UNIVERSITY *of the* WESTERN CAPE

SECONDARY METABOLITES OF THE *UROMYCLADIUM TEPPERIANUM MacAlpine* EPIPHYTIC FUNGUS.

UNIVERSITY *of the*
WESTERN CAPE

MASTERS THESIS 2021

FACULTY OF NATURAL SCIENCE

CHEMISTRY DEPARTMENT

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Abstract

Research on natural products and medicinal plants has been conducted more with each passing year due to the great interest in isolating bioactive compounds and secondary metabolites from natural products such as plants, fungi, and many other naturally occurring products. To our knowledge, the *Uromycladium tepperianum MacAlpine* fungus has not been studied in depth before thus, its organic characterization was unknown, but related species have been studied, and these have shown compounds that may be used as medicine and as health benefits. Crude and macerated extracts of the *Uromycladium tepperianum* fungus have been fractionized using various chromatographic techniques such as solvent-solvent extraction followed by dry column chromatography to achieve the required separations. Thirty-nine compounds have been isolated from the hexane(*n*-hex) extract, Ethylacetate (EtOAc) extract, and n-butanol (BuOH) extract, and they belong to the following groups, steroids, terpenoids, amino-acids, flavonoids, alkaloids, phenolics, fatty-acids, flavones, and others. Nuclear magnetic resonance (NMR) characterization of the compounds was carried based on proton NMR (^{1}H-NMR), carbon NMR (^{13}C-NMR), distortion enhancement by polarization transfer NMR (DEPT-NMR), Heteronuclear single quantum coherence spectroscopy NMR (HSQC-NMR), Heteronuclear multiple bond correlation NMR (HMBC-NMR) and Homonuclear correlation Spectroscopy NMR (COSY-NMR). All four compounds found in the hexane extract have been isolated previously from other known plants such as spinach, argan oil, cactus pear seed oil, and others, but to our knowledge, the four compounds isolated from the hexane extract were found in the *Uromycladium tepperianum* fungus for the first time. The compounds found in the other extracts, were carried out by Liquid chromatography-mass spectroscopy (LC-MS) in order to analyze compounds that where isolated, but their yields were to low for NMR detection. The compounds were then matched with databases and programs: Global natural products social molecular network, FoodDB, STOFF (environment), KNApSAcK (Natural product), PubChem (biomolecules), PlantCyc (plant), ChBI (Biomolecules), NANPDB (Natural product), UNPD (Natural Product), Elemential composition, Pubchem, Chemspider Science direct, Synapt G2 qTOF, MZmine, Chem Calc, and Science finders Scholar.

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Declaration

I, Kelly Luana Viegas Correia, hereby declare that this dissertation entitled **secondary metabolites from the epiphytic fungus Uromycladium *tepperianum MacAlpine*** is my original and of my knowledge. This research has not been submitted anywhere else for the award of a degree at any other institution or University. Other literature sources have been quoted and referenced, but the words have been re-written and rephrased.

Date 07/11/2021 Signed Kelly Correia

Acknowledgments

I would like to express my sincere gratitude to my supervisor and Head of the Department (HOD) of the department of chemistry, Prof. Wilfred T. Mabusela, who gave me the opportunity and encouragement to continue with my work in the field of organic chemistry and for allowing me to be part of his team.

I would like to sincerely thank my parents for always guiding me, believing in me, loving and supporting me in all I have wanted to accomplish, and financing my studies.

I would like to thank the Department of Chemistry for the opportunity they have provided for me to be part of the Master's program.

A special thanks to my fellow post-graduate students Masande Yalo, Masixole, Sazi, Franck, and Ndikho for always being there when I needed guidance, patience, and endless support, and for the belief they have in me as an organic chemist.

Lastly, I would like to thank my family and friends for their support, love, and guidance.

Table of Contents

Abstract ..... ***I***

Declaration ..... ***II***

Acknowledgments ..... ***III***

List of abbreviations ..... ***VI***

List of Figures ..... ***VII***

List of Spectra ..... ***VIII***

List of Tables ..... ***IX***

Scheme table ..... ***X***

List of Isolated compounds ..... ***XI***

Chapter 1 ..... ***1***

1.1 Introduction: Secondary Metabolites From the Epiphytic fungus Uromycladium Tepperianum **1**

1.2 Problem statement **3**

1.2.1 Hypothesis **3**

1.3 Aims and Objectives **4**

Chapter 2 ..... ***5***

Literature review **5**

2.1 Introduction to fungi 5

2.1.1 Natural Products and potential drug development 14

2.1.2 Mushrooms 22

2.1.3 Mushrooms and health benefits 23

2.1.4 Kinds of mushrooms 24

2.1.5 Mushrooms in cancer research 26

2.1.6 Rust fungi 30

2.1.7 Application of LC-MS and MS in plant extract analysis. 31

Chapter 3 ..... ***32***

Materials and methods **32**

3.1 Reagents and General methods 32

3.2 Spectroscopy 32

3.3 Chromatography 32

3.4 Mass spectrometry (MS) 33

3.5 *Uromycladium tepperianum* MacAlpine preparation 33

3.6 Isolation and purification of compounds 36

Chapter 4 ..... ***40***

4.1 Results and Discussion **40**

..... 41

4.1.1 Reagent tests and results 41

4.1.1 Structure elucidation of Spinasterol (compound 1) and Schottenol (compound 2) found in the

hexane extract 42
4.1.2 Structure elucidation of Palmitic Acid (compound 3) and linoleic Acid (compound 4) found in the hexane extract. 44
4.1.3 Compounds found in EtOAc and BuOH extracts 46
4.1.4 Literature review on compounds obtained through LC-MS 48
Chapter 5 ..... ***55***
5.1 Conclusion **55**
Appendix ***57***
References ***66***

List of abbreviations

EtOAc	Ethyl Acetate
DCM	Dichloromethane
BuOH	Butanol
GC-MS	Gas Chromatography – Mas Spectroscopy
MeOH	Methanol
NMR	Nuclear Magnetic Resonance
^{1}H-NMR	Proton Nuclear Magnetic Resonance
^{13}C-NMR	Carbon-13 Nuclear Magnetic Resonance
$CDCl_3$	Deuterated Chloroform
COSY	Correlation Spectroscopy
HMBC	Heteronuclear Multiple Bond Correlation
HSQC	Heteronuclear Single Quantum Coherence
TLC	Thin Layer Chromatography
IR	Infra-red spectroscopy
Hex 5	Hexane extract fractions 16-26 (mix linoleic acid and palmitic acid)
Flakes	Hexane extract fractions 35-55 (mix Spinasterol and schottenol)
STD	Standard solution
n-Hex	Hexane
LC-MS	Liquid chromatography- Mass spectroscopy

List of Figures

Figure 1: Uromycladium tepperianum MacAlpine fungus and material in powder form 33
Figure 2: solvent-solvent extraction, layer separation 35
Figure 3: Difference in chemical composition between the 3 samples 41
Figure 4:Presence of carbohydrates 41

List of Spectra

spectra 1:Spectra C13 compound 1 and 2. 57
spectra 2:Spectra DEPT compound 1 and 2 57
spectra 3:Spectra Proton compound 1 and 2. 57
spectra 4:Spectra HMBC compound 1 and 2. 58
spectra 5:Spectra Cosy compound 1 and 2. 58
spectra 6:GC-MS Spectra visualization of both compound 1 and 2. (mixture) 59
spectra 7:GC-MS Spectra molar mass of Spinasterol compound 1 59
spectra 8:GC-MS Spectra molar mass of Schottenol compound 2. 59
spectra 9:Spectra C13 compounds 3 and 4 60
spectra 10:Spectra DEPT compounds 3 and 4 60
spectra 11:Spectra Proton compounds 3 and 4 60
spectra 12:Spectra HMBC compounds 3 and 4 61
spectra 13:Spectra COSY compounds 3 and 4 61
spectra 14:GC-MS Spectra visualization of compounds 3 and 4 mixture. 62
spectra 15: GC-MS Spectra molar mass of linoleic Acid compound 4. 62
spectra 16:GC-MS Spectra molar mass of Palmitic acid compound 3. 62
spectra 17: compound 37 peaks 63
spectra 18: compound 38 peak 64
spectra 19: compound 39 spectra. 65

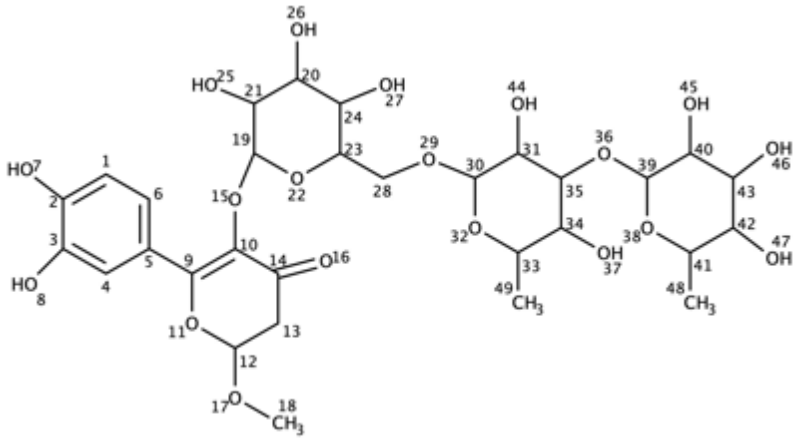
List of Tables

Table 1:NMR interpretation of compounds 1 and 2.....55
Table 2:NMR interpretation of compounds 3 and 4.....57
Table 3: EtOAc and BuOH fractions sent to LC-MS.....58
Table 5: LC-MS on EtOAc and BuOH extract compounds 47
Table 6: Global Natural products social molecular network compound finding 60

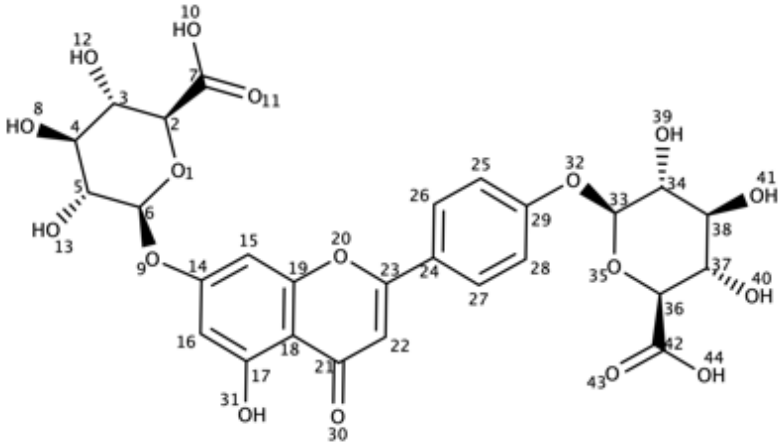
Scheme table

Scheme 1:5 steps to Hernolactone 7
Scheme 2: Syringaldehyde to Trimethoprim 8
Scheme 3: from 3,4,5-trimethoxybenzaldehyde to trimethoprim both methods 8
Scheme 4: Isolation of natural products from Uromycladium tepperianum MacAlpine Fungus 38

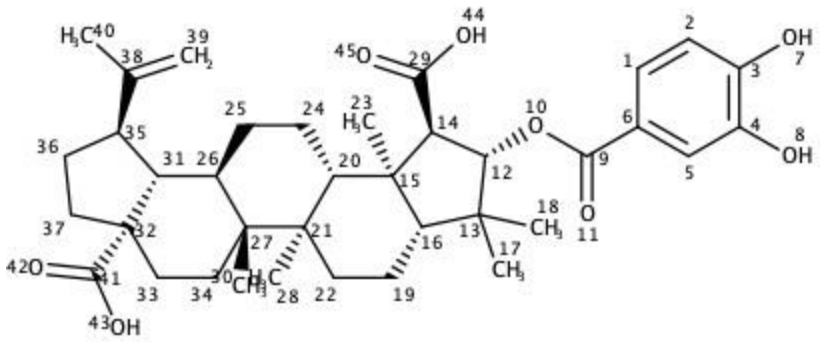
List of Isolated compounds



compound 37: 7-O-Methylquercetin-3-O-galactoside-6''-rhamanoside



compound 38: 4'-O-GlcA-7-O-GlcA Apigenin



compound 39: 3-O-protocatechuoyl ceanothic acid

Chapter 1

1.1 Introduction: Secondary Metabolites From the Epiphytic fungus Uromycladium Tepperianum

Uromycladium Tepperianum MacAlpine is a rust fungus that produces large and irregular sized galls on the leaves and stems of the plant (Wood, 2012). It is native to Australasia and has affected over 100 species of Acacia related genera, including Paraserianthes in Australia, South-East Asia, the South Pacific and New Zealand (Doungsa-ard *et al.*, 2015) The rust fungus was introduced in South Africa purposefully to control the spread of *Acacia saligna* bushes (Besaans, 2015). There is no literature on the natural product composition of this fungus. *U. Tepperianum* MacAlpine fungus consists of single-celled teliospores produced on pedicels where the ridged teliospores are produced on the surface of the galls as a brown powder and are approximately 0.02mm in diameter (Wood, no date). The wind is responsible for the spreading of the Teliospores (Newcombe, 2006). The brown powder consisting of teliospores can easily be brushed off when fresh and then infect the tree, penetrating the epidermal cells of the young phyllodes (leaves) as well as stems and flower buds (Ratio, 2017). The fungus then surrounds the plant tissue and induces the formation of the galls (Wood, 2012). The galls are quickly germinated during spring, which is the season where plants and flowers grow and reproduce the most (Aime *et al.*, 2017). The teliospores are formed between $10°C$ to $20°C$ when there is free water available on the plant's surface, which can be due to a light rain or overnight dew especially during the spring season (Wood en Morris, 2007). Since the fungus is spread rapidly by wind there is no need for artificial redistribution of the fungus (Wood, no date). New galls often develop in February to March since the fungus generally infects the plant the previous rainy season, thus new galls can be produced any time from then until late spring (Wood, 2012).

The fungus utilizes the plant's nutrients (Wood, 2012) to nourish itself, thus causing limitation of growth and seed production of that specific plant, due to the heavy galls on the plants leaves and stems (Davis en Agrios, 1970). The heavy gall makes the plant unable to cope with drought on drier months and other environmental stresses leading to the death of the plant. Once the plant has died, the fungus also dies since it lives inside the plant as a parasite (Wood, no date). Intense fires and clearing operations may lead to the extinction of the rust fungus, but later on

naturally reinvade the area, thus minimization and avoiding the clearance and burning of the areas affected would help to keep the fungus alive (Wood, 2012)

1.2 Problem statement

Among a wide range of plants, a few are classified as medicinal plants, which are widely used for the treatment of, or drug fabrication to combat or cure diseases. By utilizing modern biotechnical extraction processes, concentrated extracts of medicinal plants may be prepared for human use. In recent times, research towards the discovery of drugs and other treatments for currently incurable diseases and drug-resistant pathogens has intensified. However, the development of such new products often faces challenges which may include causation of side effects, or not being compatible with all physiological systems among others. Thus, exploring the natural product composition of the *Uromycladium Tepperianum* MacAlpine endophytic fungus, may offer opportunities for the lead discovery of new drugs as has been achieved with some fungal species. To our knowledge, no such research on the *Uromycladium tepperianum* MacAlpine endophytic fungus has been pursued to date. The host plant of the fungus, *Acacia saligna,* has been studied for its phytochemical composition, and has been found to contain compound classes such as terpenoids and phenolics. It may thus be expected that some of these compounds may be assimilated into the fungus, while the fungus may also be able to produce its own natural products for its survival.

1.2.1 Hypothesis

Some fungal species have been shown to produce secondary metabolites which possess diverse biological activities, and which have been exploited for health benefits among humankind. These chemical structures may be used in medicine as well as agricultural products, and thus exploring the natural product composition of *Uromycladium tepperianum* MacAlpine fungus, may open opportunities for the development of new medicines, drugs, or leads for new drugs. The research work is thus based on the hypothesis that *Uromycladium tepperianum* MacAlpine fungus contains secondary metabolites, some of which may display potential for development into therapeutic drugs.

1.3 Aims and Objectives

General aims and objectives

The objective of the investigation is to discover and understand the chemical composition of the *Uromycladium tepperianum* MacAlpine fungus due to the lack of background knowledge on the fungus. The investigation aims to conduct a systematic solvent extraction of the *Uromycladium tepperianum fungus* and determine its nature and composition. Preparation of both organic and aqueous solvent based maceration and crude extraction from the *Uromycladium tepperianum fungus;* isolation followed by structural elucidation of secondary metabolites.

Specific objectives

1. Use organic and aqueous solvents to perform maceration and crude extractions from the *Uromycladium tepperianum* MacAlpine fungus.
2. Carry out phytochemical screening of the *Uromycladium Tepperianum MacAlpine* extracts (Hexane [*n*-hex], Ethyl Acetate [EtOAc], Dichloromethane [DCM], Butanol [BuOH]) for various classes of compounds.
3. Carry out fractionization of organic extracts, followed by the isolation of pure compounds subsequently using various chromatographic technics.
4. Carry out compound characterization with a combination of various spectroscopic techniques, reagent tests and hydrolysis.

Chapter 2

Literature review

2.1 Introduction to fungi

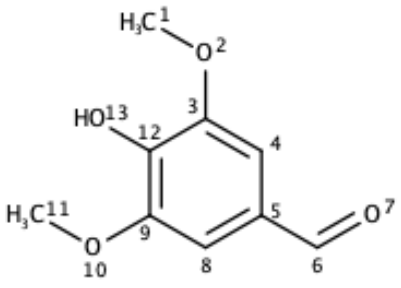
Fungi originally classified as plants, are now categorized as a separate kingdom, and considered to be among the most important organisms on earth. When combined with certain bacteria, fungi are responsible for the conversion of dead material into helpful material for soil (Mohmand *et al.*, 2011). Fungi are natural decomposers, parasites, or pathogens, were majority are beneficial partners in symbiosis with animals, plants, and algae (Reid en Webster, 1973).

The fungi kingdom consists of mushrooms, rusts, smuts, truffles, morels, moulds, and yeasts and each of these known fungi belongs to one of three groups: Eumycota, Oomycota, and Myxomycota (Gurnani *et al.*, 2014).

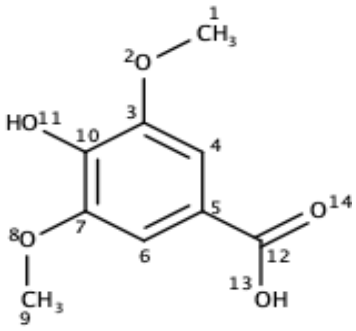
Rusts are diseases prone to grow on plants, consequently, acquiring nutrients from the plant itself and reproducing on its host (Tattar, 1989). Several species of rusts have evolved in a manner that they solely grow, infect, colonize and reproduce in specific plant species (Kolmer, Ordonez en Groth, 2009).

Smuts are cellular fungi characterized by their sizable amount of teliospores. Smuts represent the second-largest fungi cluster after rusts (Pandey, 2018).

Truffles are fruity bodies, part of the genus Tuber. These are widely used as food and medicine, wealthy in bioactive compounds such as terpenoids, flavonoids, polysaccharides, and others which supply its anti-inflammatory, anti-mutagenic, anti-cancer, and inhibition of antioxidant properties. Some of these compounds are (Reyna en Garcia-Barreda, 2014):



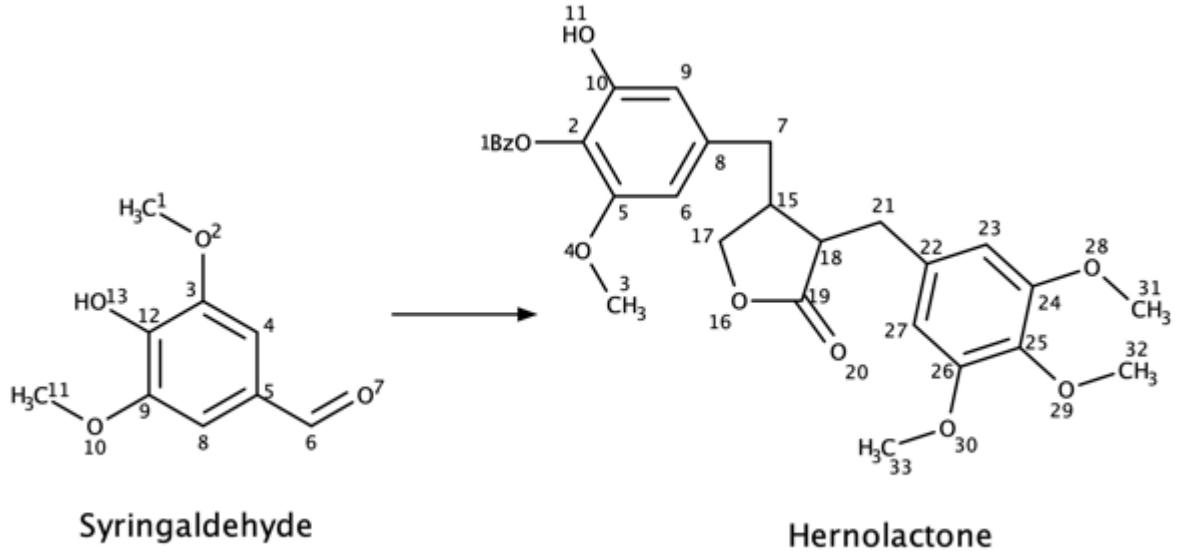
Syringaldehyde



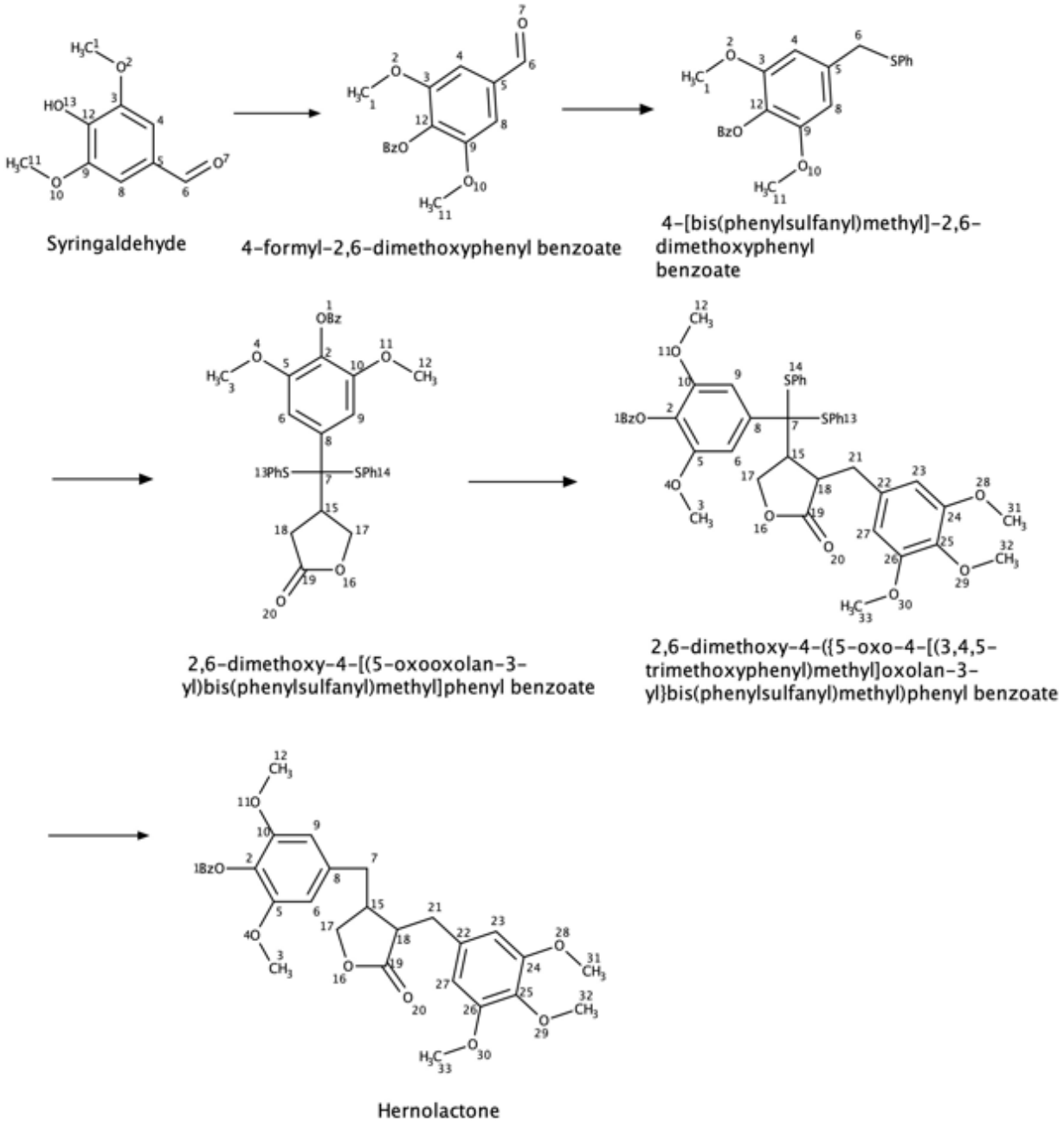
Syringic acid

Syringaldehyde, a valuable pharmaceutical starting material, is produced when lignin oxidation takes place in vanillin with molecular oxygen (Mathew *et al.*, 2018). Alcoholic hydrolysis and heating, degrade the lignin and generates aldehydes. Oxidation and the association with lignin and aldehydes produce phenolic acids, hence syringaldehyde is able to convert to syringic acid and other compounds (Aging, 2012).

Hernolactone a lignan which inhibits the activity of cyclooxygenase-2, reducing inflammation in the human body, may also be naturally isolated form the seeds of *H. Ovigera*, or is chemically synthesized with syringaldehyde as its direct precursor (Vi *et al.*, 1987).

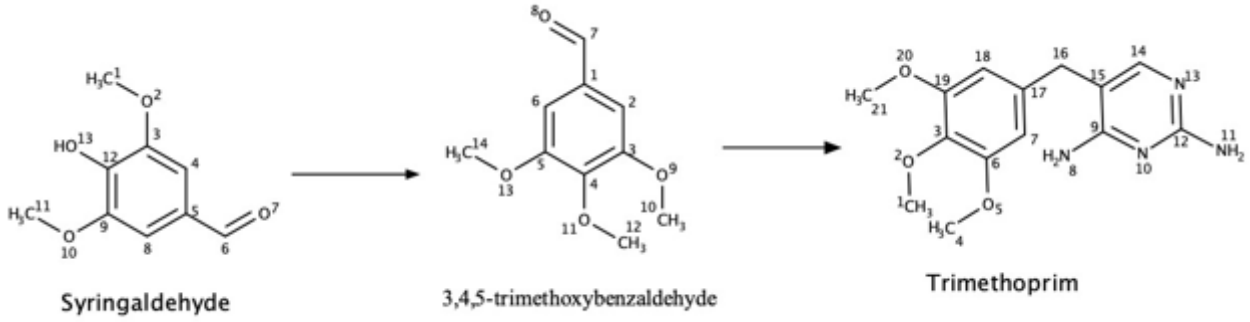


To produce hernolactone, syringaldehyde reacts with benzyl chloride under the presence of anhydrous potassium carbonate submerged in dimethylformamide, to form benzyl syringaldehyde (Chen, 2000), followed by the condensation with butanolide which occurs by Michael addition in *n*-butyllithium which followed thereafter the benzyl syringaldehyde reaction with thiophenol, converting it to its corresponding phenyldithioacetal in the presence of boron trifluoride-etherate, and finally hernolactone is obtained(Arimoto, Yamaguchi en Nishibe, 1995).



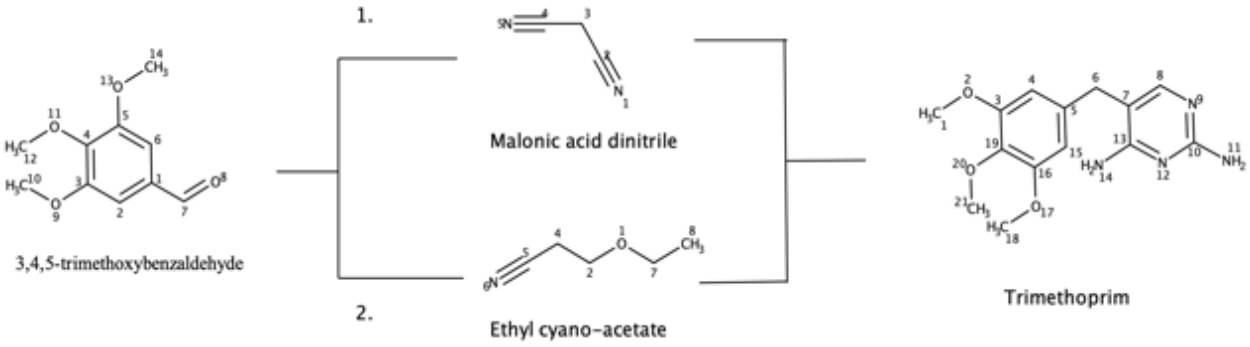
Scheme 1:5 steps to Hernolactone

Additionally, syringaldehyde is the main precursor of 3,4,5-trimethoxybenzaldehyde (Mathew *et al.*, 2018). By utilizing a copper [1]- catalyzed exchange of bromine by methoxide in DMF and separating the syringaldehyde from 3,5-dibromo-4-hydroxybenzaldehyde and 5-bromovanillin, followed by stirring the mixture containing syringaldehyde, acetone treated with dimethylsulfate, disodium carbonate and potassium hydroxide in water, 3,4,5-trimethoxybenzaldehyde is then synthesized (Kolb, 1993). 3,4,5-trimethoxybenzaldehyde is an intermediate in the synthesis of pharmaceutical drugs such as, Cintriamide, Bactrim (Biseptol), Trimethoquinol, trimethoprim and others (Sriprasanthi, 2012).



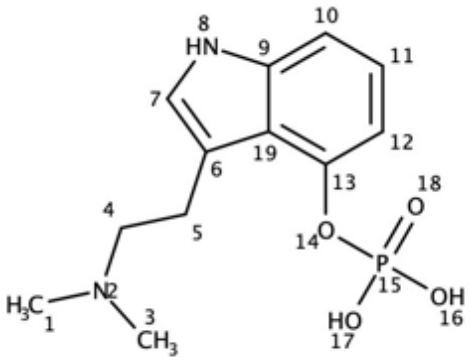
Scheme 2: Syringaldehyde to Trimethoprim

Trimethoprim synthesis with 3,4,5-trimethoxybenzaldehyde as a main precursor occurs by condensation of 3,4,5-trimethoxybenzaldehyde with malonic acid dinitrile in a Knoevenagel reaction, or Knoevenagel condensation of 3,4,5-trimethoxybenzaldehyde with ethyl cyanoacetate reaction (R.S.Vardanyan en V.J.Hruby, 2006).



Scheme 3: from 3,4,5-trimethoxybenzaldehyde to trimethoprim both methods

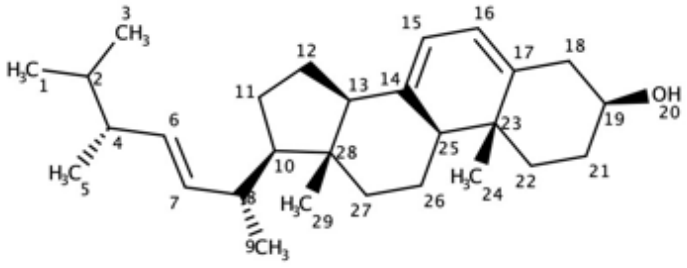
Mushrooms are fleshy spore-bearing fungi and are subdivided into, edible and non-edible mushrooms. Some mushrooms contain hallucinogenic properties such as the *Psilocybe semilanceata* mushroom that contain the compound Psilocybin which is responsible for the hallucinogenic properties within the mushroom (Wieczorek *et al.*, 2015):



Psilocybin

Psilocybin is an alkaloid psychedelic know to treat Alzheimers disease, dementia, and depression by micro-dosing which is mediated by the limbic system (Vann Jones en O'Kelly, 2020).

Morels possess bioactive compounds that permits it to possess antimicrobial, anti-fungal anti-oxidative, anti-inflammatory, anti-tumor, and immunostimulatory properties (Bulam, 2019). Ergosterol is a neutral lipid and the main component of the fungal membranes that treates severe human fungal infections (Alcazar-Fuoli en Mellado, 2012).

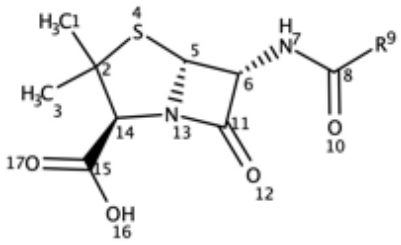


Ergosterol

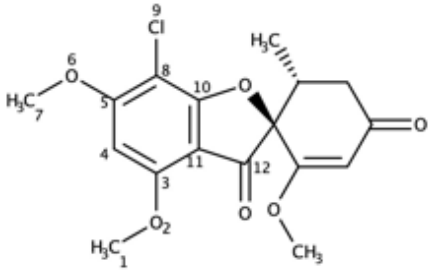
The pathway for the biosynthesis of ergosterol requires 20 enzymes and the synthesis of squalene from mevalonate (Alcazar-Fuoli en Mellado, 2012). Squalene is a cholesterol and triglyceride level lowering compound which is currently being utilized in a variety of cancer therapies (Reddy en Couvreur, 2009).

Yeasts are single-celled fungi. Yeasts such as Saccharomyces, are used in bread baking and alcohol production due to its abundance in vitamin B (Joseph en Bachhawat, 2014). Other yeasts such as *Candida albicans* are often present in the human body without causing disease unlike malignant yeasts, such as cryptococcosis, which affect the lungs, meninges, and the coverings of the brain and spinal cord of the human body (Hernday *et al.*, 2010).

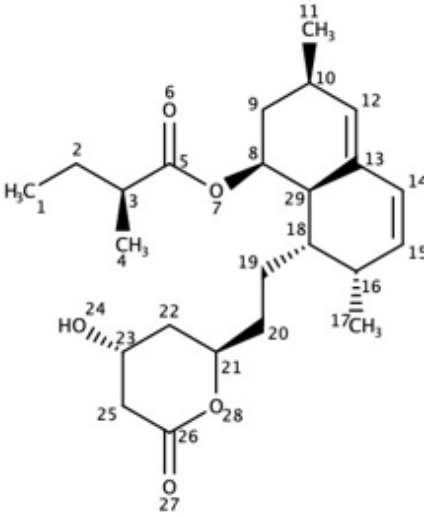
Moulds are long filamentous fungi commonly referred to as Hyphae .The *Aspergillus* and *Penicillium* fungi species are considered to be of human or animal disease importance (Houbraken, de Vries en Samson, 2014). Found as Saprophytes, both these fungi species are ubiquitous, unlike certain species viewed as immune-compromisers (Pitt, 1994). The *Aspergillaceae* fungal species produces secondary metabolites known to be pharmaceuticals, some of them being, penicillin which is an antibiotic found in *Penicillium rubens*, mycophenolic acid which is an immunosuppressant found in *Pencillium brevicompactum*, Griseofulvin which is an antifungal found in *Penicillium griseofulvum* and lovastatin known as the cholesterol lowering agent found in *Aspergillus terreus and* Streptomycin which is an antibiotic found in *actinobacterium Streptomyces griseus* (Holt, 1975) (Marinelli en Marcone, 2011) :



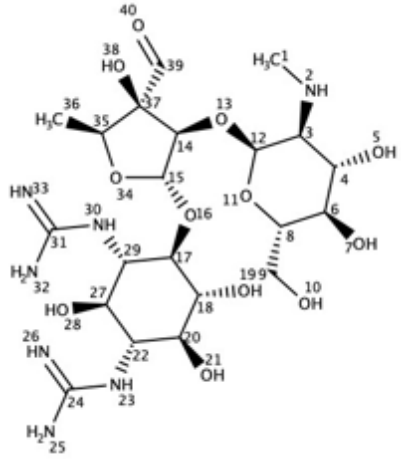
Penicillin



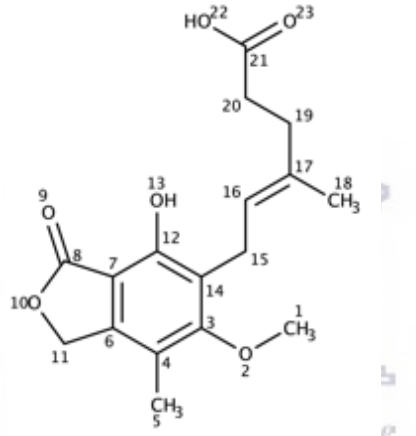
Griseofulvin



Lovastatin

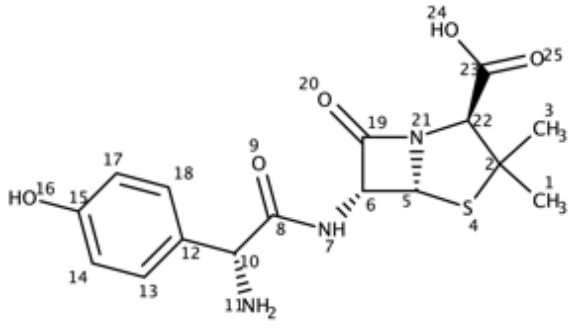


Streptomycin

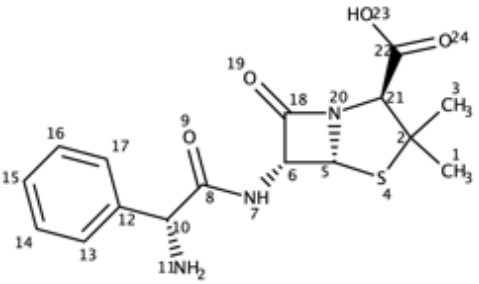


Mycophenolic acid

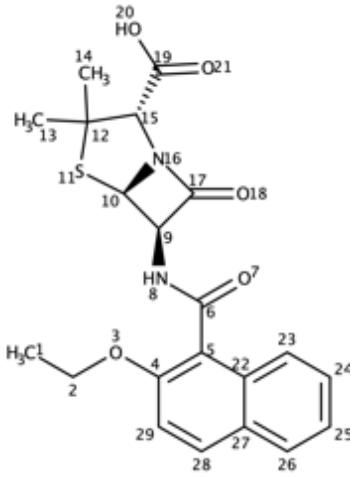
A number of medications are now derived or contain penicillin, some of them being,



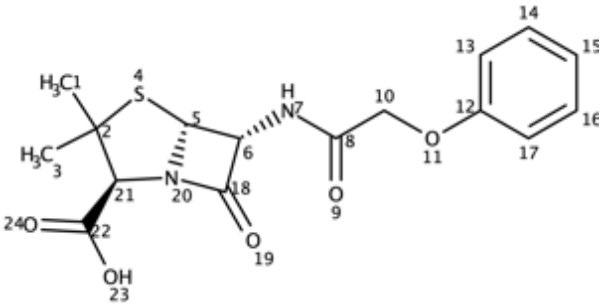
Amoxicillin,



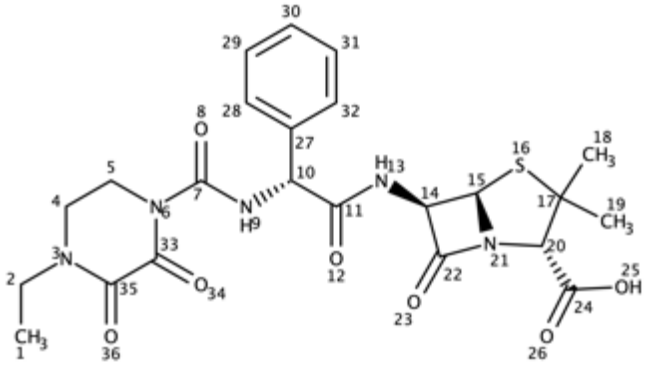
Ampicillin,



Nafcillin,

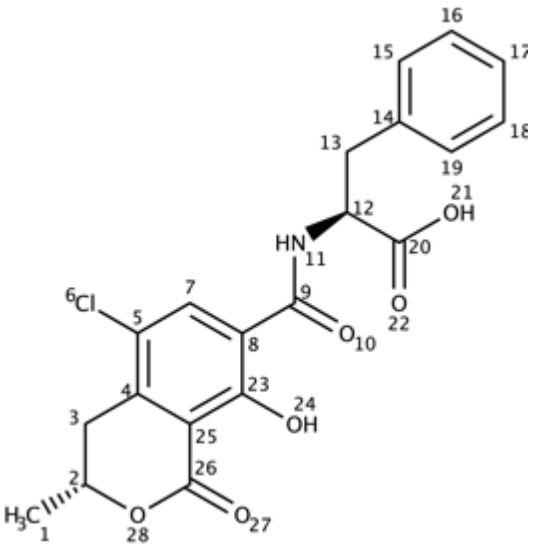


Penicillin V,

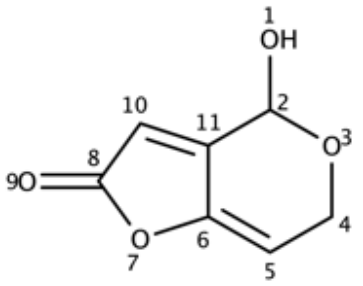


Piperacillin

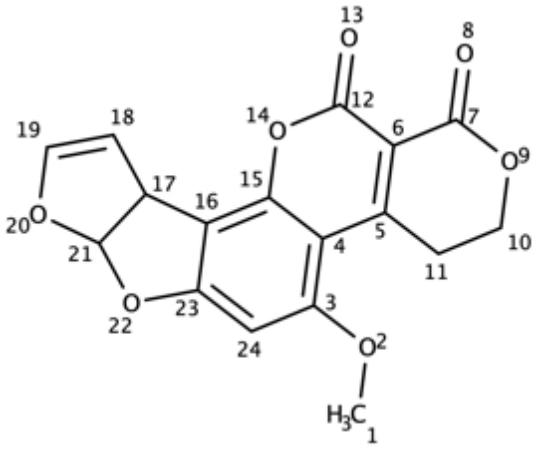
Moreover, not all secondary metabolites found in these fungi species are safe for human and animal consumption or used in medicinal uses (Frisvad, 2014). Mycotoxins are considered to be any toxic compounds, or secondary metabolites, produced by a fungus, mostly grown in food. Some of these mycotoxins found in the *Aspergillus* and *Penicillium* fungi species are (Haschek en Voss, 2013):



Ochratoxin A



Patulin



Aflatoxin

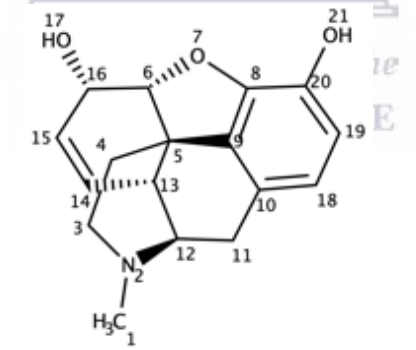
2.1.1 Natural Products and potential drug development

2.1.1.a) Plants

Natural products are any naturally occurring substances generally taken as a secondary metabolite (Gurnani *et al.*, 2014). Over 300,000 secondary metabolites exist, and their primary function is to increase the likelihood of survival of an organism by attracting or repelling other organisms (Barrios-González, 2018).

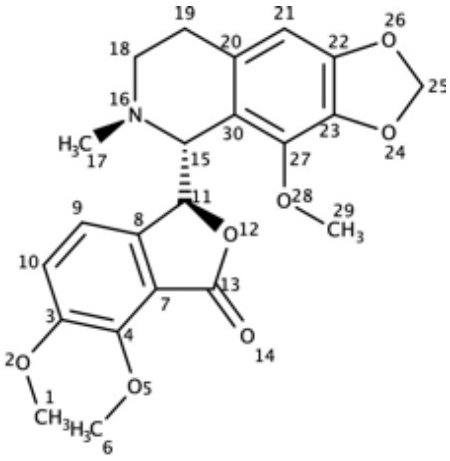
Natural products, specifically compounds and products isolated from plants, have been utilized to treat human ailments, in the forms of extracts, decoction, oils, powders, and mixtures for centuries (Mpofu *et al.*, 2014). They contain an extensive range of unknown and unexplored compounds were several are nearly impossible to emulate, thus natural products will remain a potential source of drug discovery and development in the future (Müller *et al.*, 2000).

In 1803 Wilhelm Serturner isolated a crystalline substance from *opium* and named it morphine (Huxtable en Schwarz, 2001). With formula $C_{17}H_{19}NO_3$ and IUPAC name (4R,4aR,7S,7aR,12bS)-3-methyl-2,4,4a,7,7a,13-hexahydro-1H-4,12-methanobenzofuro[3,2-e]isoquinoline-7,9-diol morphine is used as a pain reliever (Holstege, 2005).



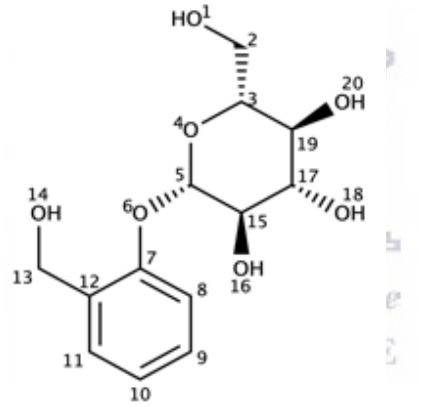
Morphine

Narcotine also known as noscapine, like morphine isolated from the *Papaveraceae* plant species or as otherwise known as *opium*, possesses painkilling, cough-suppressing , anti-cancer and various other properties (Hao, Gu en Xiao, 2015).

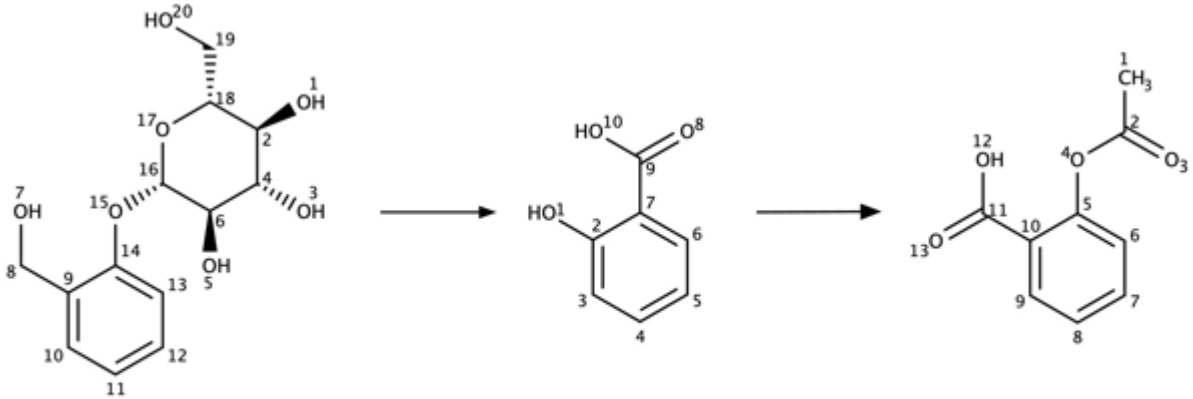


Narcotine

A few years later, in 1899, Bayer introduced aspirin which was the first semi-synthetic pure drug based on a naturally occurring compound named Salicin, obtained from the bark of the plant species *Salix* (Yunes *et al.*, 2005).

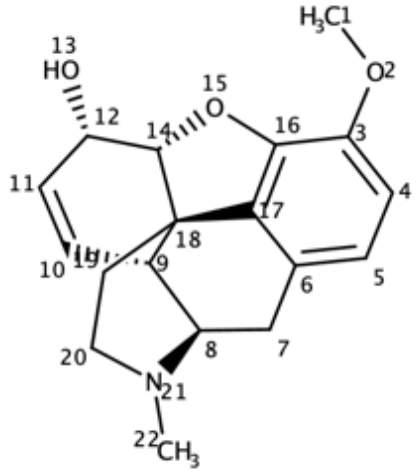


Salicin

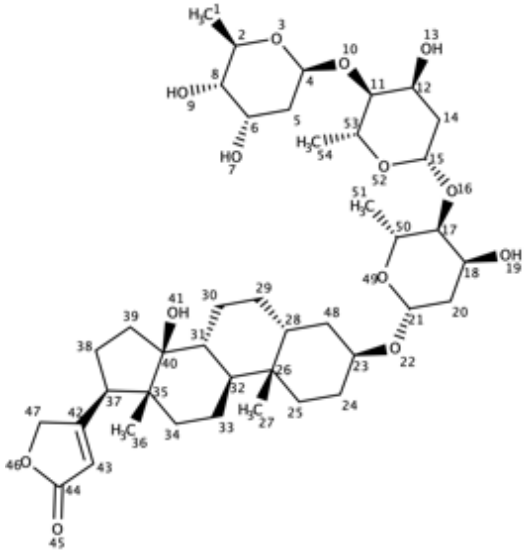


Salicin Salicylic acid Aspirin

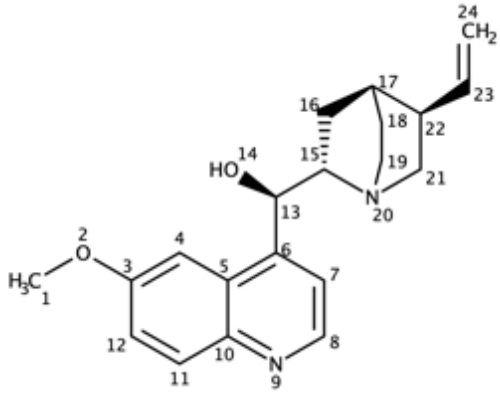
Subsequently, isolation of other drugs followed by the successful isolation of morphine and aspirin. Some of the early isolated drugs post morphine and aspirin are: (Veeresham, 2012):



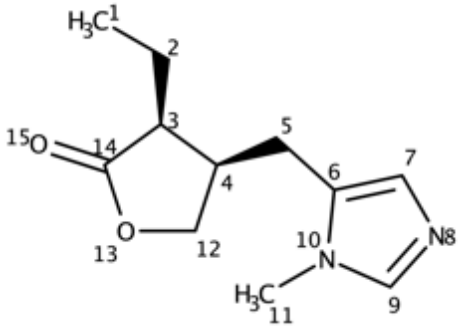
Codeine



Digitoxin



Quinine



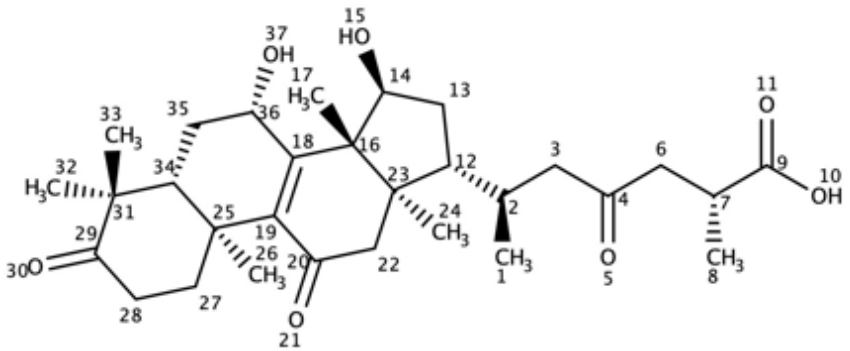
Pilocarpine

2.1.1.b) Fungi

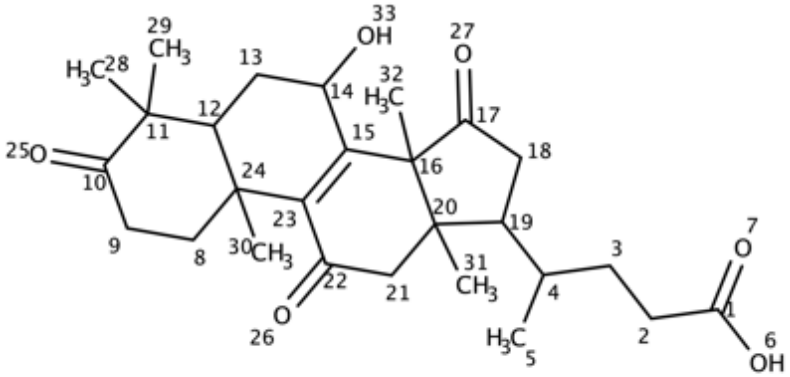
The introduction of sulphonamide antibiotics and penicillin revolutionized the drug discovery process by using natural products as a source, decreasing the fatality rates associated with bacterial infections (Groman, 2014). More than 80% of drug substances involved in drug discovery in that era were reported to be natural products, or they were inspired by natural product structures (Krause en Tobin, 2013).

Anti-cancer drugs have had natural products as their most comprehensive and single source of material since their approval in the Western countries and Japan, where 85 compounds representing 48,6% out of 100%, were natural products or compounds derived from natural products (Krause en Tobin, 2013). The study of fungi metabolites has shown that many compounds found in fungi are sources or have been proven to be antibiotics, antifungal, immunosuppressive and cholesterol-lowering agents (Kumar *et al.*, 2017).

Fungi have been extensively used as medicine due to their curative properties. Lingzhi is the rarest and most precious medicinal fruit body of the *Ganoderma* fungus in Chinese culture (Geng *et al.*, 2020). This fungus is known to prevent and mitigate a variety of clinical conditions such as amnesia, chronic bronchitis, neurasthenia, coronary heart disease, stroke, asthma, insomnia, a female endocrine disorder, cancer, arthritis, chronic hepatitis, thrombosis, and many other diseases (Moore, 2001). Some of the compounds responsible for the prevention of these diseases are: Ganoderic acid A, B and F and Lucidenic acid A, B, C, N and F with the following backbones (Haubrich, 2018).

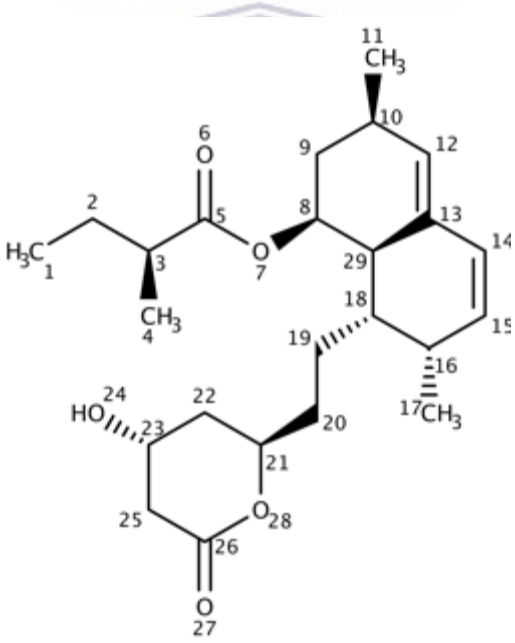


Ganoderic acid



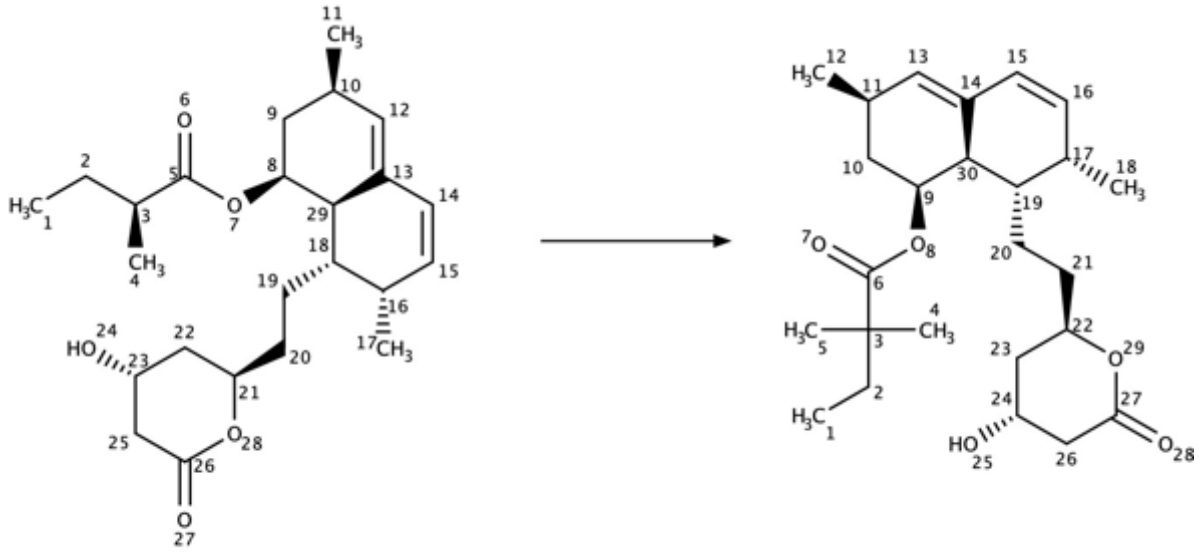
Lucidenic acid

In the western side of the world, the use of fungi increased due to their interest in fungi metabolic products in medicine after the success of penicillin (Jakubczyk en Dussart, 2020). Other successful stories of drugs developed with fungi as a source, is the cholesterol-lowering agent Mevinolin or otherwise known as Lovastatin. (Alberts *et al.*, 1980).



Mevinolin (Lovastatin)

Mevinolin was isolated from *Aspergillus terreus* and *asperlicin,* where the structure manipulation of this compound has led to the discovery of benzodiazepines, which are used to treat severe anxiety or insomnia, an example would be Simvastatin (Guina en Merrill, 2018).

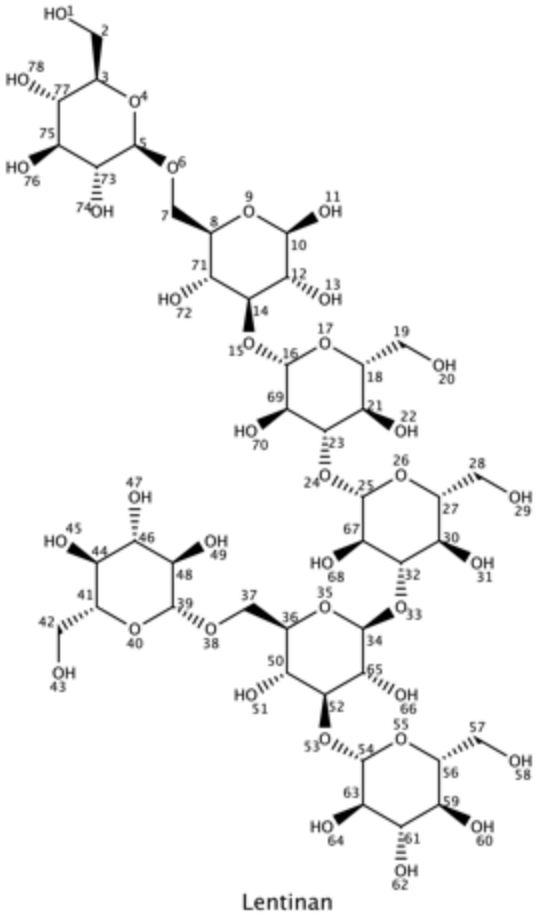


Lovastatin (Mevinolin)

Simvastation

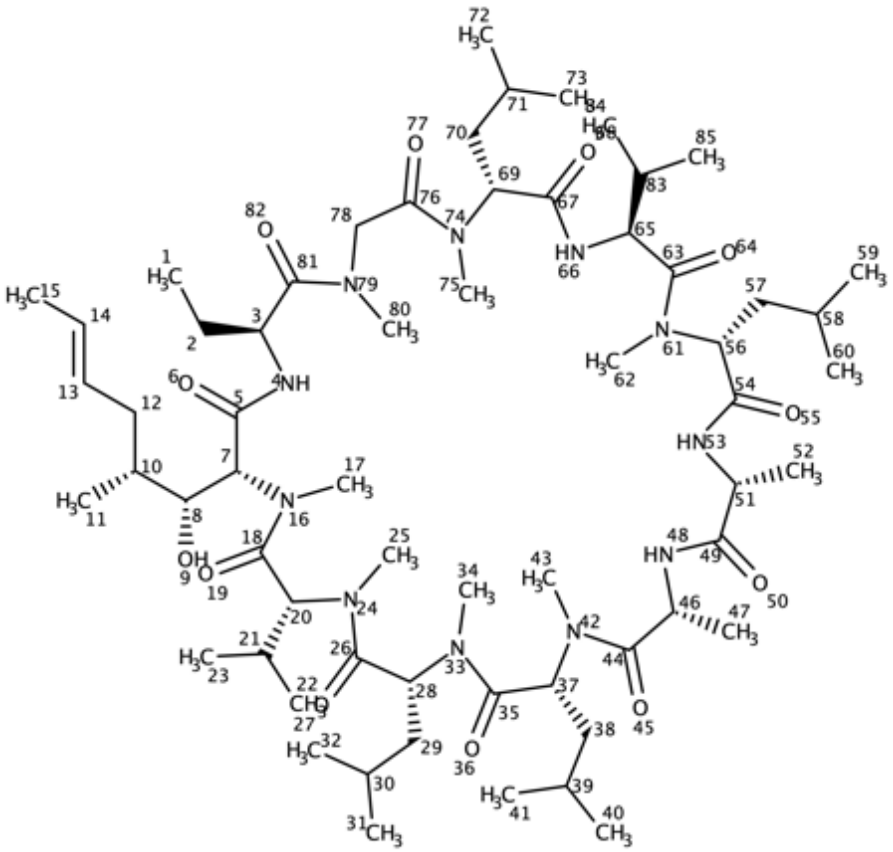
The *Cryptosporiopsis quercina* and endophytic fungus isolated from *Tripterigeum wilfordii* have shown antifungal activity against some fungal pathogens in humans such as *Candida albicans* and *Trycophyton mentagrophyte* (Strobel *et al.*, 1999).

Aspergillus parasiticus is an endophytic fungus of the coastal redwood which produces sequoiatones A and B, which show selective inhibition of human tumour cells, specifically, breast cancer cell lines (Stierle, Stierle en Bugni, 1999). Amrubicin hydrochloride was isolated from *Streptomyces peucetius.* This species is related to *Doxorubicin* which is used to treat soft tissue, acute leukaemia, lung cancer, bone sarcomas, thyroid cancer, and both Hodgkins and non-Hodgkins lymphomas (López-González *et al.*, 2013). More in cancer research, lentinan, a polysaccharide isolated from the *L. edodes mushroom* has been used to treat stomach and various other cancers types (Yang, Zhou en Zhang, 2019).



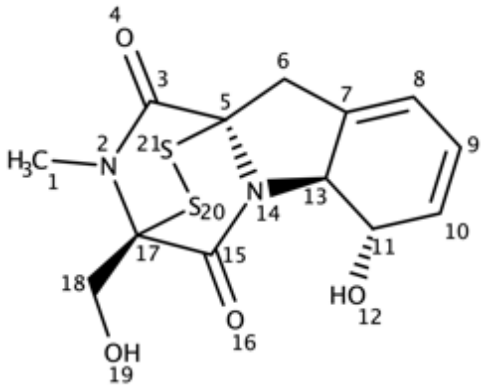
Lentinan

Natural products that were first discovered as antibiotics have recently shown to possess other activities in mammals, for example, cyclosporin (Herbrecht, Lid en Bergerat, 1990). Cyclosporin is capable of suppressing the recipient's immune response to avoid organ rejection during organ transplant (n, Eunsung Mouradian, 2008). This fungi is currently widely used and known as an immunosuppressant, and has played an essential role in improving the rate of successful transplant operations (Tabassum Khan, 2017).



Cyclosporin

Gliotoxin is a mycotoxin found in marine fungi that contain sulphur, it belongs to a naturally occurring 2,5-diketopiperazines and is also utilized to regulate the immune system and manage the postoperative recovery of transplant patients (Kumari en Srividhya, 2020).



Gliotoxin

2.1.2 Mushrooms

Mushrooms possess tonic, medical properties and attributes, generally contain higher protein contents compared to most vegetables and wild plants due to the high amounts of essential amino acids, making them the best sources of vitamins and minerals for animals and humans (Valverde, Hernández-pérez en Paredes-lópez, 2017)(Temesgen, 2018). They possess eight important amino acids, polyunsaturated fatty acids, and small amounts of saturated fatty acids (Kakon, Choudhury en Saha, 2012).

Mushroom fungi play key roles in forest ecosystems due to their unique ability to break down organic matter such as leaves, wood, and various others (Onifade *et al.*, 2019).. Wild mushrooms contain extremely high amounts of vitamin D2 and small amounts of vitamin B-complex and vitamin C (Cardwell *et al.*, 2018).

2.1.3 Mushrooms and health benefits

From a nutritional perspective, since mushrooms contain high levels of protein and fiber the cultivation of mushrooms has been considered as an alternative to protein supply in countries with high rates of malnutrition (Figueiredo en Régis, 2017).

Certain mushrooms are known to contain medicinal properties, which have been widely used and studied as possible sources of treatment or drug development for diseases such as cardiovascular , cancer, viruses, bacteria, parasites, and many others (Hyde *et al.*, 2019).

Polysaccharide-proteins extracted from medicinal mushrooms may enhance innate immune responses, resulting in antitumor activities in animals and humans (Lull, Wichers en Savelkoul, 2005).

Certain species of mushrooms are utilized as a source of healing, prevent the spread of tumor cells and AIDS (Wang, Wang en Ng, 2007), treat colds, stomach aches, headaches, hepatitis B, reduce fatigue, cholesterol levels, blood levels and strengthening of the immune system (Jong en Birmingham, 1992).

Methanolic extracts of six wild mushrooms, showed significant antimicrobial activity against *B. subtilis, S. aureus, E. coli, P. aeruginosa*, and *Candida Albicans* (Venturini *et al.*, 2008). These six mushrooms are: *Lycoperdon perlatum, Cantharellus cibarius, Romaria formosa, Marasmius oreades, Pleurotus pulmonarius and Chelonistele vermicularis* (Ahmed *et al.*, 2015).

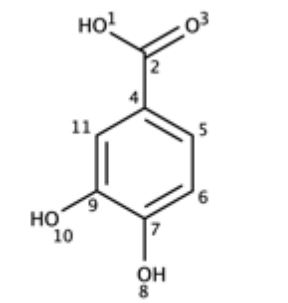
In addition mushrooms are good sources of zinc, which benefits normal fetal growth during pregnancy (Mohmand et al., 2011) . Certain species have also shown potential *in vitro* for lowering the level of oestrogen and testosterone while others demonstrte anti-inflammatory effects (Thornton, 2002).

2.1.4 Kinds of mushrooms

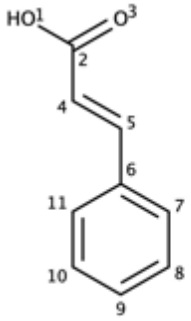
Mushrooms are divided into, edible and non-edible mushrooms. Edible mushrooms possess desirable taste and aroma and are naturally of white colour and non-edible mushrooms are naturally coloured mushrooms with the exception of certain white mushrooms. Some of these mushrooms possess compounds with properties which are useful in medicine and in human health (Fernandes et al., 2014). Some of these mushrooms are:

Macrolepiota

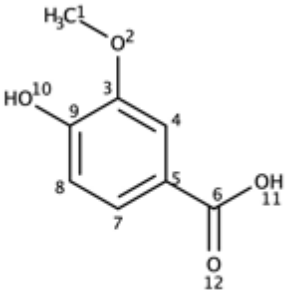
The parasol mushroom, *macrolepiota* is part of the *Agaricaceae* family. This mushroom is an edible mushroom, a basidiomycete fungus that contains bioactive compounds which are responsible for its antioxidant properties and anticancer activities against the human lung cancer cell line. Some of the compounds responsible for this antioxidant properties are the following: (Kozarski *et al.*, 2015):



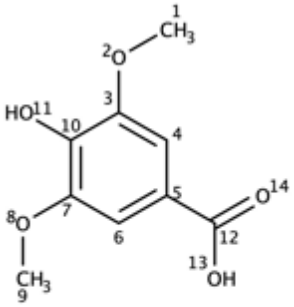
Protocatechuic acid



Cinnamic acid



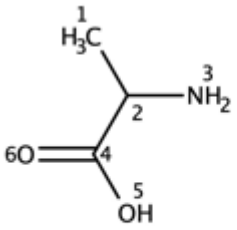
Vanillic acid



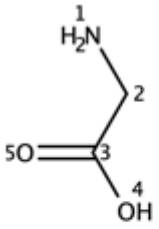
Syringic acid

Auricularia

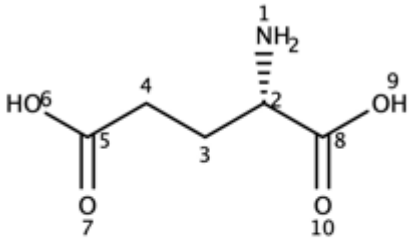
Auricularia is part of the *Auriculariaceae* family. This fungus is also an edible mushroom and it resembles an ear possessing a gelatinous texture. This fungus contains small amounts of essential amino acids such as the following and others: (Kadnikova *et al.*, 2015)



Alanine



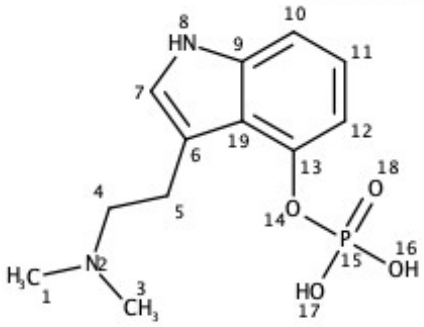
Glycine



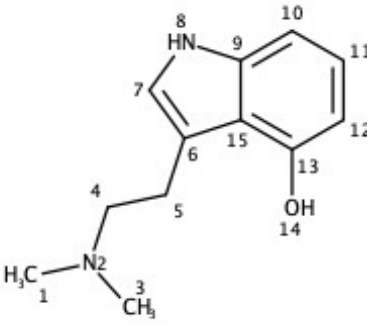
Glutamic acid

Psilocybe

Psilocybe mushrooms are also known as "magic shroom" is a poisonous mushroom, and are polyphyletic which is an informal group of fungi that contains psilocybin and psilocin (Seneca, 2007).



Psilocybin

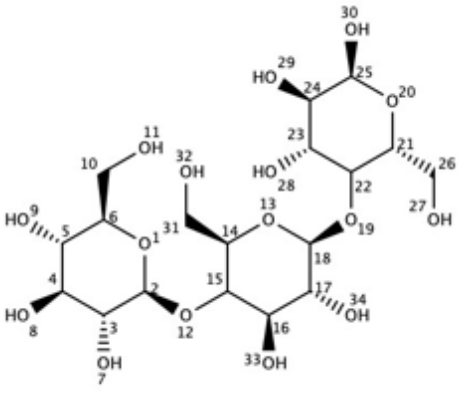


Psilocin

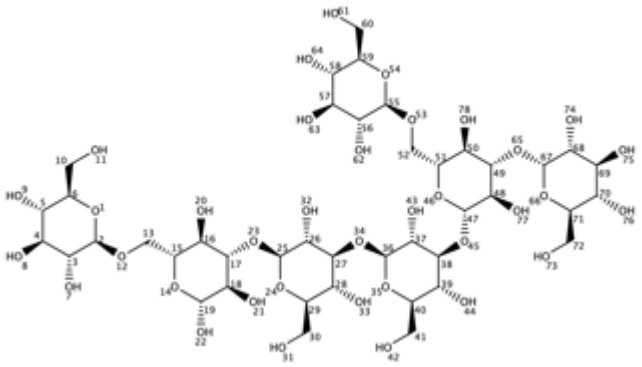
These compounds are responsible for creating the feeling of disorientation, lethargy, giddiness, euphoria, joy, depression, and even paranoia when given in high doses (Clarke, 2007) however, when taken in micro doses, psilocybin and psilocin are used to treat mental disorders (Singh, 2017).

2.1.5 Mushrooms in cancer research

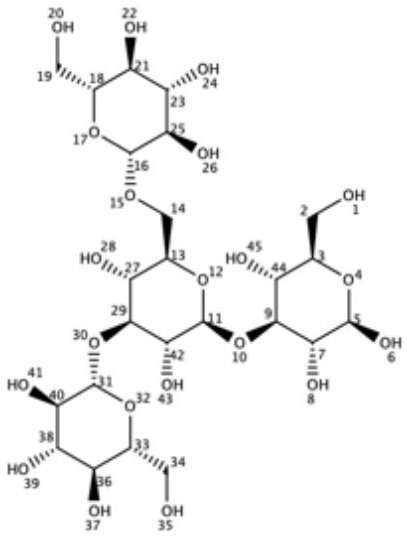
Mushrooms contain bioactive compounds and it is considered as a source of drug and nutraceutical developments, making them a functional food source (Üstün, Bulam en Pekşen, 2018). Many pharmaceutical substances that contain unique and potent properties were isolated from mushrooms (Kimura, 2013). Five mushrooms, *Lentinus edodes*, *Schizophyllum commune*, *Grifola frondosa*, *Sclerotinia*, *Pleurotus rimosus* (Chatterjee en Patel, 2016) are known to possess antitumor activities due to their SSG constituents (C.F.R. Ferreira *et al.*, 2012):



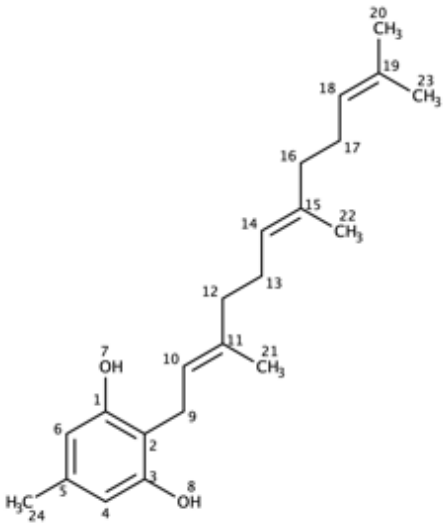
Beta-glucans



Lentinan



Schizophyllan

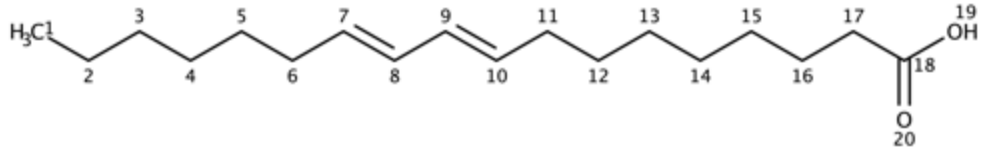


grifolan

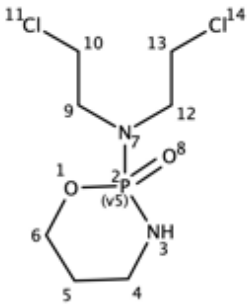
Mushrooms that contain anti-cancerous compounds, play an important role as inducers of reactive oxygen species, inhibitors of mitotic kinase, angiogenesis, topoisomerase, and anti-mitotic, leading to apoptosis and finally checking cancer proliferation (Patel en Goyal, 2012).

The ethyl acetate, methanol, and aqueous extracts of *Pleurotus rimosus* produces compounds which inhibit Dalton's Lymphoma Ascites cell line induced solid tumor and EAC cell line induced ascites tumor in mice and the antitumor effects are higher in ethyl acetate extracts compared to other extracts (Ajith en Janardhanan, 2007). In the methanol and aqueous extracts, a significant amount of antitumor properties helps inhibit tumor development (Khan *et al.*, 2020).

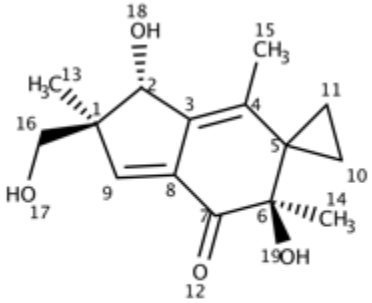
The *Agaricus bisporus* mushroom, aids on the prevention and combats breast, colorectal, ocular, and prostate cancer (Wang *et al.*, 2021). Some of the compounds responsible for the medicinal properties of the *Agaricus bisporus* are; conjugated linoleic acid (CLA), Cyclophosphamide (CP), Illudin-s and various others (Chen *et al.*, 2006).



Conjugated linoleic acid

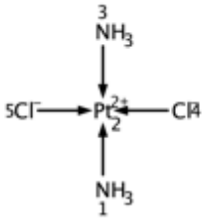


Cyclophosphamide



Illudin-S

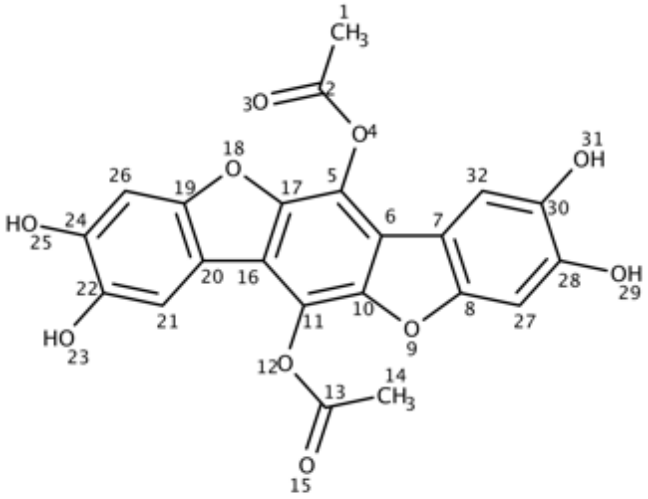
The *Maitake (D fraction)* mushroom, aids to combat breast cancer. The compounds responsible for these properties are (Alonso *et al.*, 2018): Cisplatin, sulphated polysaccharide (S-GAP-P), A novel polysaccharide-peptide GFPPS1b (Shi *et al.*, 2007).



Cisplatin

Pleurotus ostreatus, combats breast cancer. The compounds responsible are the water-soluble polysaccharide (POPS-1) (Radzki *et al.*, 2016).

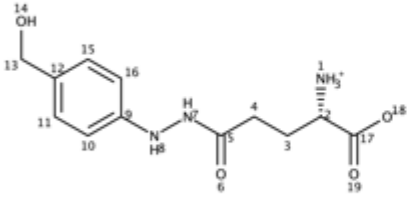
Polyozellus multiplex, known to combat hepatocellular carcinoma, colon cancer, leukemia, and gastric carcinoma, and the compound responsible is Polyozellin (Chen, Lee en Kirschner, no date).



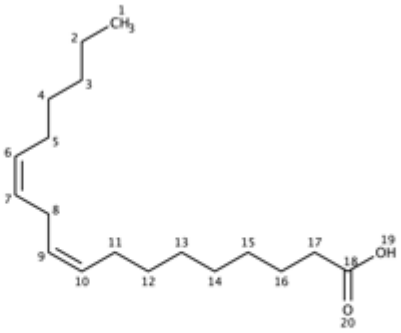
Polyozellin

Pleurotus eryngii, is known to combat hepatocellular carcinoma, and the protein responsible is lectin (Wang, Gao en Ng, 2000).

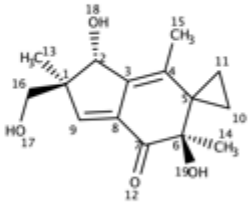
Agaricus blazei (Murill), is known to combat myeloma, hepatic cancer, leukemia, stomach and lung cancer, and the compounds and proteins responsible are, Agaritine, Agaricus bisporus lectin (ABL) Agaricus polytricha proteins (APP), Linoleic Acid and Illudin-s (Rodríguez P *et al.*, 2017).



Agaritine

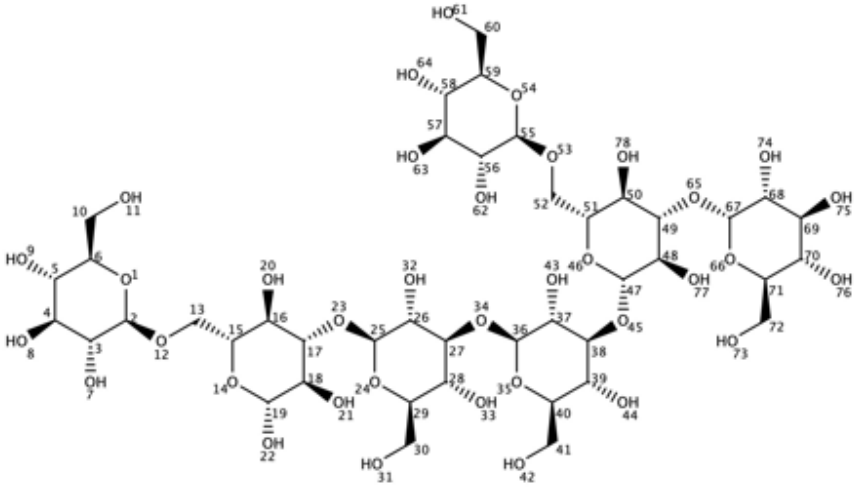


Linoleic acid



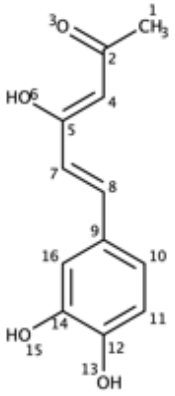
Illudin-s

Lentinula edodes is known to combat lung, cervical/ovarian, gastric and skin cancer. The compound responsible is lentinan (Zhang *et al.*, 2019).



Lentinan

Phellinus linteus it is known to combat colon cancer and the compound responsible is Hispolon (Sarfraz *et al.*, 2020).



Hispolon

2.1.6 Rust fungi

Rust fungi are parasites that grow on plants by obtaining their nutrients, reproduce and complete their life cycle on plants (Voegele en Mendgen, 2011). Rust fungi cause diseases in economically important plant species and certain species of this fungus are highly specific on what plant to infect, colonize and reproduce (Kolmer, Ordonez en Groth, 2009).

Rust fungi possess a complex life cycle called the macrocyclic cycle and it undergoes five distinctive spore stages on two unrelated hosts (Ma *et al.*, 2020).

Aecial colonies reflect the pycnial clusters, and are formed by the growth of the rust species that infect leaves on the lower leaf surface as dikaryotic hyphae (Chen *et al.*, 2014). Dikaryotic aeciospores can travel long distances and are produced in chains and quantity within pustules (Leonard en Szabo, 2005). Unlike aecial colonies, urediniospores are produced singly on stalks (Shattock et al., 2003). When the nutrients from the plant start to decline the uredinia converts to telia and produces increasing numbers of teliospores which are thickly walled and resistant to cold or drying as they begin to darken (Mendgen, 1984).

The *Uromycladium tepperianum* MacAlpine, the fungi under research, is a rust fungus that produces large, irregular-sized galls on the leaves and stems of the plant (Ratio, 2017). It was introduced in South Africa for the control and spread of the acacia saligna bushes (Control *et al.*, 2010). The *Uromycladium tepperianum* MacAlpine fungus causes the limitation in the growth and seed production of the acacia tree due to them feeding on the plants' nutrients and then forming heavy large galls around the tree (Wood en Morris, 2007).

Currently, no discoveries of bioactive compounds have been made on rust fungi, hence the investigation on the *Uromycladium tepperianum* MacAlpine fungus was conducted due to the limitation of the growth and seed production this fungus carries out on the acacia tree species.

2.1.7 Application of LC-MS and MS in plant extract analysis.

Chromatographic analysis, aids on the optimization of samples and complex plant extract mixtures containing numerous metabolites in which the chemical composition of these may differ between extracts (Keskes *et al.*, 2017). The detection of all secondary metabolites found in a plant may not be permitted when different types of chromatography detectors are utilized. Some of these chromatographic techniques are, RI (retention index), electrochemical light scattering, UV (Ultraviolet-visible spectroscopy) and various others, unlike MS (mass spectrometry) (Keskes *et al.*, 2017). MS is considered to be the most sensitive method of molecular analysis due to its power of mass separation and since all natural compounds possess a molecular weight, MS is one of the most reliable chromatographic techniques (Wolfender en Hostettmann, 1995).

LC-MS like MS is a suitable interface for secondary metabolite analysis, its interface possesses the capability of detecting weak chromophoric compounds specifically when extracts contain numerous metabolites with low yields (Wolfender en Hostettmann, 1995).

With the aid of databases these chromatographic techniques are utilized to separate and characterize complex compound mixtures and isomers. In the field of organic chemistry, the database Global natural products social molecular network is one of the most reliable natural product databases due to it being a mass spectrometry ecosystem based database, possessing open access, to annotated fragmentation mass spectrometry data, aiding on the identification of data and compound characterization.

Chapter 3

Materials and methods

3.1 Reagents and General methods

3.1.1 Chemicals and reagents

Solvents: dichloromethane (DCM), *n*-hexane (*n*-hex), ethyl acetate (EtOAc), butanol (BuOH), methanol (MeOH), Chloroform ($CHCl_3$), Acetone, vanillin, standard solution, phenol, sulphuric acid, dragendorff reagent, and ninhydrin reagent.

3.1.2 General experimental methods

The employed experimental methods in the study included, NMR Spectroscopy, Mass Spectrometry, column chromatography, Liquid Chromatography - Mass Spectroscopy, Gas Chromatography- Mass Spectroscopy, ninhydrin and Dragendorff reagents, hydrolysis, phenol sulphuric acid test and UV-lamp.

3.1.3 Evaporation of the solvents

Solvent evaporation was performed on a Buchi Rotavapor R-114. The water bath temperature was maintained at 50 ^oC for plurality of evaporations, however, kept at 60 ^oC when evaporating BuOH.

3.2 Spectroscopy

3.2.1 Nuclear magnetic resonance (NMR) spectroscopy

NMR Spectroscopic analysis was conducted in 1 and 2 dimensions and recorded at 25 ^oC on a 400 MHz Bruker advanced IIIHD Nanobay Spectrometer, using a 5mm BBO probe, deuterated chloroform $CDCl_3$ as the solvent solution. The coupling constants and chemical shifts were expressed as (J, Hz) and δ ppm respectively.

3.3 Chromatography

3.3.1 Column Chromatography (CC)

Silica gel 60 (0.040 - 0.063 mm) 230 - 400 mesh particle size (Merck) were packed in glass columns (10 - 30 mm diameter) for column chromatography.

3.3.2 Thin layer Chromatography (TLC)

Thin layer chromatography was carried out on the pre-coated silica gel of 0.2 mm layer thickness 60 F_{254} plates (Merck). TLC spot visualization was carried out under UV light at 254 nm and 366 nm, followed by the spraying of the TLC plate with vanillin spray reagent, prepared by dissolving 15 g of vanillin in 250 ml ethanol and 2.5 ml concentrated sulphuric acid. After the TLC plates were sprayed heating of the plates revealed the spots of colour.

3.4 Mass spectrometry (MS)

3.4.1 Gas Chromatography Mass Spectrometry (GC-MS)

GC-MS spectra were recorded by an Agilent technology gas chromatography coupled to 5977E MSO model mass spectrometer at an isothermal temperature of 250 ^{o}C , equipped with a flame ionisation detector (FID) on a DB 225 capillary column. Helium was the carrier gas used. A temperature of 225 ^{o}C was programmed once the samples of 10 µl were injected into the GC analyser on a flow rate of 1.4 ml/min.

3.4.1 Liquid Chromatography-Mass Spectrometry (LC-MS)

LC-MS analysis was performed at the University of Stellenbosch. LC-MS spectra were recorded by Waters Synapt G2, using modes ESI probe injecting into a stream of water and into a stream of acetonitrile, data was collected in ESI positive and negative modes with cone voltage 15 V.

3.5 *Uromycladium tepperianum* MacAlpine preparation

3.5.1 Plant Material

The *Uromycladium tepperianum* plant material provided by the Chemistry department and Professor Mabusela weighed 42,4 g, harvested in Khayelitsha suburb of Cape Town, South Africa GPS coordinates (34˚01’05.4”S 18˚36’26.1“E). A second and third collection was required to conduct the experiment due to small product yields. The second collection of the plant material weighed 772.49 g, and the final collection weighed 5 kg (5000 g).

Figure 1: Uromycladium tepperianum MacAlpine fungus and material in powder form

3.5.2 Extract preparation

Maceration and crude extraction of the material were the two methods of preparation conducted. Part of the freshly ground material was macerated using 80% methanol and 20% water over a period of 24 hours (process was repeated five times), followed by evaporation of the methanol and freeze-drying of the residue. The surplus ground material was utilized to perform crude extraction by the addition of *n*-Hex, DCM, EtOAc and BuOH (singularly, leaving each solvent submersed in the plant material over a period of 24 hours) followed by filtration of the material and evaporation of the solvents utilizing the Buchi Rotavapor R-114, allowing the samples to dry into powder form. After acquiring the desired amounts of extract, the extracts were then singularly placed in a column, where fractions were collected, purified, isolated, and characterized utilizing chromatographic techniques, NMR, TLC, GC-MS and LC-MS.

3.5.3 Maceration Method (the first collection of material used 42,4g and the second batch used 300g)

Post maceration (80% MeOH and 20% water), filtration, and solvent evaporation (Buchi rotavapor), the sample was freeze dried, followed by solvent-solvent extractions (the process was repeated three times). The solvent-solvent extractions were conducted by four different solvents, *n*-hex , DCM, EtOAc, and BuOH. The dissolution of the dried samples was conducted by distilled water and transferred into a separating funnel were *n*-Hex, EtOAc, DCM, and BuOH were singularly added, followed by shaking of the funnel (three times). The aqueous and organic layers were left to separate over a period of four hours, followed by the separation of the organic layer.

3.5.4 Crude extraction method (second collection of material used 472,49 g)

The ground material was submersed in either: *n*-hex, DCM, EtOAc, or BuOH singularly, and left immersed overnight. The process was repeated 3-4 times followed by evaporation of the solvent with aid of a Buchi Rotavapor. After dry, the samples were weighed and screened using TLC.

3.5.5 Tannin containing compounds and its removal by acetone precipitation

The aqueous extract, left post solvent-solvent extraction and the separation of the organic layer was divided into two.

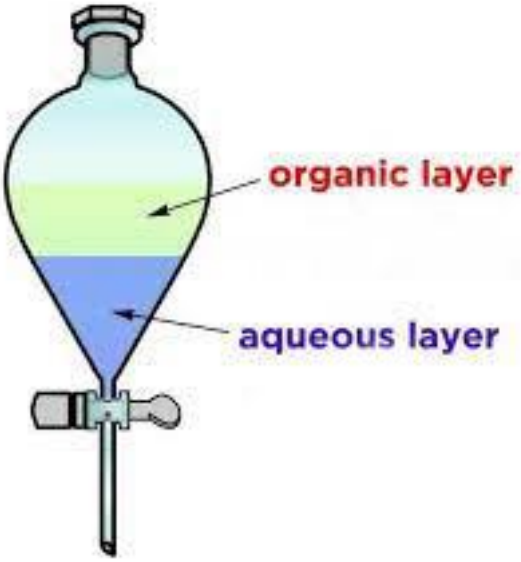


Figure 2: solvent-solvent extraction, layer separation

11,15 g (first experiment), and 6,23 g (second experiment) were utilized to conduct this experiment. 40 % water (80 ml) was added to a flask for material dissolution, followed by the gradual addition of 60 % acetone (120 ml), while stirring. The solution was left overnight to stir. The stirred solution was then placed in a centrifuge at 2300 speed for 20 minutes. The precipitate separated from the aqueous tannin-containing solution, was decanted and dissolved in deionized water, followed by the evaporation of the remaining acetone and freeze-drying of the material. Subsequently, the sample was prepared using hydrolysis methodology, for LC-MS and NMR characterization.

3.5.6 Hydrolysis

The proportions of the polysaccharides and other carbohydrates were determined by firstly, conducting hydrolysis of the crude material, where 100 mg of the sample and 10 ml of 2 M trifluoroacetic acid was added into a test tube and heated in a hot water bath at 100 ºC for 24 hours, followed by LC-MS and NMR characterization (Van Wychen en Laurens, 2015).

3.5.7 Reagents test

Dragendorff reagent was utilized to detect the presence of alkaloids in the sample, by revealing a bright orange spot on TLC after submerging the plate spotted with the crude extract into the orange solution/reagent.

Ninhydrin reagent was used to detect the presence of amino acid in the sample, by revealing a bright purple spot on TLC after submerging the plate spotted with the crude extract into the solution/reagent.

Phenol/sulfuric acid test was conducted in order to detect the presence of carbohydrates in the sample post hydrolysis. A colour change to orange indicated the presence of carbohydrates in the sample (Ismail, 2017).

3.6 Isolation and purification of compounds

3.6.1 Fractionation of the hexane extract

The *n*-hex extract acquired from the second collection was merged with the first collection *n*-hex extract, and it was utilized to conduct this experiment due to the possession of higher yields. The *n*-hex extracts (2,34 g + 0,845 g+ 0,125g = 3,31g) was placed in a glass column for fractionization, by adsorbtion on silica gel by gravity elution . The following *n*-hex elution were utilized: 2 L of 100% *n*-hex for initial elution, and 2 L mixtures of hexane and EtOAc in the following ratios (*n*-hex 9,5: EtOAc 0,5), (*n*-hex 9: EtOAc 1), (*n*-hex 8: EtOAc 2) , (*n*-hex 7: EtOAc 3), (*n*-hex 6: EtOAc 4), (*n*-hex 5: EtOAc 5), (*n*-hex 4: EtOAc 6), (*n*-hex 3: EtOAc 7), (*n*-hex 2: EtOAc 8), (*n*-hex 1: EtOAc 9) and 100 % EtOAc. The collected fractions were analysed by TLC using 10 ml of *n*-hex and EtOAc mixtures as tank solution in the following ratios: (*n*-hex 9: EtOAc 1), (*n*-hex 8: EtOAc 2), (*n*-hex 7: EtOAc 3), (*n*-hex 6: EtOAc 4). Fractions with similar retention factors were merged. Fractions 35 to 55 were combined and sent to NMR for characterization leading to the isolation of compounds 1 and 2. Fractions 16-26 were combined for bearing the same retention factor. Subsequently purification of the fractions utilizing a silica gel column was conducted using MeOH as the eluting solvent. Fractions 4, 5 and 6 were obtained, combined according to retention factor, sent to NMR for characterization, thus leading to compounds 3 and 4 isolation.

3.6.2 Fractionation of the EtOAc extract

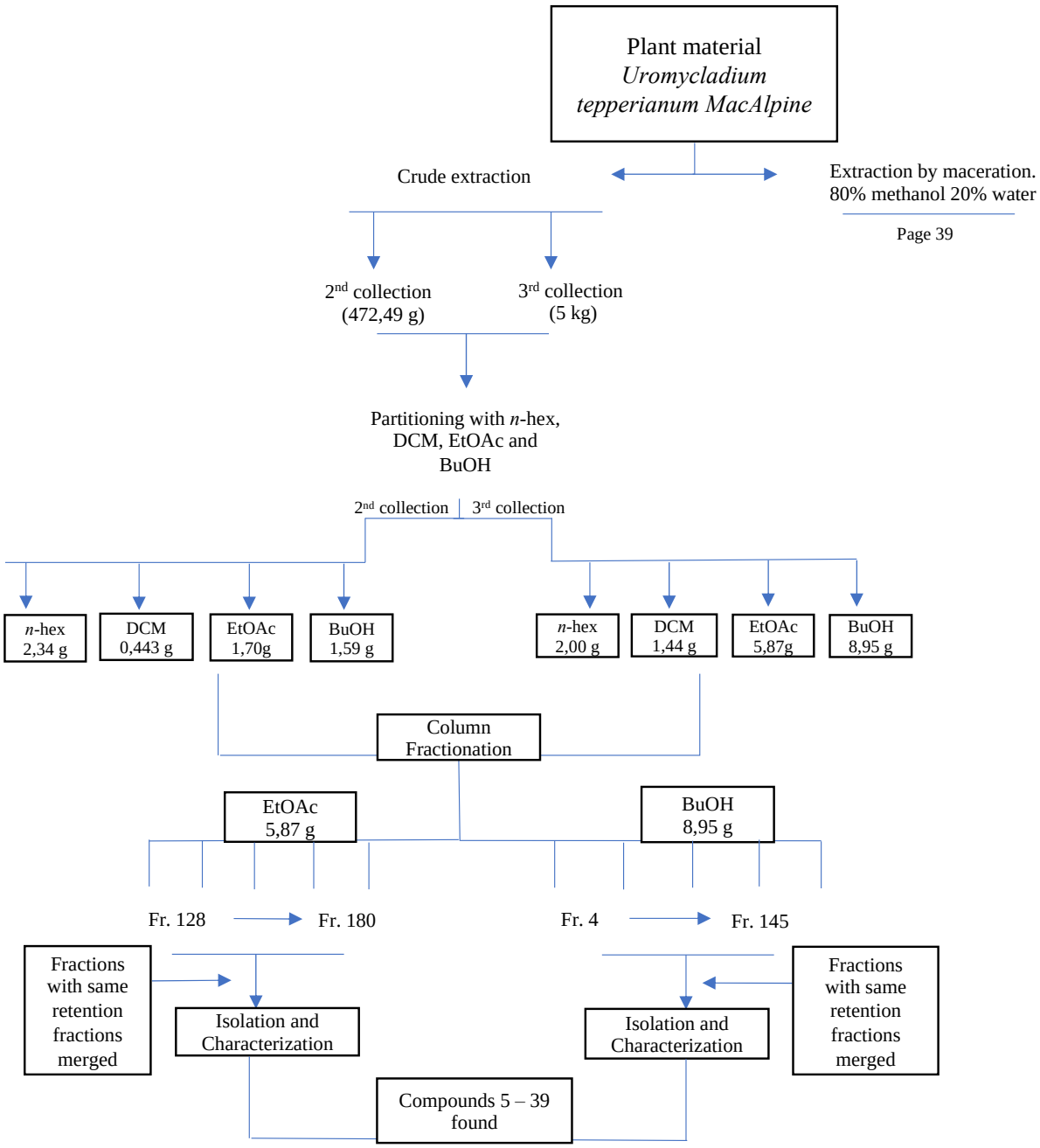
The EtOAc extract acquired from the third collection was utilized to conduct this experiment due to the possession of higher yield. The EtOAc extract (5,87 g) was placed in a glass column for fractionization, and adsorbed on silica gel by gravity elution. EtOAc elution were utilized: 2 L of 100 % DCM for initial elution , and 2 L of mixtures of DCM and EtOAc in the following ratios (9:1), (8:2), (7:3) , (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and 100 % EtOAc. The collected fractions were analysed by TLC using *n*-hex and EtOAc mixtures as tank solutions in the following ratios: (9:1), (8:2), (7:3), (5:5). Fractions with similar retention factors were merged together such as 128-140 , 181-190 , 201-207, 208-215, and 161-180. The first four merged fractions (128-140 , 181-190 , 201-207, 208-215) were prepared and sent to LC-MS for further

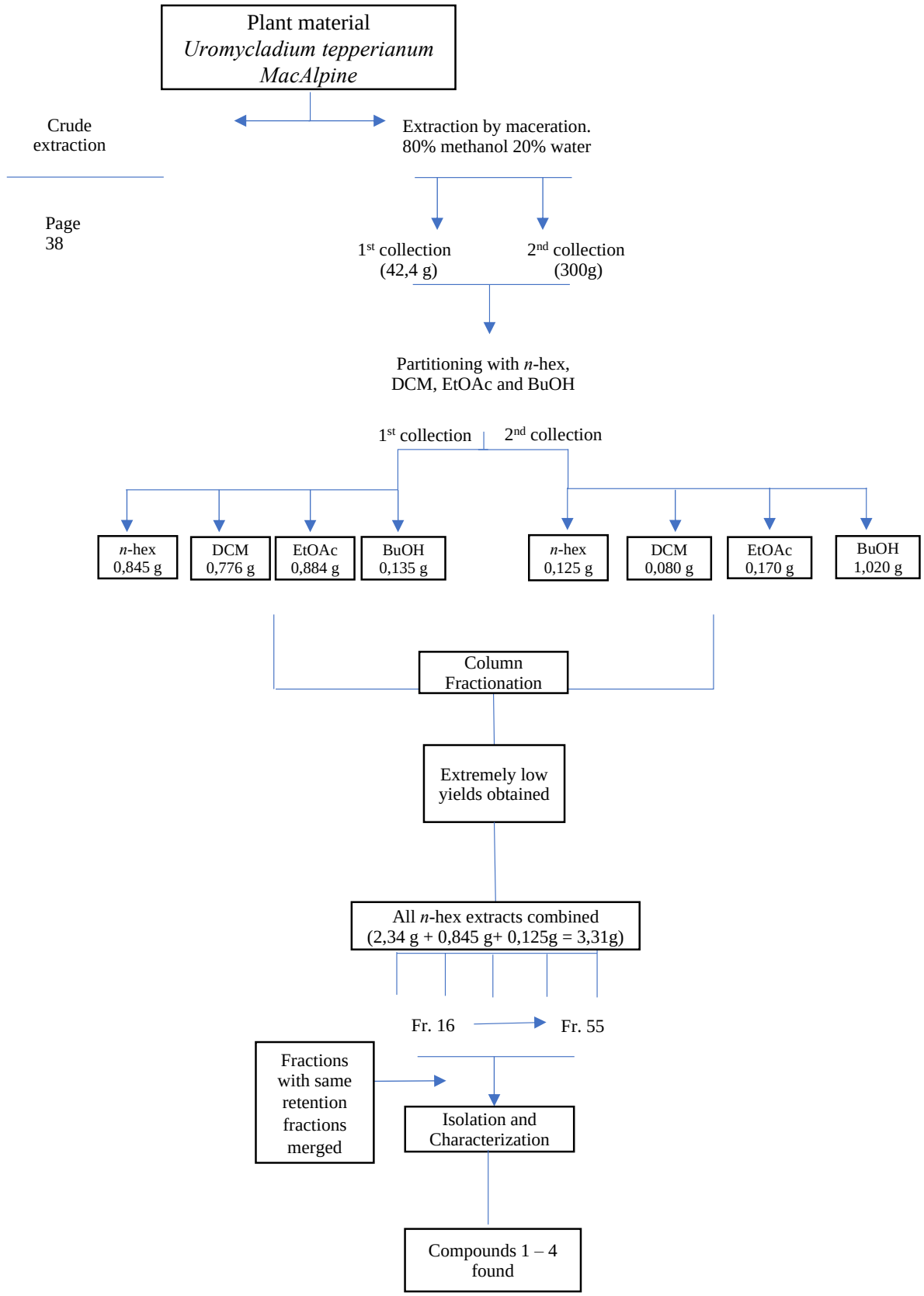
analysis followed by database characterization. Fractions 161-180 were merged and loaded onto a silica gel column for further separation, with elution mixtures of 2 L of 100 % DCM for initial elution , and 2 L of mixtures of DCM and EtOAc in ratios of (9:1), (8:2), (7:3) , (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and 100 % EtOAc, obtaining fractions 43-51 which were merged due to possessing the same retention factor, followed by LC-MS analysis at the University of Stellenbosch.

3.6.3 Fractionation of the BuOH extract

The BuOH extract acquired from the third collection was utilized to conduct this experiment due to the possession of higher yield. The BuOH extract (8,95 g) was placed in a glass column for fractionization, adsorbed on silica gel by gravity elution. DCM elution was utilized: 2 L of 100 % DCM for initial elution, and 2 L of mixtures of DCM and EtOAc in the following ratios (9:1), (8:2), (7:3) , (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and 100 % EtOAc. The collected fractions were analysed by TLC using chloroform and methanol mixtures as tank solutions in the following ratios: (9,5:0,5), (9:1).Fractions with similar retention factors were merged, such as fractions 103-145 and 96-102. The second set of fractions merged (96-102) were prepared and sent to LC-MS for further analysis. Fractions 103-145 were merged and loaded onto a silica gel column for further separation with elution mixtures of; 2 L of 100 % DCM for initial elution, and 2 L of mixtures of DCM and EtOAc in the following ratios (9:1), (8:2), (7:3) , (6:4), (5:5), (4:6), (3:7), (2:8), (1:9) and 100 % EtOAc, obtaining fractions 4-7, and 91-110 which were merged due to bearing the same retention factor and sent to LC-MS for analysis at the University of Stellenbosch.

Scheme 4: Isolation of natural products from *Uromycladium tepperianum* MacAlpine Fungus





Chapter 4

4.1 Results and Discussion

Subsequently to both methods of extraction, all samples underwent dry column chromatography. The results recorded are to be found in **Table 1**.

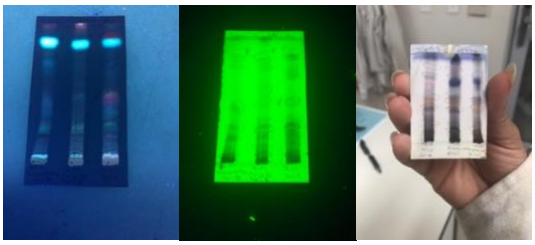
Table 1: Collections and Extract quantities

Solvents	1st collection material weight: 42,4 g	2nd collection material weight:300g	2nd collection Crude material weight: 472,49g	3rd collection material weight:5kg
Yield post maceration	4% yield 1,70 g post freeze dry	4% yield 13,27 g post freeze dry	N/A	10% yield 401,52 g post freeze dry
Hexane	0,845 g	0,125g	2,34 g	2,00 g
EtOAc	0,884 g	0,17g	1,70 g	5,87 g
DCM	0,776 g	0,08g	0,443 g	1,44 g
BuOH	0,135 g	1,02g	1,587 g	8,95 g

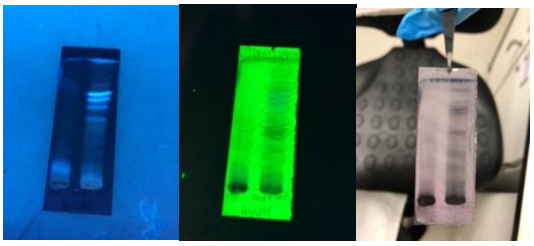
The primary collection, the plant material was a mature, dry gall, with brown and powdery texture. Two extra collections were required to perform this experiment due to small product yields post maceration and crude extractions. The second collection weighed 772,49 g and it was divided into two parts, 300 g was utilized to conduct maceration and 472,49 g was utilized to conduct crude extraction. Moreover, the plant material was mature, but not as dry, brown, yellow, and powdery within. The last collection weighed 5 kg. Unlike the first collection the galls were not brown and dry, instead, within the gall, white and light green colours where observed, resembling an apple or a pear, it also possessed a damp and tough texture. It is suspected that due to climate, season and environmental conditions the phytochemical characteristics of the material were different for every collection, and this can be observed below on the TLC plates.

Lane representation

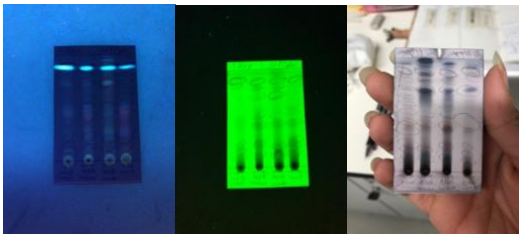
Lane 1: 1st collection, Lane 2: 2nd collection, Lane 3: 3rd collection



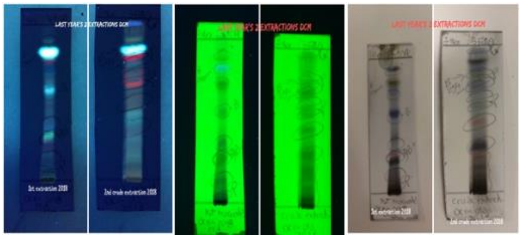
DCM extracts



BuOH extracts (collection 1 was not tested due to lack of product)



EtOAc extracts (4th lane same as lane 1)



Hexane extracts (collection 3 was not tested, not required)

Figure 3: Difference in chemical composition

4.1.1 Reagent tests and results

The **Dragendorff reagent** was applied in order to detect the presence of alkaloids in the sample. Orange spots were observed post spraying the TLC plate with the reagent, displaying a positive result for the presence of alkaloids in the sample.

The **ninhydrin reagent** was applied in order to detect the presence of amino acid in the sample, no purple spots were seen post spraying the TLC plate with the reagent, displaying a negative or inconclusive result for the presence of amino acids in the sample.

The **phenol; sulfuric acid** test was conducted in order to detect the presence of carbohydrates in the sample. A colour change to orange indicated the presence of carbohydrates in the sample.

Figure 4:Presence of carbohydrates

4.1.1 Structure elucidation of Spinasterol (compound 1) and Schottenol (compound 2) found in the hexane extract

Flake like compounds (compound 1 and 2) were obtained from fractions 35-55 directly from the main hexane extract column, followed by NMR characterization. No further purification was needed. The compounds were isolated as a mixture due to their isomeric characteristic to one another. Spinasterol was the most abundant compound out of the two, making Schottenol the least abundant compound, thus the focus was on Spinasterol when analysing the NMR and GC-MS spectra. From the NMR data using ^{1}H-NMR, ^{13}C-NMR, COSY, and HMBC spectra the compounds and structures were identified and the data showed typical steroidal characteristics. The compounds in the mixture differ on the double bond positions since Spinasterol possesses a double bond at position 22 and Schottenol only contains one double bond at position 7 such as Spinasterol, and no other double bonds. The proton NMR spectral data displayed a signal at (δc 21) δ-0,962ppm (3H, d), J=6,8 Hz Me, a signal at (δc 22) δ5,09 ppm (H, dd) 8, 8 Hz & 15,2 Hz, a signal at (δc 23) δ4,96 (H, brs) 8,4Hz & 15,2Hz, another signal at (δc 26) δ0,78 (3H, d) J=6,4 Hz Me, another signal at (δc 27) 0,73 (3H,s), (δc 29) 0,75 (3H,t) J=7,2Hz Me, where found. These are the main structural characteristics of Spinasterol. ^{13}C NMR spectrum has 29 carbons for the Spinasterol structure. From this spectrum, two double bonds were found present as well as a secondary alcohol. A DEPT spectrum showed 3 quaternary carbon atoms and, 6 methyl resonances. See appendix for NMR Spectra of compounds 1, 2,3,4 and 5 and GCMS spectra for compounds 6,7 and 8.

Table 2: NMR interpretation of compound 1

Position/ position on the structure.	^{13}C-shift (ppm)	^{1}H-shift (integration, multiplicity), (ppm)	Most important *J* coupling	HMBC
1/C8	139,6 (C)	-	-	H-9, H-14
2/C22	138,1 (CH)	5,09 (H,dd) (mixture)	8,8Hz & 15,2Hz	H-20, H_3-21, H-23
3/C23	129,5 (CH)	4,96 (H, brs)	8,4Hz & 15,2Hz	H-20, H-22, H-24, H-25, H_2-28_a
4/C7	117,5 (CH)	5,09 (3H, dd)		H-a, H-14
5/C3	71,1 (CH)	3,53 (H, m)		H-1b, H-2b, H-4
6/C17	55,9 (CH)	1,187 (H, m)		H_2-12, H-15a, H_2-16, H_3-21, H-22
7/C14	55,1 (CH)	1,74 (H, m)		H-12b, H-15b, H_3-18
8/C24	51,2 (CH)	1,47 (H, m)		H-22, H-23, H-25, H-28, $H_3-26, H_3-26, H_3-27, H_3-29$
9/C9	49,5 (CH)	1,58 (H, m)		H-7, $H_2-12, H-14, H_3-19$
10/C13	43,3 (C)	-	-	$H_2-11, H12a, H-15b, H_2-16, H_3-18$
11/C20	40,8 (CH)	1,95 (H, m)		$H-21, H-22, H-23$
12/C5	40,3 (CH)	1,33 (H, m)		H-1b, $H_2-4, H-7, H_3-19$
13/C12	39,6 (CH_2)	1,14 & 1,93 (H, m)		H-9,H_3-18
14/C4	38,0 (CH_2)	1,65 & 1,20 (H, m)		H-5, H-2b
15/C1	37,2 (CH_2)	1,755 & 1,01 (H, m)		H_2-9, H_3-19
16/C10	34,2 (C)	-	-	H-1b, H-4b, H-2b, H_3-19
17/C25	31,9 (CH)	1,47 (H, m)		H-23, $H_3-26, H_3-27, H-28a$
18/C2	31,5 (CH_2)	1,73 & 1,31 (H, m)		H-1b, H_2-4
19/C6	29,7 (CH_2)	1,198 & 1,69 (H, m)		H-7
20/C16	28,5(CH_2)	1,68 & 1,201 (H, m)		H-12b, H-15a, H-17
21/C28	25,4 (CH_2)	1,34(H, m)		H-23, H_3-29
22/C15	23,0 (CH_2)	1,43 & 1,32(H, m)		$H_2-16, H-17$
23/C11	21,6 (CH_2)	1,51 & 1,40(H, m)		H-9, H_2-12
24/C21	21,4 (CH_3)	0,962 (3H, d)	J=6,8Hz Me	H-17, H-20, H-22
25/C26	21,1 (CH_3)	0,78 (3H, d)	J=6,4Hz Me	H_3-27
26/C27	19,0 (CH_3)	0,76 (3H, d)	J=6,0Hz Me	H_3-26
27/C19	13,0 (CH_3)	0,73 (3H, s)		H-1a
28/C29	12,2 (CH_3)	0,75 (3H, t)	J=7,2Hz Me	H_2-28
29/C18	12,0 (CH_3)	0,48 (3H, s)		H-12a, H-14, H-17

Research on phytoestrogens found in the *Pueraria* roots was conducted by Gook-che and associates (Daejeon, 2005). The roots were found to be mainly used as a rejuvenating folk medicine. Investigations on the toxicity of the Spinasterol in several cancer cell lines was conducted, and it was found that the *Pueraria* roots possess anti-tumor, anticancer activities and antiproliferative effects (Shattock, 2003).

4.1.2 Structure elucidation of Palmitic Acid (compound 3) and linoleic Acid (compound 4) found in the hexane extract.

Hex 5 fraction (compound 3 and 4) was obtained from fractions 16-26 taken from the main hexane extract column. Compounds with the same retention factor were combined followed by evaporation. The sample was further purified in order to isolate the desired compounds. To perform the isocratic elution, a smaller column was utilized and run using 100 % MeOH (methanol), where fractions 4, 5 and 6 were obtained, and screened with a solvent system of 80 % Hexane and 20 % EtOAc in the TLC plate tank. Moreover NMR characterization post isolation was conducted. The compounds were isolated as a mixture. In the mixture there were two compounds found, palmitic acid and linoleic acid. Palmitic acid was the most abundant compound out of the two, hence, the focus was on the palmitic acid when analysing the NMR Spectra and GC-MS spectra. From the ^{1}H-NMR, ^{13}C-NMR, DEPT, COSY, and HMBC spectra the compounds were identified to be of typical terpenes. The peaks identified as the "typical" fatty acid peaks are 2 triplets, a singlet, and a multiplet. at (δc 2) δ-1.40ppm (2H,t), (δc 16) δ-0,91ppm (3H,t), (δc 3) δ-2.32ppm (2H,s), (δc 15) δ-1.39ppm (2H, M). Spectra 9, 10,11,12 and 13 for NMR and spectra 14, 15 and 16 for GC-MS.

Table 3: NMR Interpretation of compound 3

Position/ position on the structure.	^{13}C-shift (multiplicity), (ppm)	^{1}H-shift (integration, multiplicity), (ppm)
1	178,39 (C)	-
2/C16	130,24 (CH_3)	0.91 (3H,t)
3/C15	33,76(CH_2)	1.39 (2H,m)
4/C14	31,90(CH_2)	1.31 (2H,m)
5/C13	31,54(CH_2)	1.31(2H,m)
6/C12	27,22(CH_2)	1.31(2H,m)
7/C11	29,15(CH_2)	1.31(2H,m)
8/C10	29,15(CH_2)	1.31(2H,m)
9/C9	29,15(CH_2)	1.31(2H,m)
10/C8	29,15(CH_2)	1.31(2H,m)
11/C7	29,15(CH_2)	1.31(2H,m)
12/C6	29,15(CH_2)	1.31(2H,m)
13/C5	29,15(CH_2)	1.31(2H,m)
14/C4	29,15(CH_2)	1.31(2H,m)
15/C2	29,15(CH_2)	1.40 (2H,t)
16/C3	29,15(CH_2)	2.32 (2H,s)
17	OH	9.78

Palmitic acid was found in marine red algae, *Amphiroa zonata*. It was found to contain selective cytotoxicity to human leukemic cells by inducing apoptosis. It was also found that palmitic acid does not affect DNA topoisomerase II, which then suggests that palmitic acid may be a lead for anti-cancer drugs (Jeon *et al.*, 2005).

4.1.3 Compounds found in EtOAc and BuOH extracts

Post fractionization of the columns, eight fractions were chosen. Withing these eight, five fractions were collected from the column eluent with EtOAc, and three were collected from the column eluted with BuOH. The fractions were then prepared, and submitted for LC-MS analysis. The below fractions were selected according to the number of compounds viewed on TLC. These possessed low value yields, after the attempt on the isolation of compounds. View **Table 4** .

Table 4: EtOAc and BuOH fractions sent to LC-MS

Fraction name	Extract	Weight
C13-C14 (91-110)	BuOH	5,5mg
K9 (208-215)	EtOAc	5,6mg
C17 (96-102)	BuOH	8,5mg
K29 (128-140)	EtOAc	9,2mg
K32 (181-190)	EtOAc	9,9mg
K33 (201-207)	EtOAc	7,4mg
LV3	EtOAc	0,6mg
C13-C14 (4-7)	BuOH	0,03mg

A set of LC-MS data was received from the University of Stellenbosch post analysis of the eight given samples. Compounds were characterized utilizing the following data bases: Global natural products social molecular network, FoodDB, STOFF (environment), KNApSAcK (Natural product), PubChem (biomolecules), PlantCyc (plant), ChBI (Biomolecules), NANPDB (Natural product), UNPD (Natural Product)

Table 5: LC-MS of EtOAc and BuOH extracts

Compounds Found			
Compound number	**Average Rt (min)**	**Average Mz**	**Structure Rank**
5	3.289	121.02876	Benzoic Acid
6	4.621	225.11133	Tuberonic acid
7	4.621	243.12206	Pandangolide 1a
8	5.632	267.02768	Coumesterol
9	4.338	269.04468	Apigenin
10	4.977	281.08237	5,7-Dimethoxyflavone
11	5.082	285.03842	Kaempferol
12	4.977	293.08362	Dehydrocycloguanandin
13	4.389	299.05392	Chrysoeriol
14	4.674	313.07132	Cirsimaritin
15	6.561	313.12134	Cribrostatin 5
16	4.992	341.10123	4',5,6,7-Tetramethoxyflavone
17	4.988	353.10263	(+)-Sesamin
18	4.988	371.11349	Sesamolinol
19	4.108	403.1041	Chalconaringenin 2'-xyloside
20	4.07	419.17023	Eupaformosanin
21	5.029	431.13525	Medicocarpin
22	3.378	431.20508	Schizandrin;(+)-Schizandrin;Schisandrol A;Wuweizichun A
23	3.697	441.215	Jatamanvaltrate G;(-)-Jatamanvaltrate G
24	10.819	457.32974	Platanic acid
25	2.918	467.16714	Varixanthone
26	3.543	471.2012	Kushenol H
27	5.609	477.11978	Phelligridin F;(-)-Phelligridin F
28	4.108	483.16541	Diversolide G
29	5.497	495.12512	(-)-(7"R,8"S)-4",5,7-trihydroxy-3',5'-dimethoxy-4',8"-oxyflavonolignan-7",9"-diol
30	6.775	495.26041	Ninandrographolide;Andropanoside
31	10.166	499.3389	Poricoic acid H;(+)-Poricoic acid H
32	7.19	503.33783	Myrianthic Acid
33	4.116	513.17542	Icariside II;Baohuoside 1;3-[(6-Deoxy-alpha-L-mannopyranosyl)oxy]-5,7-dihydroxy-2-(4-methoxyphenyl)-8-(3-methyl-2-butenyl)-4H-1-benzopyran-4-one
34	4.337	539.09674	Volkensiflavone
35	4.812	555.21954	Rubriflorin A
36	5.236	585.2337	Baccatin III;(-)-Baccatin III

After obtaining the LC-MS data sheet, the raw files provided by Mr Taylor from the University of Stellenbosch were placed in the Global natural products social molecular network database and the following compounds were found.

Table 6: Global Natural Products Social Molecular Network database compounds

Compound number	Average Mz	Peak	Structure rank	Spectra number
37	621.109	269.0	4'-O-GlcA-7-OGlcA Apigenin	17
38	769.22	300.2	7-O-Methylquercetin-3-O-galactoside-6''-rhamnoside	18
39	621.341	109.0	3-O-protocatechuoyl ceanothic acid	19

Insufficient literature on 4'-O-GlcA-7-OGlcA apigenin and 7-O-methylquercetin-3-O-galactoside-6''-rhamnoside was provided on the database and various others besides the certitude of them being natural products.

4.1.4 Literature review on compounds obtained through LC-MS

3-O-protocatechuoyl ceanothic acid

3-O-protocatechuoyl ceanothic acid,is found in plants and foods such as olives. This compound possesses antioxidant, anti-inflammatory, anti-apoptotic, antihyperglycemic activities (Semaming *et al.*, 2015).

Benzoic Acid

Benzoic Acid is also known as phenylformic acid. It is an aromatic carboxylic acid naturally present in plants and animal tissues. This may be found in some berries(cranberries, strawberries), prunes, apples, yogurt, cayenne pepper, and other foods. Benzoic Aacid prevents

infections caused by bacteria hence acting as an antibacterial and antifungal. Benzoic acid can treat skin irritations and inflammation caused by insect bites, burns, or eczema, they also possess antioxidant properties, are used as flavouring agents in food, used in the production of cosmetics, hygiene and other pharmaceutical products. They are active ingredients in pesticide products and biocidal products in veterinary hygiene .(Khotimchenko, Vaskovsky en Titlyanova, 2002).

Tuberonic Acid

Tuberonic acid is an oxo monocarboxylic acid, and it can be found in jasmonic acid in potatoes. They act as antimicrobials, possess insecticidal functions and they regulate the defence mechanisms (del Olmo, Calzada en Nuñez, 2017).

Pandangolide 1a

Pandangolide 1a has been isolated from the Cladosporium fungus which was extracted from the red sea sponge *Niphates rowi*, and it is said to possess anti-cancer and anti-inflammatory activities (Wakuta *et al.*, 2010).

Coumesterol

Coumesterol is a phytochemical organic compound, first identified in *ladino clover* and *alfalfa*. It can also be found in soybeans, brussels sprouts, spinach, and many other different legumes. Coumesterol mimics the bioactivity of estrogen, it has antioxidant properties, and it's an anti-inflammatory agent. Coumesterol also has the ability to decrease bone resorption and promote mineralization of the bone. Daily injections of coumesterol have shown to reduce bone loss in rats. It also decreases ovarian weight and increases apoptotic cell death in ovaries of adult rats exposed during lactation (Hill, 2006).

Apigenin

Apigenin is found in a variety of plants such as, fruits, vegetables, and Chinese herbs. It possesses anti-inflammatory, antioxidant, antibacterial, and antiviral functions and it is also used as a blood pressure reducer. Apigenin also triggers cell apoptosis and cell autophagy, induces cell cycle arrest, suppresses the cell migration and invasion in the organs and it helps stimulate immune responses being a great candidate for an anti-cancer agent (Moon *et al.*, 2009).

5,7-Dimethoxyflavone

5,7-Dimethoxyflavone, is found in *Kaempferia parviflora*. The *Kaempferia parviflora* has been found to possess various types of activities such as; cellular metabolism-regulating, anti-cancer, cardioprotective, neuroprotective, antiallergic, anti-inflammatory, due to the 5,7-dimethoxyflavone which is the specific compound responsible for its anti-inflammatory activities (Yan *et al.*, 2017).

Kaempferol

Kaempferol is a polyphenol flavonoid found in a variety of plants such as, kale, beans, spinach, and broccoli. It reduces the risk of chronic diseases such as cancer by augmenting the human body's antioxidant defences against free radicals. It also inhibits apoptosis, angiogenesis, inflammation, and metastasis (Chen *et al.*, 2018).

Dehydrocycloguanandin

Dehydrocycloguanandin was isolated from the mangrove plant and it is known to have antinociceptive, anti-inflammatory, and antipyretic activities (Chen en Chen, 2013).

Chrysoeriol

Chrysoeriol is an anti-inflammatory and diabetic control activity containing flavonoid found in the methanolic extract of the *Cardiospermum halicacabum* and *Gladularia selloi.* This compound also shows activities of plasma reduction in glucose, hemoglobin, and glycosylated hemoglobin, while rising the plasma insulin sensitivity (Shilpi *et al.*, 2012).

Cirsimaritin

Cirsimaritin is a strong antioxidant, antimutagen, antibacterial compound found in *Baccharis trimera less* also known as Carqueja, a folk medicine commonly used in Brazil to prevent cancer and increase antimutagens and antitumor promoting activities, it is also found in *Rosmarinus officinalis L*. and other medicinal plants (Krishnan *et al.*, 2020).

Cribrostatin 5

Cribrostatin 5 is one of the five Cribostratin compounds found and isolated from the marine sponge *Cribrochalina* species in the Republic of the Maldives. Compounds, Cribostratins 3, 4, and 5 provide cancer cell line inhibitory activities, antibacterial and antifungal activities. Cribostratins 1-5 are also sources for strong antibiotics production due to their antibiotic activities. (Nakasugi en Komai, 1998)

4',5,6,7-Tetramethoxyflavone

4',5,6,7-Tetramethoxyflavone, isolated from the peels of the *Citrus aurantium* and *Citrus reticulata blanco cv. Ponkan,* possesses antimutagenic, anticarcinogenic, and antitumor activity (Pettit *et al.*, 2000) which show antiproliferative activities against cancer cells (Chen, Montanari en Widmer, 1997).

(+)-Sesamin

Sesamin, isolated from the *Sesamum indicum* also known as sesame seeds/ sesame oil, possesses antioxidant, anti-diabetic, anti-obesity, anti-thrombotic, anti-hypertensive, and anti-inflammatory properties preventing cardiovascular diseases. (Du en Chen, 2010).

Sesamolinol

Sesamolinol like Sesamin isolated from the *Sesamum indicum,* possesses antioxidant, and anti-inflammatory properties. (Dalibalta, Majdalawieh en Manjikian, 2020).

Chalconaringenin 2'-xyloside

Chalconaringenin 2'-xyloside was isolated from the ethanol extract of the Uzbek medicinal plant *Helichrysum maracandicum*, it contains anticarcinogenic properties and antiproliferative activity (Taylor, no date).

Eupaformosanin

Eupaformosanin, isolated from *Eupatorium formosanum* (Lee *et al.*, 1977), is an antitumor, antileukemic, and antisarcoma agent with the purpose of affecting the nucleic acid and the protein of Ehrlich Ascites carcinome cells(Yagura *et al.*, 2008). Eupaformasanin also acts as

an antineoplastic agent in the cells metabolisms inhibiting deoxyribonucleic acid synthesis (Lee *et al.*, 1977).

Medicocarpin

Medicocarpin is a flavonoid derivative from the *Ononis Sicula Guss* species mainly found in Egypt and from the *Glycyrrhiza pallidiflora* hairy root species. It is utilized in skin care products due to its anti-aging, anticancer and antioxidant properties (Oliveira en Rodrigues, 2018).

(+)-Schisandrin;Schisandrol A;Wuweizichun A

Schisandrol A has been isolated from the Schisandra Chinensis bail fruit (Kwon *et al.*, 2018). Schisandrin or otherwise called Schisandrol A is the main active ingredient in the fruit, and it has been studied and found to possess anti-inflammatory, and antioxidant effects, it has a protective effect on induced sepsis lipopolysaccharides (Hall *et al.*, 1980).

Jatamanvaltrate G

Jatamanvaltrate G was isolated from the *Valeriana jatamansi* plant species and it is considered an iridoid compound (Ma *et al.*, 2021). Iridoids from the *Valeriana jatamansi* contain anti-inflammatory and anti-proliferative properties Jatamanvaltrate G being one of them. These are also known to inhibit the proliferation of human glioma stem cell lines (Yu *et al.*, 2008).

Platanic Acid

Platanic Acid, isolated from the *Syzigium claviflorum* leaves, and found to contain anti-HIV activity (Kahnt *et al.*, 2018) . It is an inhibitor of HIV replication in the H9 lymphocyte cells in the human body and an anti-AIDS agent(Liu *et al.*, 2021).

Varixanthone

Varixanthone, isolated from the *Emericella variecolor* marine Fungus and this compound shows antimicrobial activity (From en Clavzflqrum, 1994).

Kushenol H

Kushenol H, isolated from the dried roots named Kushen ("Kujinn") of the *Sophorae flavescens* plant species, original to Japan, have been utilized as Chinese and Japanese traditional medicine for centuries. The Kushenols O - A are known flavonoids known to constitute antibacterial and antiandrogen activities (Malmstrøm *et al.,* 2002).

Phelligridin F;(-)-Phelligridin F

Phelligridin F, isolated from the *Inonotus xeranticus* mushroom species is a new hispidin derivative. This compound was found to have antioxidant activities (Kuroyanagi *et al.,* 1999).

Diversolide G

Diversolide G isolated from the *Ferula diversivittata,* is a cancer chemo-preventive agent in humans. (Lee *et al.,* 2006).

(-)-(7''R,8''S)-4'',5,7-trihydroxy-3',5'-dimethoxy-4',8''-oxyflavonolignan-7'',9''-diol

(-)-(7"R,8"S)-4",5,7-trihydroxy-3',5'-dimethoxy-4',8"-oxyflavonolignan-7",9"-diol is a flavonolignan found in the *Calamus quiquesetinervius* plant/vegetable native of Northern Taiwan. This phenolic compound was found to have cardiovascular protective activities (Iranshahi *et al.,* 2010).

Ninandrographolide;Andropanoside

Andropanoside or otherwise known as Ninandrographolide is a compound found in the Andrographis paniculate species and these were produced by glucosylation at the C19-hydroxyl. This compound is known to be an anti-inflammatory, anti-cancer, immunomodulatory, antimalarial, antidiabetic, fever combating, laryngitis, gastric infections reduction agent, and many more(Chang *et al.,* 2010).

Poricoic acid H;(+)-Poricoic acid H

Poricoic acid H has been isolated from the Poria cocos herb species which originated from China and is used as a prescribed diuretic and as a sedative. Poricoic acid H was found to inhibit

cytotoxicity to all cancer cell lines and other tumor-promoting effects in conjunction with the other poricoic acids (Sun *et al.*, 2019).

Myrianthic Acid

Myrianthic Acid is a terpenoid isolated from the *Vaccinium emarginatum* plant species and it is found to have cytotoxic and anti-inflammatory activities (Ukiya *et al.*, 2002).

Icariside II;Baohuoside 1;3-[(6-Deoxy-alpha-L-mannopyranosyl)oxy]-5,7-dihydroxy-2-(4-methoxyphenyl)-8-(3-methyl-2-butenyl)-4H-1-benzopyran-4-one

Icariside II is an icariin metabolite that derives from the *herba Epimedii*. This compound is known to induce apoptosis in the A431 human epidermoid carcinoma cells (Tu *et al.*, 2020).

Volkensiflavone

Volkensiflavone was isolated from both *Garcinia multiflora* and *Rhus succedanea* plant species. This compound is known to have antiviral activities against respiratory viruses such as influenza A and B, parainfluenza type 3, measles, respiratory syncytial and adenovirus type 5, and herpes viruses(Wu *et al.*, 2013).

Rubriflorin A

Rubriflorin A has been isolated from the stems of the *Schisandra rubriflora* and it is a dibenzocyclooctene lignan. This compound is known to possess anti-tumor and anti-cancer activities (Lin *et al.*, 1998).

Baccatin III;(-)-Baccatin III

Baccatin III was isolated from the heartwood of *Taxus baccata L.* and it's considered to be a taxoid. Anti-inflammatory and antinociceptive activities are medicinal characteristics of this compound. This was tested in vivo (Li *et al.*, 2004).

Chapter 5

5.1 Conclusion

The assessment of the phytochemistry and the identification of several of the compounds present in the *Uromycladium tepperianum* MacAlpine fungus was important, due to the lack of knowledge and, literature reports on this fungus. Since rust fungi are mostly known to be diseases which affect plants, this investigation may be deemed as a first of its kind and may now be documented.

Moreover, the investigation conducted on the *Uromycladium Tepperianum* MacAlpine fungus was found to provide unexpected results since, apart from certain yeasts and moulds, mushrooms are the only types of fungi widely reported on the contribution towards medicinal treatments, and drug developments, unlike rust fungi, which are considered to be plant diseases with no medicinal uses. A detailed investigation on the phytochemistry of this fungus was conducted, leading to the isolation of 39 simple and complex compound structures. Compounds 1, 2, 3 and 4 were characterized by utilizing NMR GC-MS and MS (Spinasterol, Schottenol, Palmitic acid and Linoleic acid), compounds 37, 38 and 39 (4'-O-GlcA-7-OGlcA apigenin , 7-O-Methylquercetin-3-O-galactoside-6''-rhamnoside and 3-O-protocatechuoyl ceanothic acid) were characterized by utilizing LC-MS and the Global natural products social molecular network database. Lastly compounds 5 to 36 were characterized by utilizing LC-Ms with the aid of various other databases.

The majority of the bioactive compounds found in this fungus possessed isomeric characteristics with extremely low yields, making isolation and characterization in NMR exceedingly difficult. Since NMR requires at least 5mg of sample to confirm the organic purity and characterization of the product, LC-MS and various data bases [Global natural products social molecular network, FoodDB, STOFF (environment), KNApSAcK (Natural product), PubChem (biomolecules), PlantCyc (plant), ChBI (Biomolecules), NANPDB (Natural product), UNPD (Natural Product), Elemental composition, Pubchem, Chemspider Science direct, Synapt G2 qTOF, MZmine, Chem Calc, and Science finders Scholar] were utilized to isolate and characterize the remaining compounds.

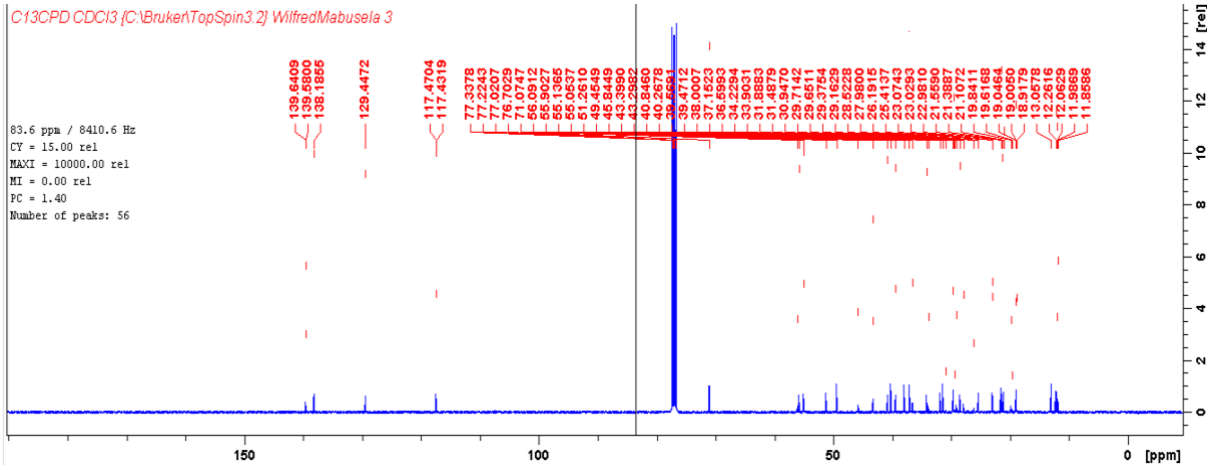
3.5.5 Future recommendations

A sizeable amount of material is required (of at least 20 kgs) in the future for further investigation or confirmation of compound structures, as to be able to obtain higher amounts of the organic constituents present in the fungus for better separation, isolation, purification and characterization.

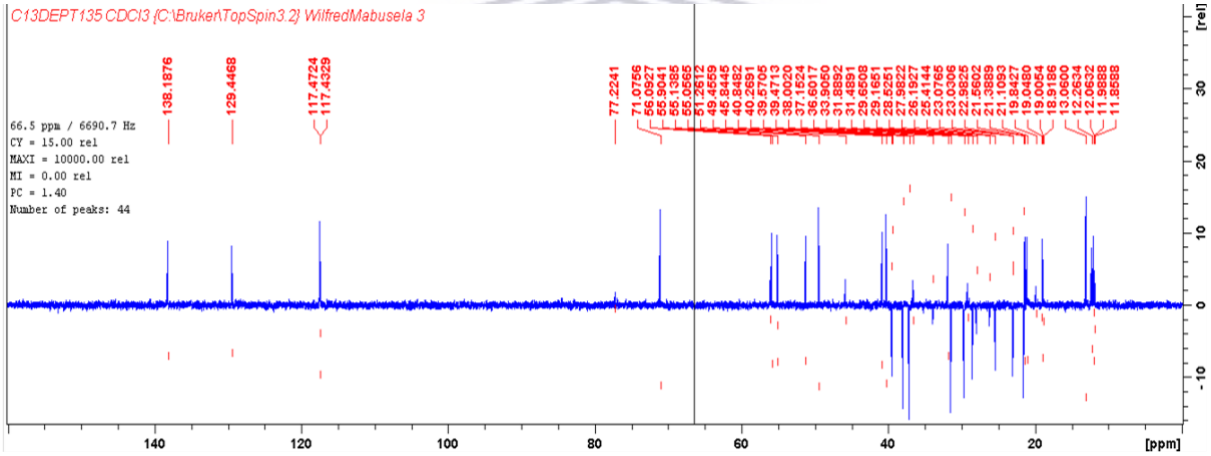
Further research is necessary to investigate and compare the phytochemistry and chemical composition of this fungus when collected in different seasons and environments on all four extracts (*n*-hex, EtOAc, DCM and BuOH) using HPLC as a method for seasonal profiling of the compounds present in the fungus.

Appendix

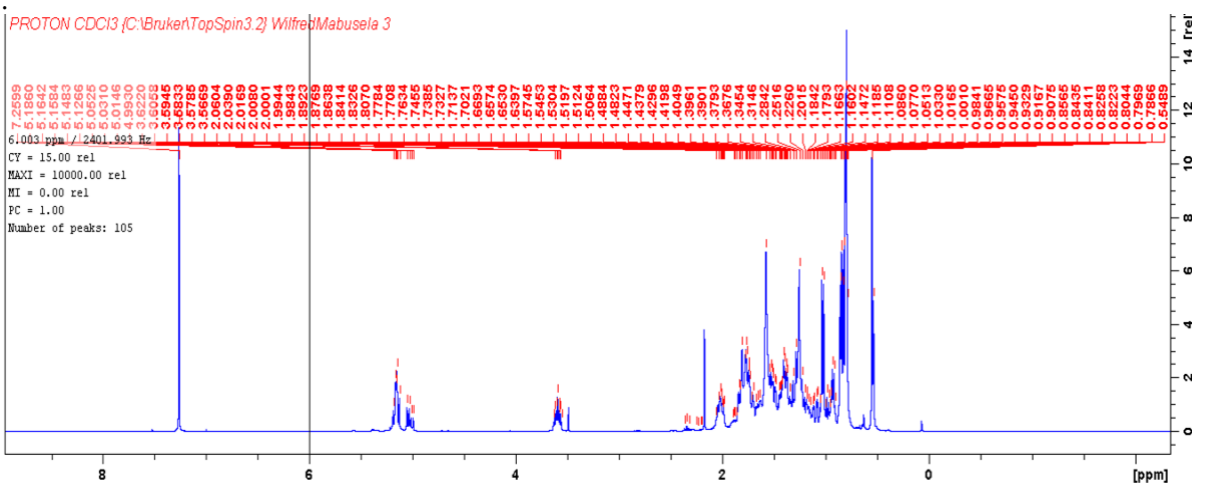
NMR Spectra (compound 1 and 2)



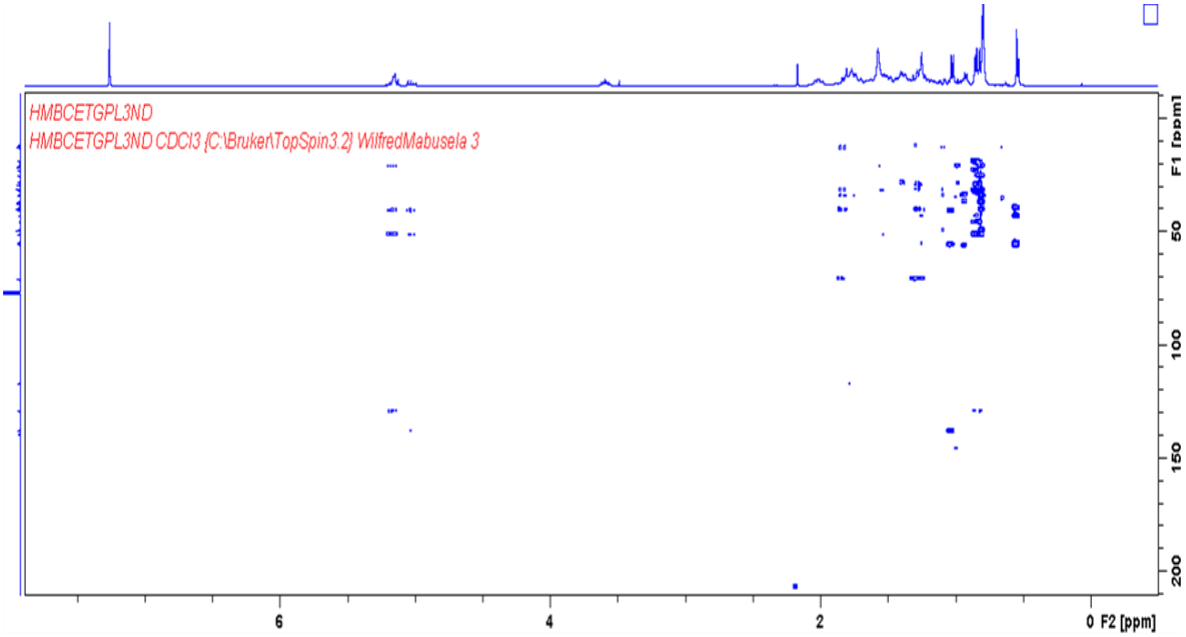
spectra 1:Spectra C13 compound 1 and 2.



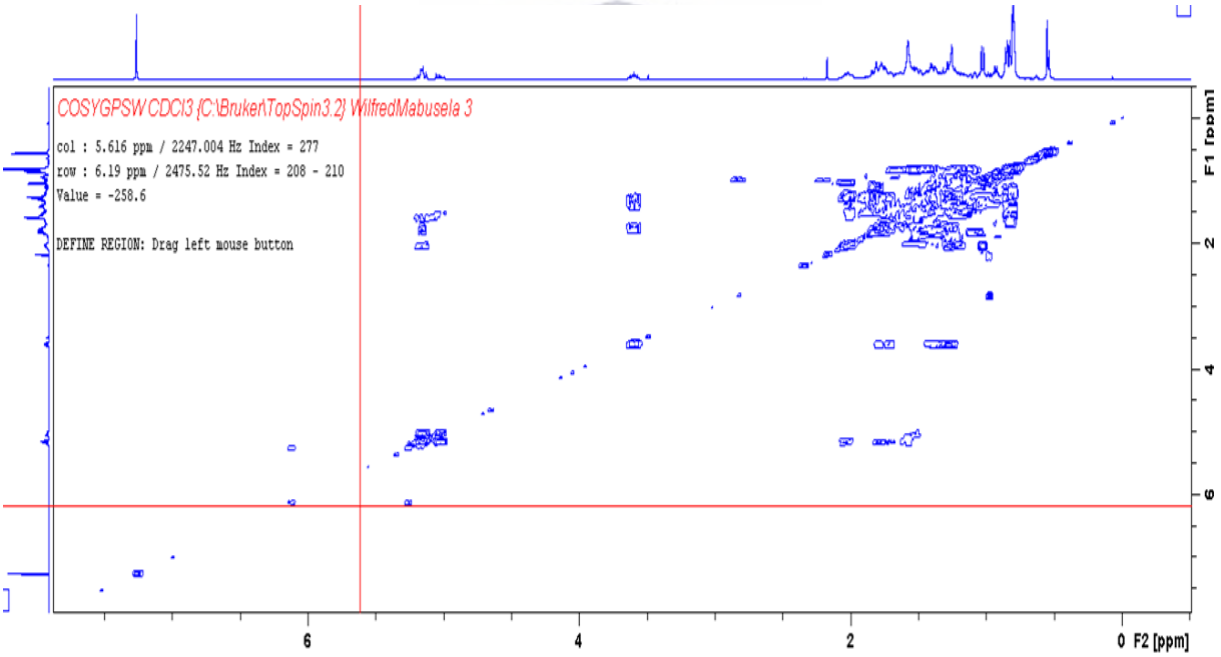
spectra 2:Spectra DEPT compound 1 and 2



spectra 3:Spectra Proton compound 1 and 2.

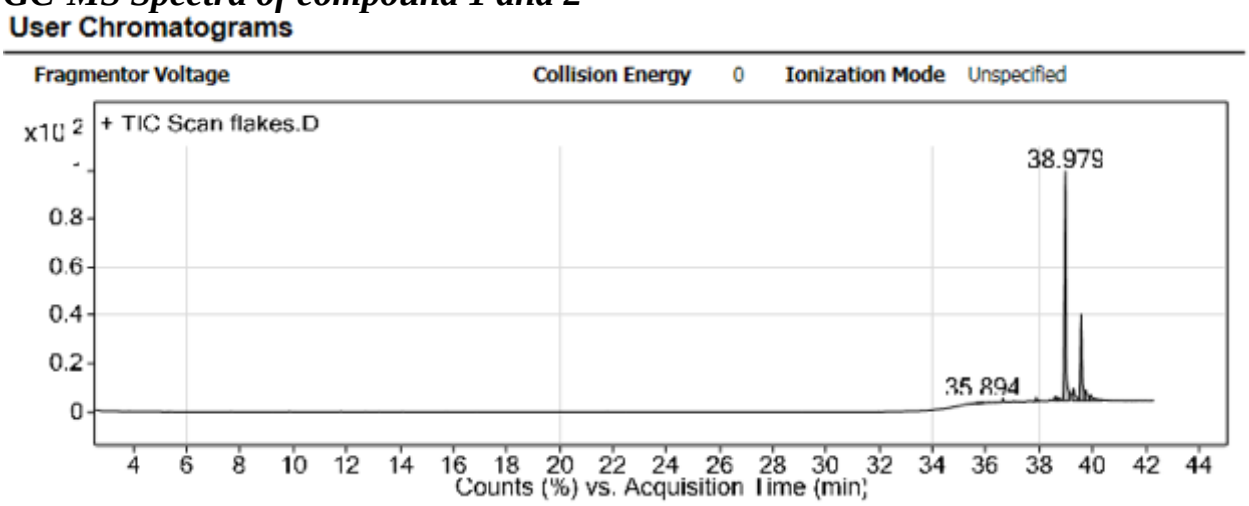


spectra 4:Spectra HMBC compound 1 and 2.

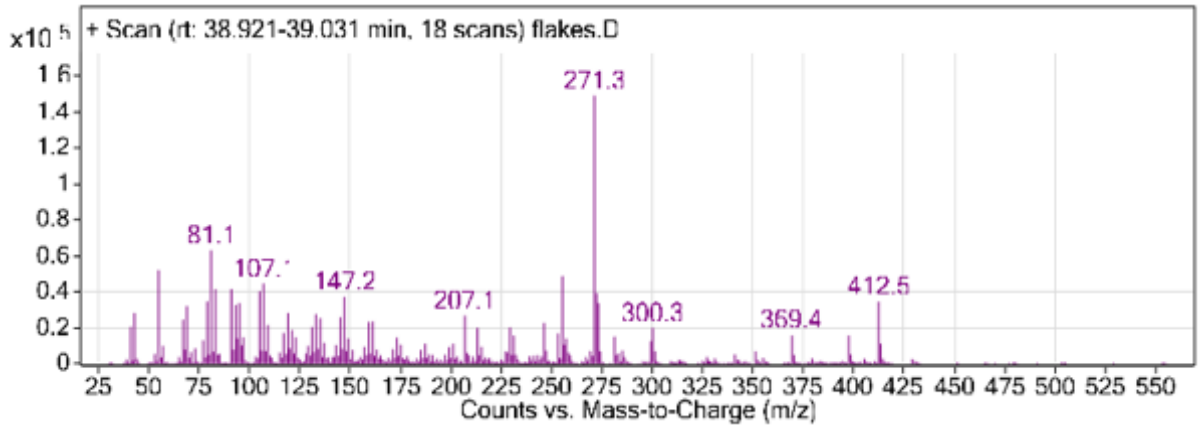


spectra 5:Spectra Cosy compound 1 and 2.

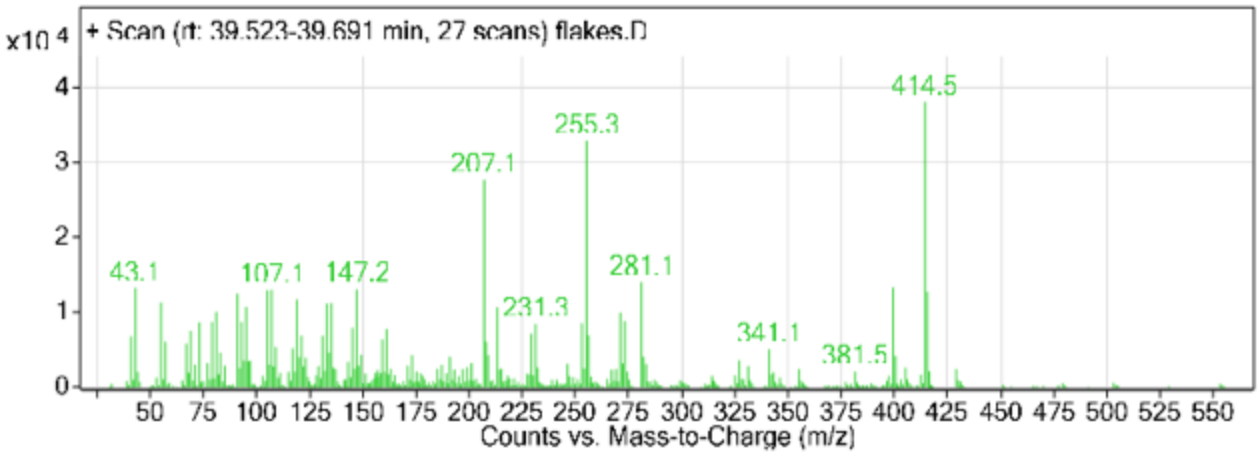
GC-MS Spectra of compound 1 and 2



spectra 6:GC-MS Spectra visualization of both compound 1 and 2. (mixture)

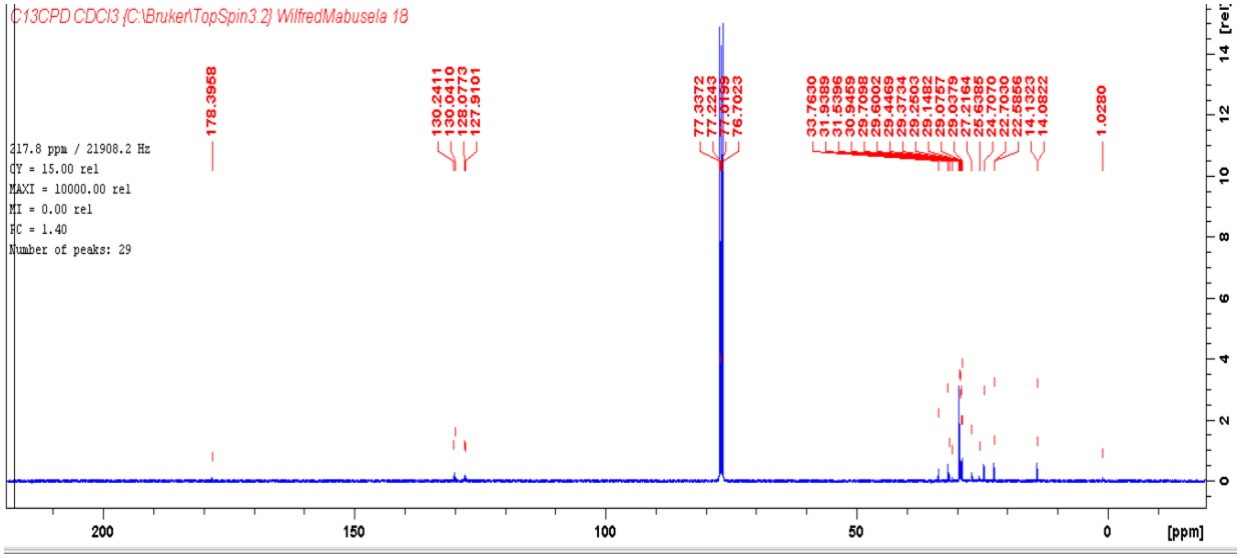


spectra 7:GC-MS Spectra molar mass of Spinasterol compound 1

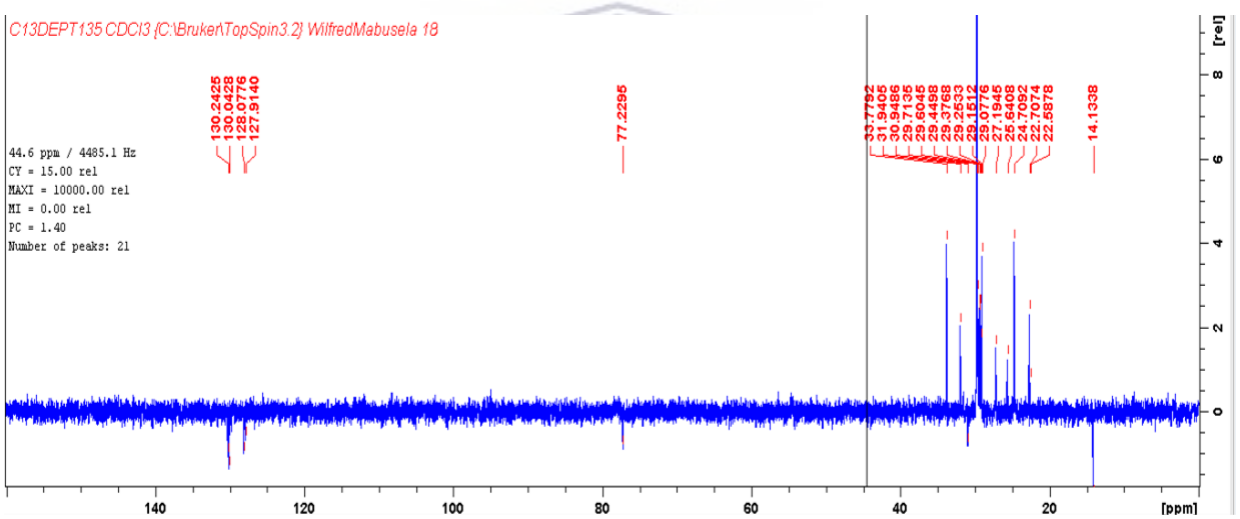


spectra 8:GC-MS Spectra molar mass of Schottenol compound 2.

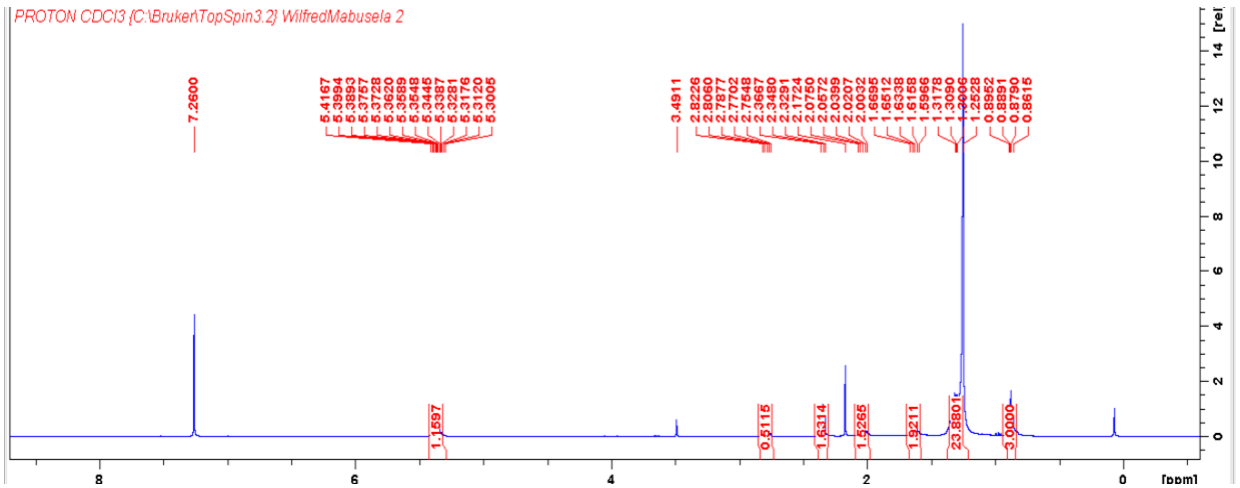
NMR Spectra compound 3 and 4



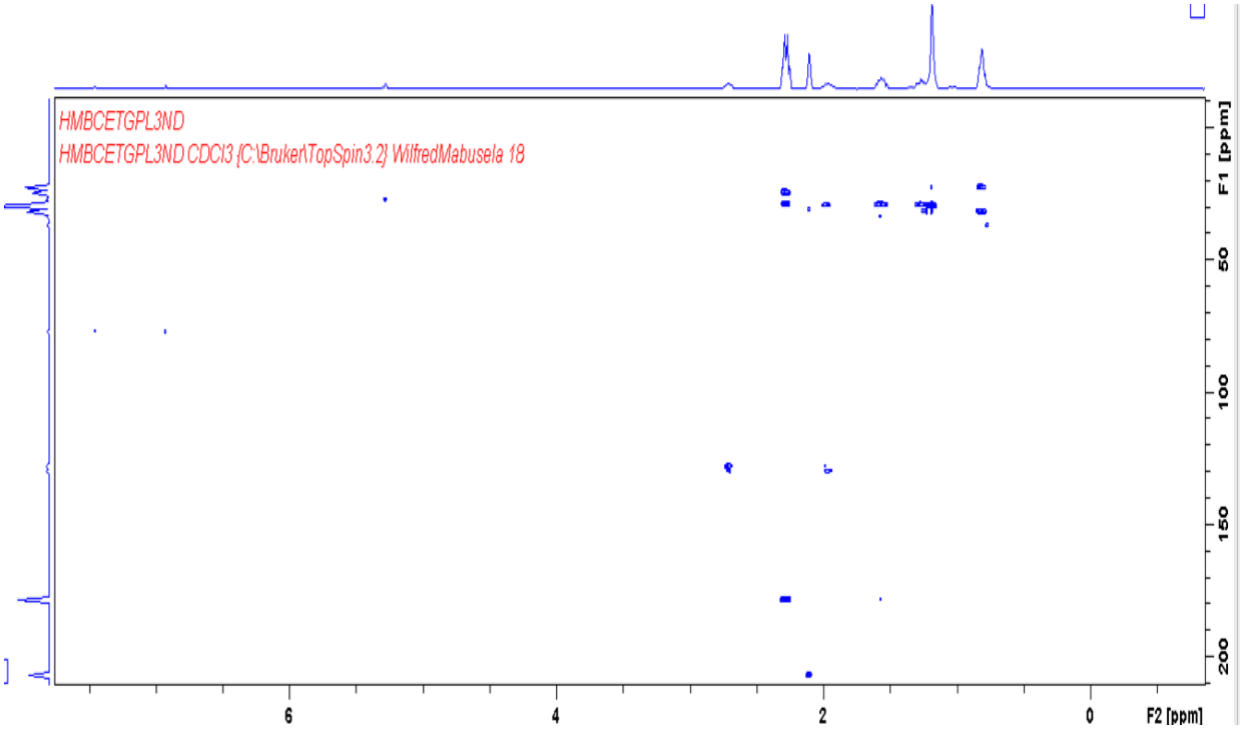
spectra 9:Spectra C13 compounds 3 and 4



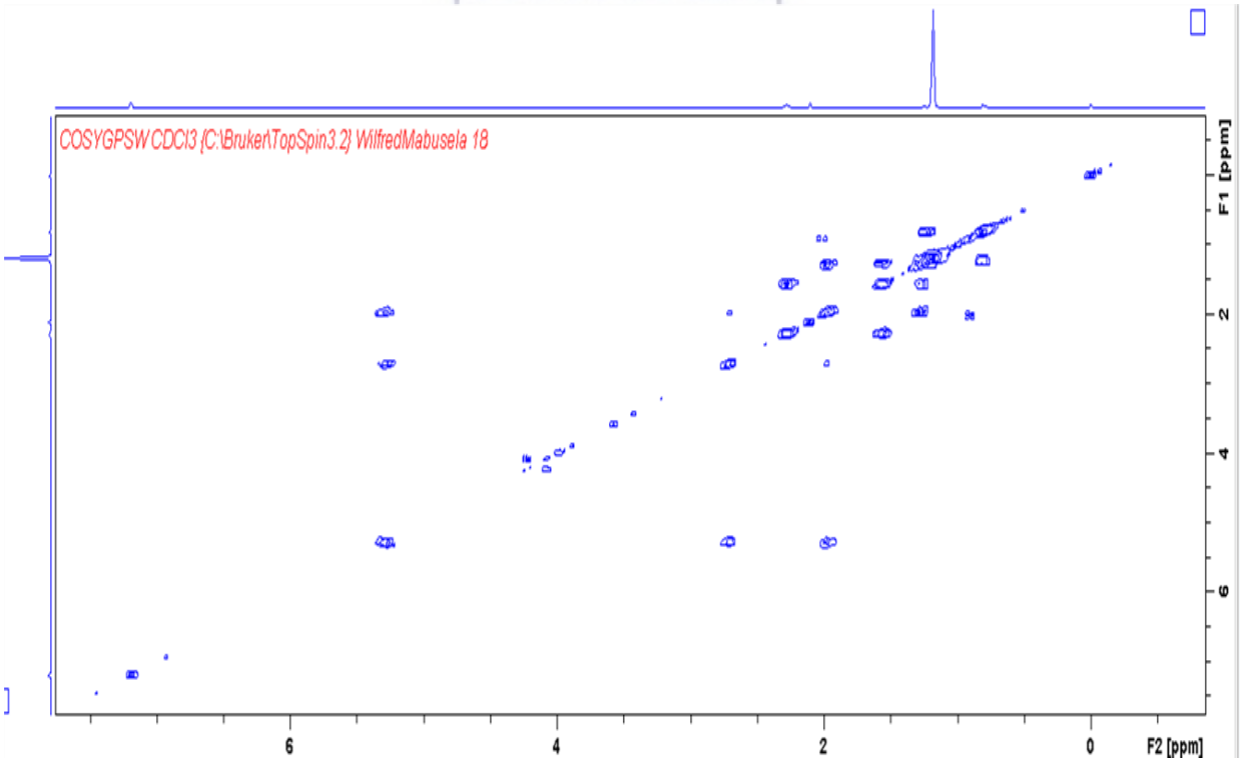
spectra 10:Spectra DEPT compounds 3 and 4



spectra 11:Spectra Proton compounds 3 and 4

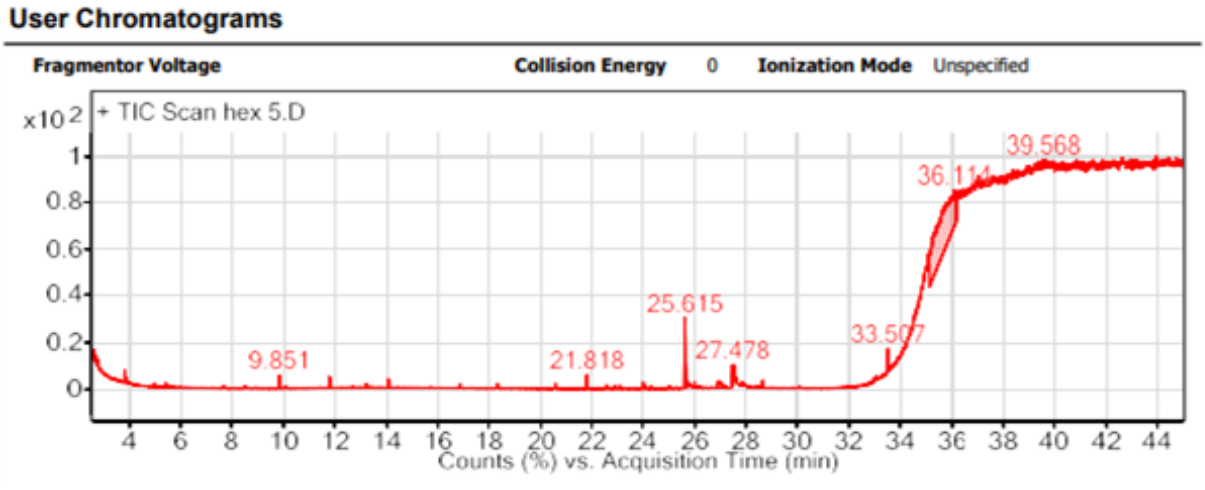


spectra 12:Spectra HMBC compounds 3 and 4

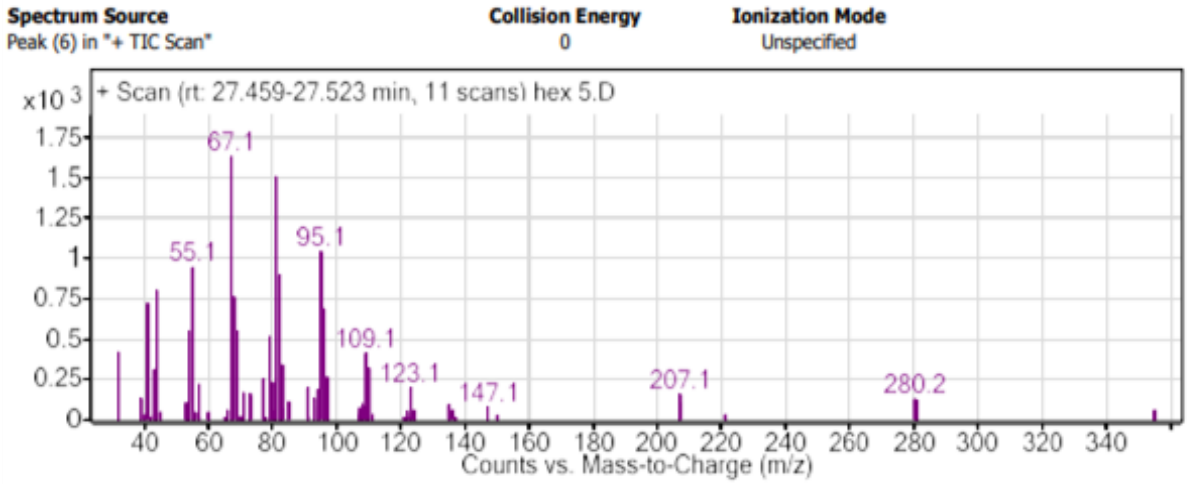


spectra 13:Spectra COSY compounds 3 and 4

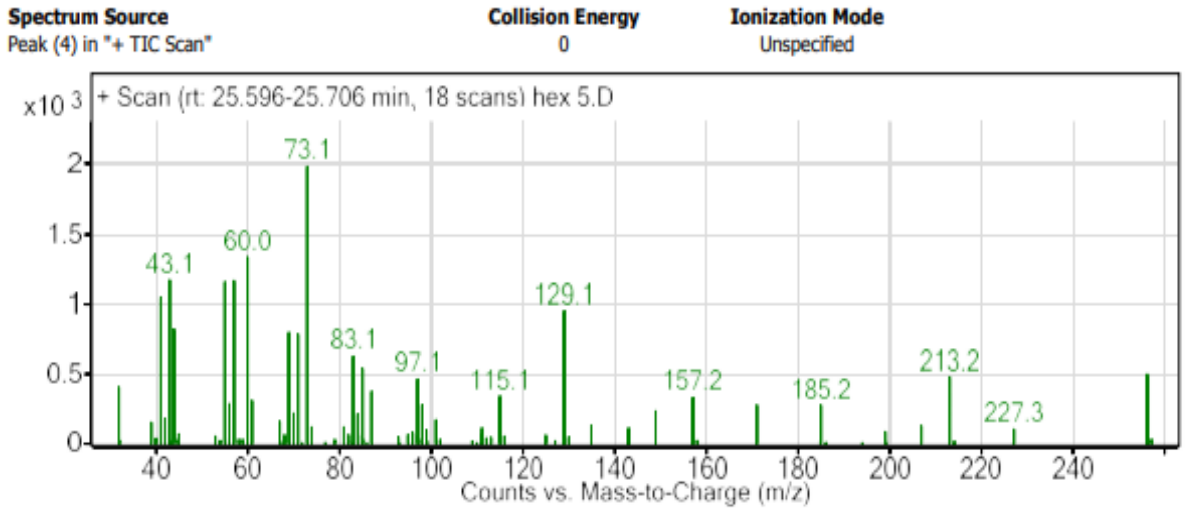
GC-MS spectra compounds 3 and 4



spectra 14:GC-MS Spectra visualization of compounds 3 and 4 mixture.



spectra 15: GC-MS Spectra molar mass of linoleic Acid compound 4.



spectra 16:GC-MS Spectra molar mass of Palmitic acid compound 3.

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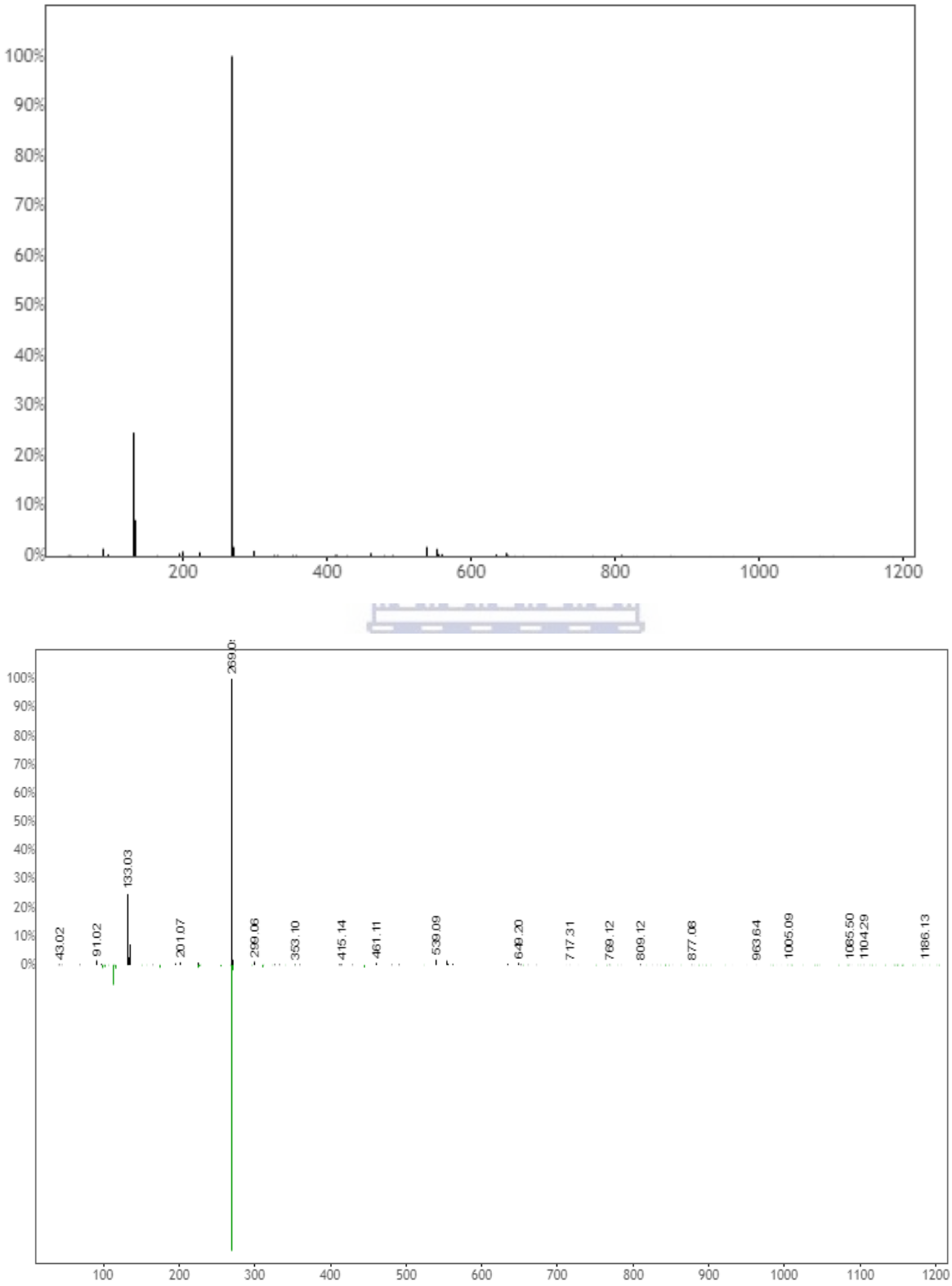
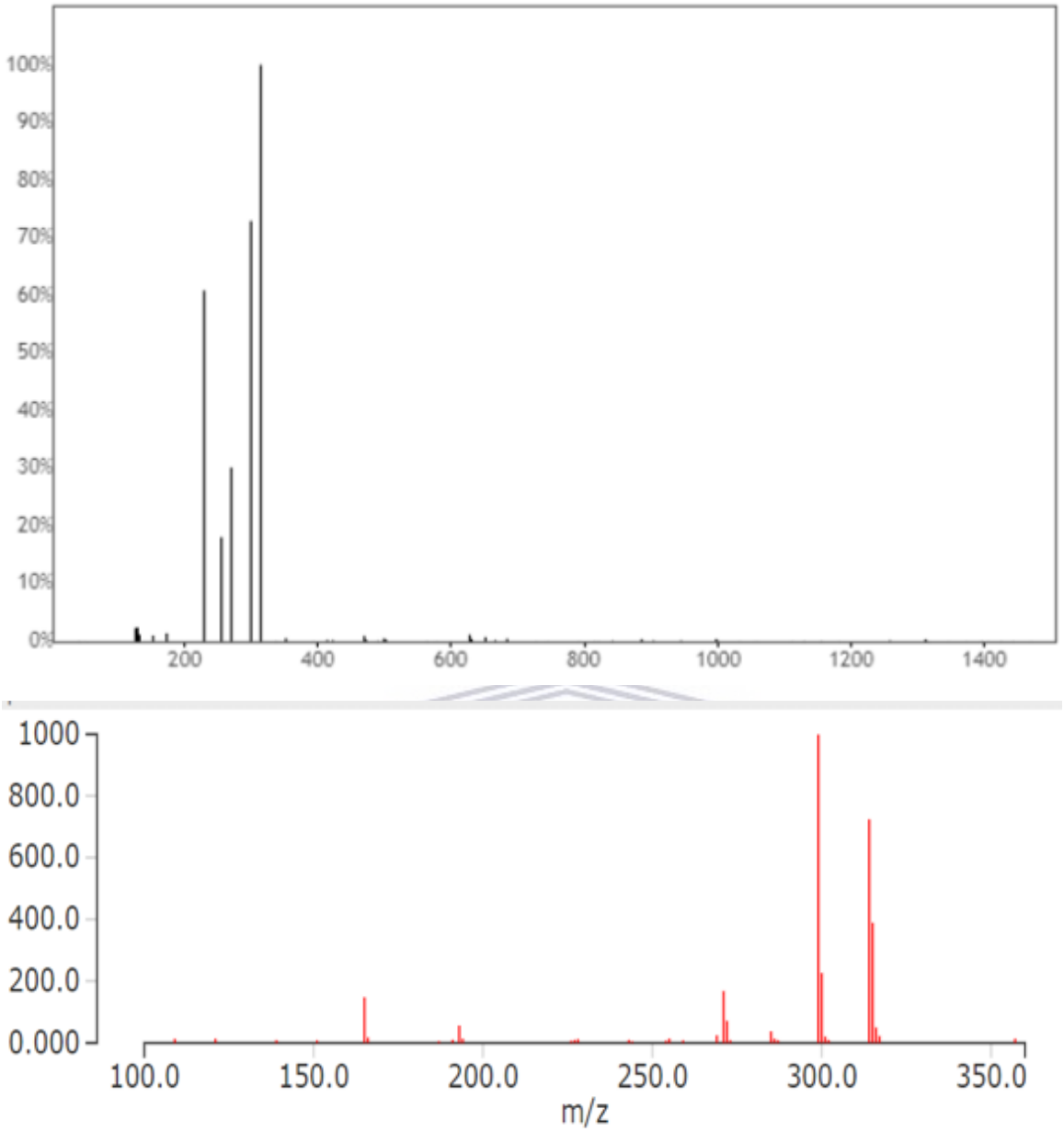
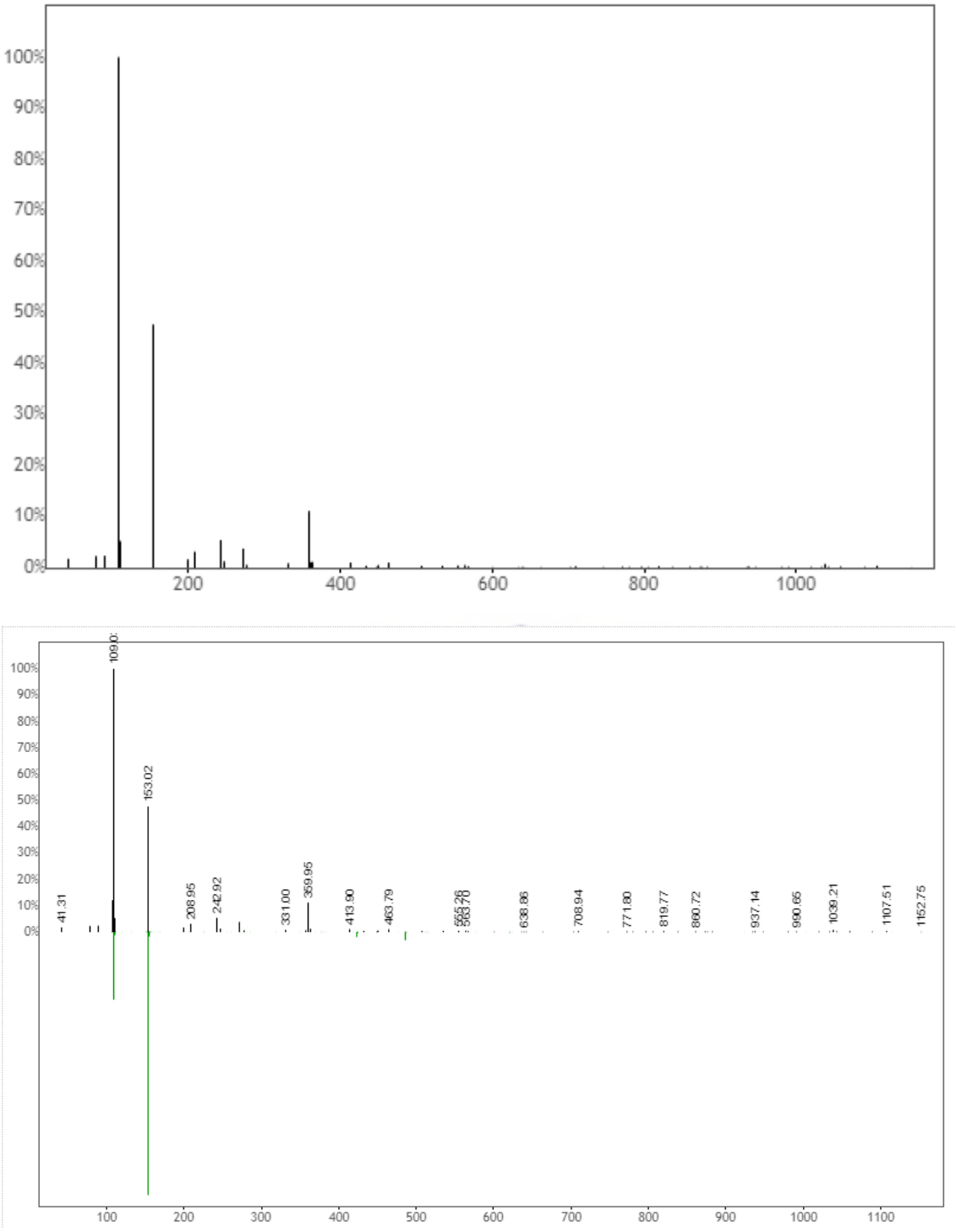


spectra 17: compound 37 peaks



spectra 18: compound 38 peak



spectra 19: compound 39 spectra.

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