounds were different from each other.

4.6.2 Biological Assays of *Ganoderma lucidum*

All crude extracts (*n*-hexane, ethyl acetate and methanol), the isolated compound, ergosta-5,7,22-triene-3β,14α-diol(22*Z*) **(72)** and controls were investigated for both antibacterial and antifungal activities (Table 7).

The results in Table 7 revealed that the extracts of *G. lucidum* were significantly active against MR*SA* (Methicillin-Resistance *Staphylococcus aureus* ($p<0.022$) (Appendix 3H), *Streptococcus pyogenes* ($p<0.05$) and *Creptococcus neoformans* ($p<0.05$) (Appendix 3I). No extract caused inhibition of *P. aeruginosa*, *E. coli* and *K. pneumoniae*. This shows that the concentrations of the pure compound were either low, the active compound may not have been there or the issue of antagony might have existed in the compounds. These results agree to those by (Nwachukwu *et al*., 2017).

The results showed that *n*-hexane and methanol extracts of *G. lucidum* exhibited significant inhibitions against MR*SA* ($p=0.022$) while ethylacetate against *Streptococcus pyogenes* ($p<0.05$). *Escherichia coli*, *Pseudomonas aeruginosa* and *Klebsiella*

pneumoniae did not respond to any of the extracts including the control used. Gram negative bacteria usually resist most conventional drugs. The bacteria have additional external membrane composed of phospholipids and polysaccharides which prevents the drugs from attacking those (Clayton and Mah, 2017). Non responsive effects of the micro-organisms to crude extracts (*n*-hexane, ethyl acetate and methanol), **77** and controls did not mean that they could not respond totally to the tests applied. Antagony could have been a factor that might have led to the negative effect. This means that the activity of one compound was interfered with another compound in the crude extracts. Concentrations of bioactive compounds in crude, isolates and controls could have been low. Another factor is that there might have been complete absence of compounds that could cause activity. Antimicrobial activities of other *Ganoderma lucidum* species have also been tested in the past (Ranjith *et al*., 2015). The data for the bioactivity of local *G. lucidum* is represented by the bar graph shown in figure 17 below.

Table 7: Antimicrobial activity of the crude extracts, compound 72 from *G. lucidum* and controls

Micro-org.	Amp/ Nys	Hex. Extract	EtOAc Extract	MeOH Extract	Comp. **(72)**	*P*-value
E. coli	NI	NI	NI	NI	NI	
P. aeruginosa	NI	NI	NI	NI	NI	
K. pneumoniae	NI	NI	NI	NI	NI	
MRSA	31±0.3	13.3±0.3	10.3±0.3	10.3±0.3	10.3±0.3	*P*=0.022
S. pyogenes	40.3±0.3	10.3±0.3	11.7±0.3	NI	10.3±0.3	*P*<0.05
C. neoformans	30.7±0.3	NI	NI	NI	NI	*P*<0.05

Table represents means ± SD at N=3 (three replicates) different in a row means they are significantly different at p<0.05.Key: Amp: Ampicillin, Nys: Nystatin, Hex.-Hexane extract, EtOAc: Ethyl acetate extract, MeOH: Methanol extract; NI: No Inhibition

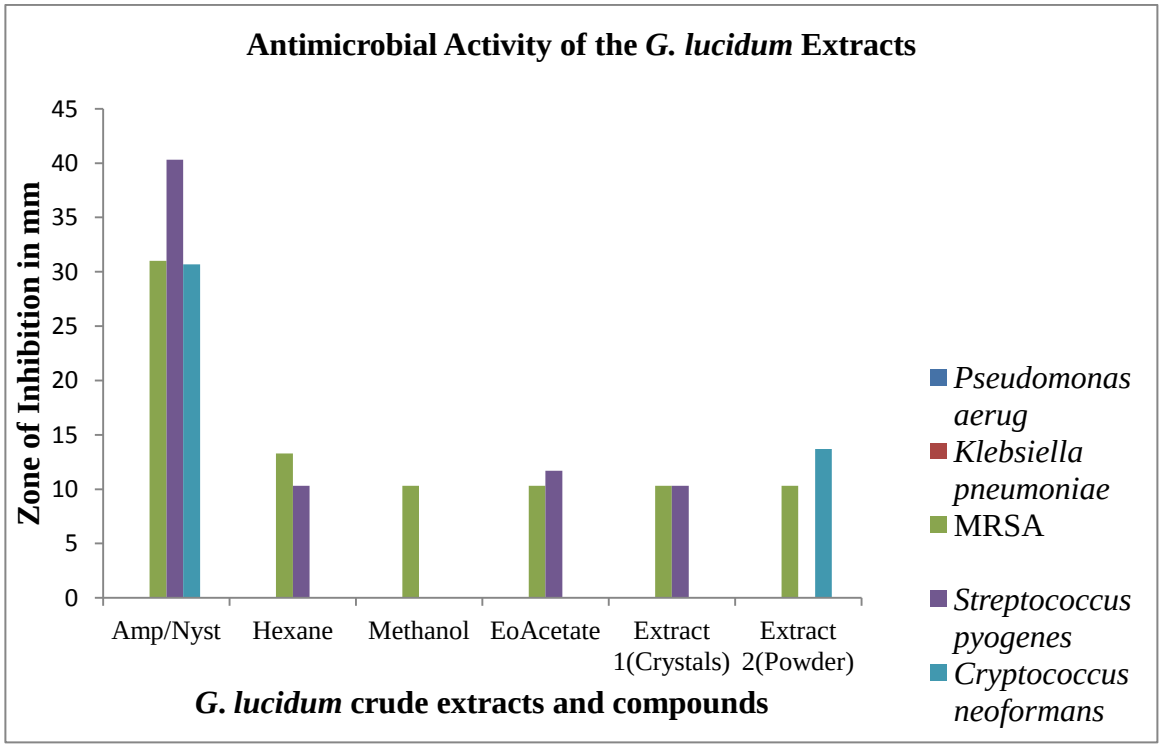

Figure 17: Bar graph showing antimicrobial activities between controls crude extracts and isolated compounds from *G. lucidum*

CHAPTER FIVE

CONCLUSION AND RECOMMENDATION

5.1 CONCLUSION

The following phytochemicals were present in both ethylacetate and methanol extracts of *Termitomyces letestui*; steroids, triterpenoids, saponins, alkaloids, carbohydrates, phenolics, flavonoids and polyoses. Glycosides were absent in both extracts of *Termitomyces letestui*. In *Ganoderma lucidum*, *n*- hexane, ethyl acetate and methanol extracts contained steroids, triterpenoids, reducing sugar, glycosides and flavonoids. Saponins and alkaloids were absent in all the three extracts. Ethylacetate and methanol extracts had phenolics while they were absent in *n*- hexane extract.

Isolation of compounds from *Termitomyces letestui* and *Ganoderma lucidum* was achieved. Ergosta-5,7,22-triene-3β-ol-(22*Z*) was isolated from *Termitomyces letestui* while ergosta-5,7,22-triene-3β,14α-diol-(22*Z*) was obtained from *Ganoderma lucidum*. Bio-inactivity was observed in *T. letestui* but not in *G. lucidum*. The bio-assay tests showed that ergosta-5,7,22-triene-3β,14α-diol (22*Z*), hexane and ethylacetate extracts from *Ganoderma lucidum* were active against MRSA and *Creptococcus pyogenes*. Crude extracts (EtOAc and MeOH) and the isolated compound, ergosta-5,7,22-triene-3β-diol (22*Z*), from *T. letestui* did not show any activity against the microbes tested.

5.2 RECOMMEDATIONS

Both crude extracts of *Termitomyces letestui* and *Ganoderma lucidum* contained many oily components rendering separation difficult. More advanced research techniques should be applied so as to separate the oily materials. These oils could be useful in the problem of micro-organisms resistance to drugs. Bioactivity of crude extracts and their

isolated compounds against some other pathogens should be carried out. More research should be done to determine better antimicrobial compounds to develop better drugs.

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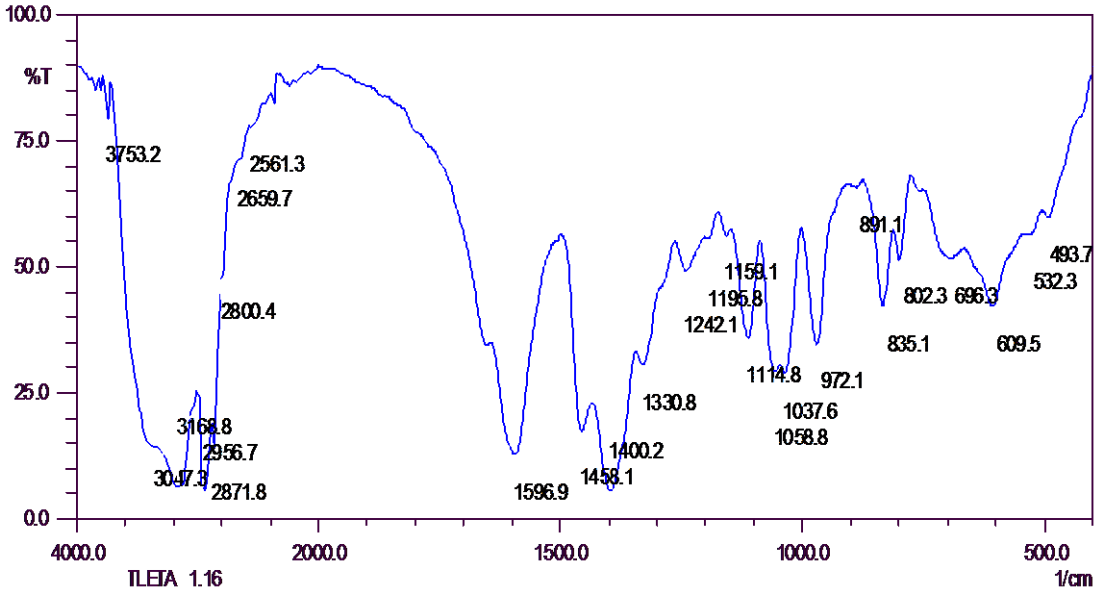
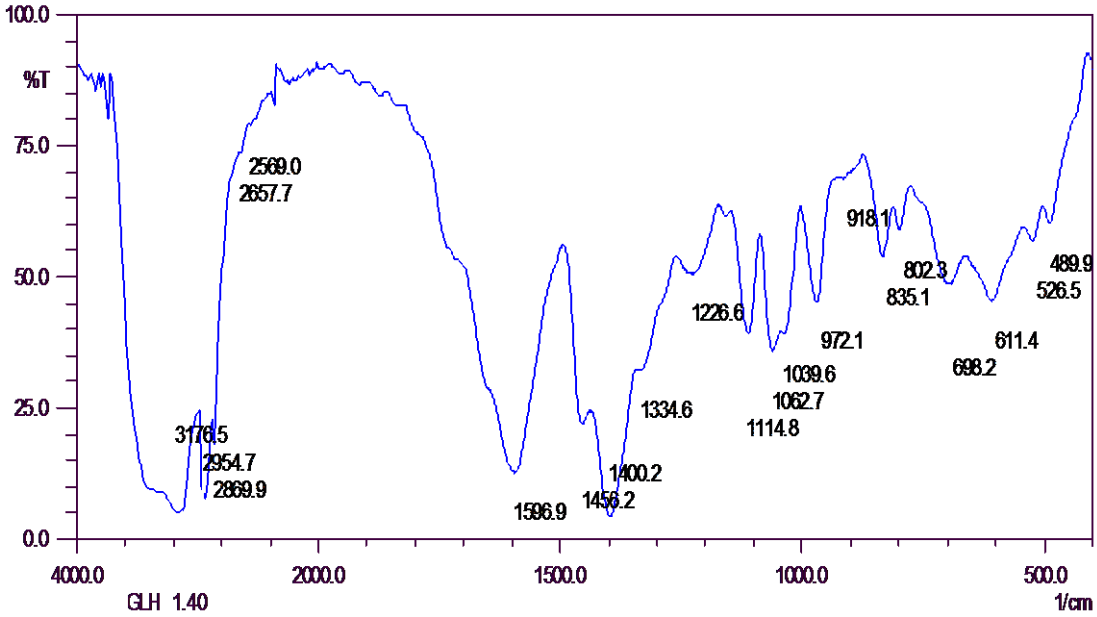
APPENDICES

APPENDIX 1: Infra-red spectra for Compounds 69 and 72



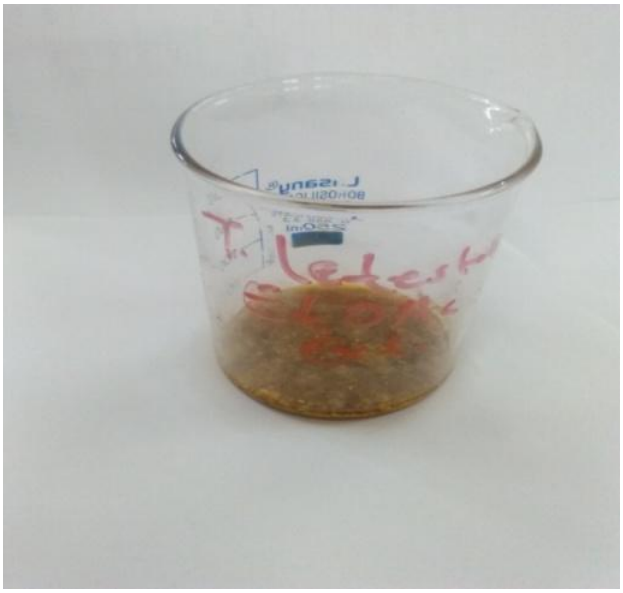
APPENDIX 1A: Infra-red spectrum of ergosta-5,7,22-triene-3β-ol (22*Z*) **(69)**



APPENDIX 1B: Infra-red spectrum of ergosta-5,7,22-triene-3β,14α-diol (22*Z*) **(72)**

APPENDIX 2: Pictures of *T. letestui* extracts, Isolates and their Anti-microbial activities

APPENDIX 2A: *Termitomyces letestui* Kenyan species

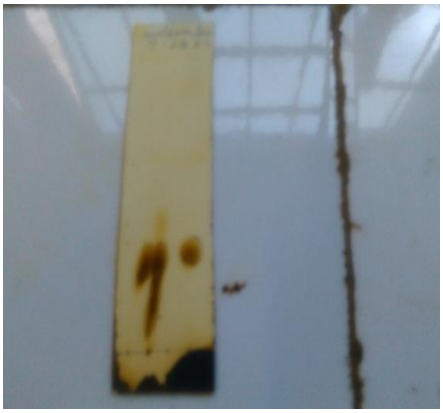


APPENDIX 2B: *T. letestui* ethylacetate extract

APPENDIX 2C: *T. letestui* methanol extract

APPENDIX 2D: TLC for EtOAc extract of *T. letestui*

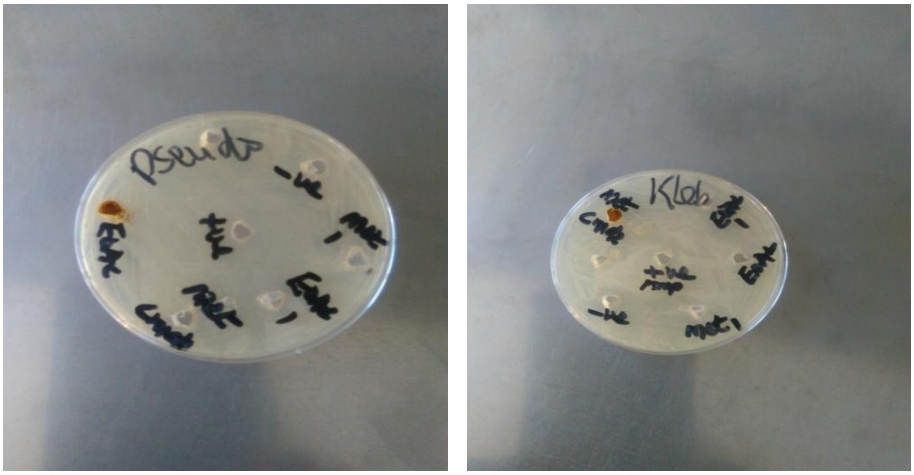
APPENDIX 2E: TLC for methanol extract of *T. letestui*



APPENDIX 2F: A single spot on TLC plates shows ergosta-5,7,22-triene-3*β*-ol (22Z) isolated from EtOAc of *T. letestui*

APPENDIX 2G: White needle-like crystal compounds (ergosta-5,7,22-triene-3β-ol (22Z) from Ethylacetate extract of Kenyan *Termitomyces letestui*

APPENDIX 2H: Anti-microbial activity of crude and pure compounds from *T. letestui*



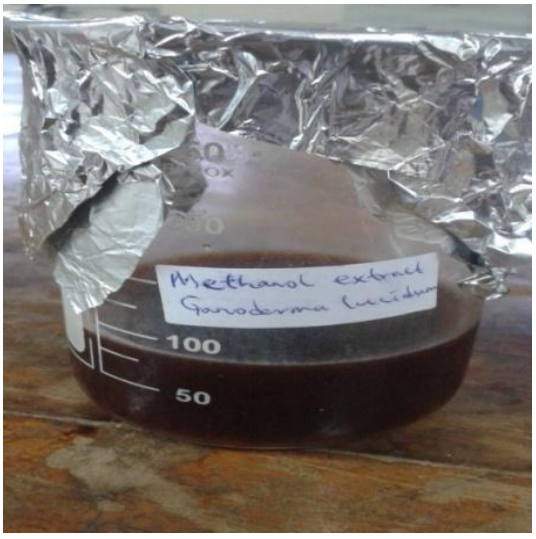
APPENDIX 2I: Bio-assays of *T.letestui*'s extracts and pure compounds against *P.aeruginosa* and *K. pneumoniae*

APPENDIX 3: Pictures of *G. lucidum* extracts, Isolates and their Anti-microbial activities

APPENDIX 3A: Kenyan *G. lucidum* when grinded

APPENDIX 3B: *G. lucidum* hexane extract

APPENDIX 3C: *G. lucidum* ethylacetate extract



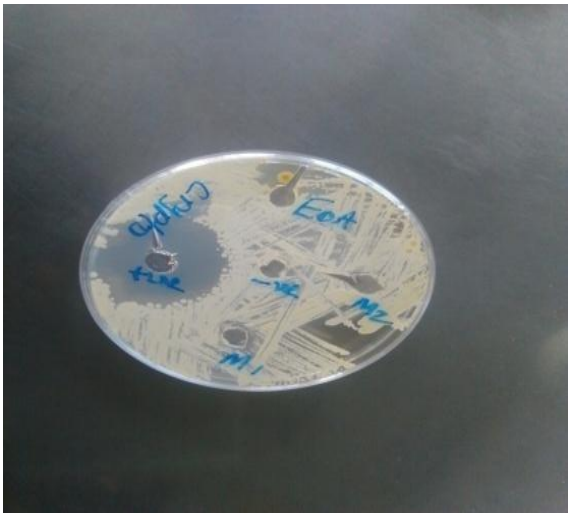
APPENDIX 3D: *G. lucidum* methanol extract

APPENDIX 3E: TLC plates for hexane extract

APPENDIX 3F: TLC plates for ethylacetate extract

APPENDIX 3G: TLC for ergosta-5,7,22-triene-3*β,* 14*α*- diol (22*Z*) (R_f = 0.61)

APPENDIX 3H: Anti-microbial activity of crude, pure compound and controls*(MRSA)*



APPENDIX 3I: Anti-microbial activity of crude, pure compound and controls *(C.neoformans)*