IDENTIFICATION OF CHEMICAL CONSTITUENTS IN THE EXTRACTS OF *PLEUROTUS GIGANTEUS* (BERK.) KARUNARATHNA & K. D. HYDE

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ABSTRACT

Pleurotus giganteus mushroom is used for culinary purposes by the indigenous people (Orang Asli) in Malaysia for food. The present study was undertaken to extract and profile the bioactive metabolites in the fruiting bodies of the cultivated mushroom. Various chemical solvents were used in this investigation because different compounds were extracted by different solvents based on the solvent's polarity. The solvents employed were methanol, hexane, ethyl acetate, distilled water and butanol. The ethyl acetate and hexane extracts were then subjected to flash column chromatography with stepwise addition of hexane and acetone. The identification of compounds in the sub-fractions from ethyl acetate and hexane extracts were done using GC-MS and LC-MS/MS analyses. Twenty seven secondary metabolites namely fatty acids such as palmitic acid and oleic acid; fatty acid methyl esters such as methyl linoleate; phenolics such as caffeic acid and cinnamic acid; sterols such as ergosterol and neoergosterol; organic acids such as succinic acid and alkaloids such as *N*-acetylphenylethylamine were identified. Extraction of the flavour components (essential oil) was also performed. Benzyl alcohol was identified to be present in the essential oil. To our knowledge this is the first report of chemical components from the fruiting bodies of *Pleurotus giganteus.*

ABSTRAK

Cendawan *Pleurotus giganteus* digunakan oleh orang asli di Malaysia sebagai makanan. Kajian ini telah dijalankan untuk mengekstrak dan memprofilkan metabolit aktif daripada janabuah cendawan ini. Pelbagai pelarut kimia pengekstrakan telah digunakan dalam penyiasatan ini kerana metabolit yang berlainan telah diekstrak berdasarkan kepolaran pelarut tersebut. Pelarut yang digunakkan adalah metanol,heksana, etil asetat, air suling dan butanol. Ekstrak etil asetat dan heksana difraksikan menggunakan kaedah kromatografi kilat dengan penambahan heksana dan aseton secara berperingkat. Komponen-komponen dalam sub-fraksi etil asetat dan heksana telah dikenalpasti dengan menggunakan analisis KG-SJ dan KC-SJSJ. Dua puluh tujuh metabolit yang terdiri daripada asid lemak seperti asid palmitik dan oleik; ester metil asid lemak seperti metil linoleat; sebalian fenolik seperti asid kafeik dan asid sinamik; sebatian sterol seperti ergosterol dan neoergosterol; asid organik seperti asid suksinik dan alkaloid seperti asetilfemiletilamina telah dikeanlpasti. Pengekstrakan komponen perisa (minyak pati) juga dilaksanakan. Sebatian seperti benzyl alkohol telah dikenalpasti dalam minyak pati ini. Ini adalah laporan pertama tentang komponen-komponen bioaktif daripada janabuah *Pleurotus giganteus*.

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LIST OF SYMBOLS AND ABBREVIATIONS

DPPH	2, 2-diphenyl-1-picrylhydrazyl
DNA	deoxyribonucleic Acid
NMR	nuclear magnetic resonance
MCF	michigan cancer foundation
CCL4	chemokine (C-C motif) ligand 4
m	metre
nm	nanometre
μm	micrometre
mm	millimetre
L	litre
g	grams
%	percentage
h/A	hexane/acetone
v/v	volume/volume
min	minutes
mM	millimole
mg	milligram
kg	kilogram
NIST	national institute of standards and technology
eV	electron volt
m/z	mass-to-charge ratio
M	molecular ion
LDL	low-density lipoprotein
HCL	hydrochloric acid
LPS	lipopolysaccharides
NADH	nicotinamide adenine dinucleotide hydrogen

ADP	adenosine diphosphate
C	carbon
O	oxygen
N	nitrogen
H	hydrogen
$FeCl_3$	iron (III) chloride
WHO	world health organization
GC-MS	gas chromatography mass spectrometry
LC-MS/MS	liquid chromatography mass spectrometry mass spectrometry
RNS	reaction nitrogen species
TLC	thin layer chromatography
°C	celsius
UV	ultra violet
ml	mili-liter
Lib	libary
sp.	specie
spp.	specices

CHAPTER 1

INTRODUCTION

In recent times consumption of mushroom species has greatly increased. This is due to continuous developments in cultivation, harvest, postharvest, processing and storage treatments, which facilitates the consumption throughout the year. Besides the nutritional properties, mushrooms have been demonstrated to possess activities that contribute to health (Palacios *et al.,* 2011). For millennia, humankind has valued mushrooms as an important edible and medical resource. Medicinal mushrooms have a long history of use in folk medicine. The use of mushroom - based medicines for healing is as ancient and universal as medicine itself. Until the dawn of this century, natural products from mushrooms have served as the mainstay of all medicines world-wide. Although herbalism has declined in the west, it continues to exist throughout the developing world. Over 70% of the world population still relies on herbal remedies for their health care needs. In particular, mushrooms that are useful against cancers of the stomach, esophagus and lungs are known in China, Russia, Japan, Korea, America and Canada (Wasser , 2002). There are about 200 species of mushrooms that have been found to markedly inhibit the growth of different kinds of tumors. Hitherto, several antitumor polysaccharides such as hetero-beta-glucans and their protein complexes (e.g., xyloglucans and acidic beta-glucan-containing uronic acid), dietary fibers, lectins, and terpenoids have been isolated from medicinal mushrooms (Wasser, 2002). Moreover, in Japan, Russia, China, and America several different polysaccharide antitumor agents have been developed from the fruiting body, mycelia, and culture medium of various medicinal mushrooms such as *Lentinula edodes, Ganoderma lucidum, Schizophyllum commune, Trametes versicolor, Inonotus obliquus,* and *Flammulina velutipes* (Wasser , 2002).

The use of mushrooms, past and present, and practices, represent an important cultural heritage as they have been used since times immemorial as food and medicine according to traditional ecological knowledge transmitted along generations. Mushrooms have long been valued as highly tasty/nutritional foods. In some fields, including the food and pharmaceutical industries, mushrooms are an important and valued commodity (Pereira *et al.,* 2011). In 2004, the estimated value of wild edible mushroom gathering was $2 billion US dollars. Therefore, their chemical and biological characteristics attract significant interest as they are natural bioreactors for the production of compounds with human interest (Pereira *et al.,* 2011).

Mushrooms accumulate a variety of bioactive metabolites (e.g. phenolic compounds, polyketides, terpenes, steroids, and polysaccharides) with immunomodulatory, cardiovascular, liver protective, anti-fibrotic, anti-inflammatory, anti-diabetic, anti-viral, antimicrobial activities, and antitumor properties (Pereira *et al.,* 2011). Purified bioactive compounds derived from medicinal mushrooms are potentially important new source of natural antioxidants that positively influence oxidative stress related diseases such as cancer (Pereira *et al.,* 2011). Thus, they might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present (Reis *et al.,* 2011).

Mushrooms have also become one of the most desirable food in oriental cuisine because of their low caloric value, characteristic smell, taste and texture. Thus, mushrooms are considered as highly valuable bio-engineering resources for development of food material (functional foods as well as for use of starting material in the production of drugs (Mizuno, 1999). Functional compounds in mushrooms have recently been highlighted as they are able to lower cholesterolemia, modulate the immune system and inhibit tumoral growth (Zhang *et al.,* 2001).

Many species of wild mushrooms such as *Pleurotus porrigens* (angel's wings), *Schizophyllum commune* (Split gill), *Hygrocybe conica* (witch's hat), *Hygrophorus sp.* and *Polyporus sp.* (Bracket fungi) are sold in local Malaysian markets, with great economical interest for local people as a source of income. Although research has focused mainly on the therapeutic effects and cultivation methods of mushrooms. Little information is available about their biological properties. In addition, while many studies have focused on cultivated and wild edible mushrooms in the northern hemisphere, there are limited data in the literature concerning the antioxidant properties of the edible wild mushrooms from Southeast Asia, especially Malaysia (Wong & Chye, 2009).

Pleurotus giganteus (Berk.) Karunarathna & K.D. Hyde previously known as *Panus giganteus* (Berk.) Corner is a medicinal and a culinary mushroom consumed by selected indigenous communities in Malaysia. It is domesticated strain from china which currently being considered for large scale cultivation. This study was performed to profile chemically a potentially interesting species *Pleurotus giganteus* which has not yet been characterized in the literature. The most common method used in the isolation of bioactive compounds from natural products is column chromatography which based on absorption chromatography. The mobile phase (e.g. solvents) moves a mixture of substances through the stationary phase (e.g. silica gel beads). The different compounds in the sample have different affinities for the mobile and stationary phase, and appear from the stationary phase at different times. The stationary phase and mobile phase are chosen based on the nature of the sample mixture in order to achieve the best possible separations of compounds. Column chromatography is advantageous over most other chromatographic techniques because it can be used in both analytical and preparative applications. Besides determining the number of components of a mixture, column chromatography can also be used to separate and purify substantial quantities of those components for subsequent analysis. Thus, in this study, the above mentioned strategy

for identifying new lead compounds from the fruiting bodies of *Pleurotus giganteus* is adopted.

The objectives of the present study were:

i. To assess the chemical profile of *Pleurotus giganteus* from the aqueous, hexane, butanol, ethyl acetate and methanol extracts.
ii. To identify the essential oils in the essential oil extract.

CHAPTER 2

LITERATURE REVIEW

2.1 Mushrooms

Mushrooms have a great nutritional value since they are quite rich in protein, with an important content of essential amino acids and fiber, and poor in fat. Edible mushrooms also provide a nutritionally significant content of vitamins (B1, B2, B12, C, D, and E). Edible mushrooms could be a source of many different nutraceuticals such as unsaturated fatty acids, phenolic compounds, tocopherols, ascorbic acid and carotenoids. Thus, they might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present. More than 3000 mushrooms are said to be ‘‘the main edible species’’, of which only 100 are cultivated commercially, and only 10 of those on an industrial scale (Reis *et al.,* 2011). Their global economic value is nevertheless now staggering, and a prime reason for the rise in consumption is the above mentioned combination of their value as a food as well as their medicinal and nutraceutical values. Production of mushrooms continuously increases over time with China being the biggest producer (more than 1.5 million metric tons in 2007). The most cultivated mushroom worldwide is *Agaricus bisporus,* followed by *Lentinula edodes, Pleurotus spp.* and *Flammulina velutipes* (Reis *et al.,* 2011).With increasing cultivation of previously uncultivated mushrooms it has become important to perform scientific studies which contribute to the elaboration of nutritional databases of the consumed fungi species worldwide, allowing comparison between them.

2.2 Antioxidants in mushrooms

Maintenance of equilibrium between free radical production and antioxidant defences (enzymatic and non enzymatic) is an essential condition for normal organism function-

ing. When this equilibrium has a tendency for the production of free radicals we say that the organism is in oxidative stress. In this situation, excess free radicals may damage cellular lipids, proteins and DNA, affecting normal function and leading to various diseases. In aerobic organisms, the free radicals are constantly produced during the normal cellular metabolism, mainly in the form of Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) (Ferreira *et al.,* 2009). Exposition of the organism to free radicals has led to the development of endogenous defence mechanisms to eliminate them. These defences were the response of evolution to the inevitability of ROS production in aerobic conditions. Natural products with antioxidant activity may help the endogenous defence system. In this perspective the antioxidants present in the diet assume a major importance as possible protector agents reducing oxidative damage. Particularly, the antioxidant properties of wild mushrooms have been extensively studied and many antioxidant compounds extracted from these sources have been identified, such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids. Wild mushrooms might be used directly in diet and promote health, taking advantage of the additive and synergistic effects of all the bioactive compounds present. The antioxidants found in mushrooms are mainly phenolic compounds (phenolic acids and flavonoids), followed by tocopherols, ascorbic acid and carotenoids (Ferreira *et al.*, 2009). Phenolic acids can be divided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids, which are derived from non-phenolic molecules benzoic and cinnamic acid, respectively (Ferreira *et al.,* 2009). The overall effectiveness of a natural phenolic antioxidant depends on the involvement of the phenolic hydrogen in radical reactions, the stability of the natural antioxidant radical formed during radical reactions, and the chemical substitutions present on the structure. The substitutions on the structure are probably the most significant with respect to the ability of a natural antioxidant to participate in the control of radical reactions and to form resonance-stabilized natural antioxidant radicals. Previ-

ous studies concerning the analysis of the phenolic components of wild mushrooms can be found in the literature (Puttaraju *et al.,* 2006 and Ribeiro *et al.,* 2006).

2.3 Antioxidant properties of tropical wild edible mushrooms in Malaysia

In a previous study (Wong & Chye, 2009), selected species of wild edible mushrooms were obtained from the interior areas of East Malaysia to determine the total phenolics and antioxidant properties, including free radical scavenging, reducing power and metal chelating activities. Selected species of edible wild mushrooms were obtained throughout the year from indigenous people who collect edible forest resources in various districts of Sabah, East Malaysia. The wild mushrooms were identified as *Pleurotus porrigens*, *Hygrocybe conica*, *Xerula furfuracea* (Rooted oude), *Schizophyllum commune* and *Polyporus tenuiculus* (Pore fungus). Based on the results obtained, petroleum ether (PE) and methanolic extracts from these edible wild mushrooms were effective in DPPH radical scavenging and metal chelating ability (Wong & Chye, 2009).

2.4 Antioxidant properties of commercially cultivated mushrooms in Malaysia

Pleurotus sajor-caju is a mushroom which is commerially cultivated in Malaysia but is not native to Malaysia**.** In a recent study**,** the chemical composition and *in vitro* antioxidant activity of aqueous butanol and ethyl acetate extracts of *Pleurotus sajor-caju* were investigated. Twenty-two compounds comprising methyl esters, hydrocarbon fatty acids, ethyl esters, and sterols were identified in ethyl acetate extracts, while cinnamic acid, nicotinamide, benzeneacetamide, and 4-hydroxybenzaldyhde were identified in butanol extracts by gas chromatography−mass spectrometry and NMR analysis. The antioxidant activity was determined by a β-carotene bleaching method, ferric reducing

antioxidant power, trolox equivalent antioxidant capacity, and lipid peroxidation assays, while the total phenolic content in *P. sajor-caju* was assessed by Folin−Ciocalteau's method. The aqueous and butanol extracts exhibited the highest antioxidant activity, corresponding to the total phenolic content. The subfractions from the ethyl acetate extract (EP1, EP2, EP3, and EP4), however, showed moderate antioxidant activity. The regular consumption of *P. sajor-caju* as a part of our diet may render nutritional and nutraceuticals benefits for good health (Kanagasabapathy *et al.,* 2011).

2.5 Anticancer activities of mushrooms

Compounds from mushrooms may enhance innate immune responses, resulting in anti-tumor activities. Several mushroom polysaccharide have proceeded through phases I, II, and III clinical trials and are used extensively and successfully in Asia to treat various cancers and other diseases (Wasser, 2010). There are more than two dozen species of endophytic fungi can synthesize paclitaxel, one of the most important anticancer drugs known (Ng, 1998).

In a previous study (Chen *et al.,* 2006),white button mushrooms (*Agaricus bisporous*) are a potential breast cancer chemopreventive agent, as they suppress aromatase activity and estrogen biosynthesis. The mushroom extract activity was evaluated in the estrogen receptor-positive/aromatase-positive MCF-7aro cell line *in vitro* and *in vivo*. Mushroom extract decreased testosterone-induced cell proliferation in MCF-7aro cells but had no effect on MCF-10A, a nontumorigenic cell line. The most potent mushroom chemicals are soluble in ethyl acetate. The major active compounds found in the ethyl acetate fraction are unsaturated fatty acids such as linoleic acid, linolenic acid, and conjugated linoleic acid (Chen *et al.,* 2006). The interaction of linoleic acid and conjugated linoleic acid with aromatase mutants expressed in Chinese

hamster ovary cells showed that these fatty acids inhibit aromatase with similar potency and that mutations at the active site regions affect its interaction with these two fatty acids. Whereas these results suggest that these two compounds bind to the active site of aromatase, the inhibition kinetic analysis indicates that they are noncompetitive inhibitors with respect to androstenedione. Because only conjugated linoleic acid was found to inhibit the testosterone-dependent proliferation of MCF-7aro cells, the physiologically relevant aromatase inhibitors in mushrooms are most likely conjugated linoleic acid and its derivatives. The *in vivo* action of mushroom chemicals was shown using nude mice injected with MCF-7aro cells. The studies showed that mushroom extract decreased both tumor cell proliferation and tumor weight with no effect on rate of apoptosis. Therefore the study illustrated the anticancer activity *in vitro* and *in vivo* of mushroom extract and its major fatty acid constituents (Chen *et al.*, 2006).

"Lingzhi" (*Ganoderma lucidum*), a popular medicinal mushroom, has been used in China for longevity and health promotion since ancient times. Investigations into the anticancer activity of lingzhi have been performed in both *in vitro* and *in vivo* studies, supporting its application for cancer treatment and prevention. The proposed anticancer activity of lingzhi has prompted its usage by cancer patients. It remains debatable as to whether lingzhi is a food supplement for health maintenance or actually a therapeutic "drug" for medical proposes. Thus far there has been no report of human trials using lingzhi as a direct anticancer agent, despite some evidence showing the usage of lingzhi as a potential supplement to cancer patients. Cellular immune responses and mitogenic reactivity of cancer patients have been enhanced by lingzhi, as reported in two randomized and one nonrandomized trials, and the quality of life of 65% of lung cancer patients improved in one study (Yuen & Gohel, 2005). The direct cytotoxic and anti-angiogenesis mechanisms of lingzhi have been established by *in vitro* studies; however, clinical studies should not be neglected to define the applicable dosage *in vivo*. At

present, lingzhi is a health food supplement to support cancer patients, yet the evidence supporting the potential of direct in vivo anticancer effects should not be underestimated. Lingzhi or its products can be classified as an anticancer agent when current and more direct scientific evidence becomes available (Yuen & Gohel, 2005).

In an effort to translate the Asian traditional medicines into western-accepted therapies, scientists have demonstrated that the extracts from fruit-bodies or mycelium of *Phellinus linteus* not only stimulate the hormonal and cell-mediated immune function and quench the inflammatory reactions caused by a variety of stimuli, but also suppress the tumor growth and metastasis. Mounting evidence from different research groups has shown that *Phellinus linteus* induces apoptosis in a host of murine and human carcinomas without causing any measurable toxic effects to their normal counterparts. Recently, research has been focused on the anti-tumor effect of *Phellinus linteus*, and in particular, on its ability to enhance some conventional chemotherapeutic drugs. These studies suggest *Phellinus linteus* to be a promising candidate as an alternative anticancer agent or a synergizer for existing antitumor drugs (Zhu *et al.*, 2008).

2.6 Anti-cholesterol activity and statins of mushrooms

Mevastatin, lovastatin and monacolins L are members of the drug class of statins, used in combination with diet, weight-loss, and exercise for lowering cholesterol (hypolipidemic agent) in those with hypercholesterolemia to reduce risk of cardiovascular disease (Lewington *et al.*, 2007). The mevastatin and lovastatin were the first statins discovered in fungi. Lovastatin was later found being produced in *Pleurotus ostreatus* (oyster mushroom) (Campbell & Vedaras, 2010). Red yeast rice, produced by the fungus *Monascus purpureus*, naturally contains lovastatin, mevastatin, and monacolins L and *J. Lentinula edodes* (shiitake) contains the anticholesterol compound

eritadenine (Bisen *et al.,* 2010). Zaragozic acids which also have been shown to be able to treat hypercholesterolemia were first discovered in *S. intermedia* and *L. elatius* (Bergstrom *et al.,* 1995).

2.7 Anti-hormone and anti-inflammatory activity of mushrooms

A small clinical study showed the extract of *Agaricus subrufescens* (agaricus blazei) had an anti-inflammatory effect. Animal studies noted extracts of *Fomes fomentarius* (amadou) could reduce inflammation. In cell culture, extracts of *Geastrum saccatum* (rounded earthstar) demonstrated anti-inflammatory activity (Forland *et al.,* 2011, Guerradore *et al.,* 2007, Park *et al.,* 2004). *Phellinus linteus* is a medicinal mushroom that has been practiced in oriental countries for centuries to prevent ailments as diverse as gastroenteric dysfunction, diarrhea, haemorrhage and cancers. *Niuchangchih* is a basidiomycete endemic to Taiwan. It is well known as a Traditional Chinese Medicine, and Taiwanese aborigines used this species to treat liver diseases and food and drug intoxication. The compounds identified in *Niuchangchih* are predominantly polysaccharides, triterpenoids, steroids, benzenoids and maleic/succinic acid derivatives (Ao *et al,* 2009). The fruiting bodies and fermented products of *Niuchangchih* have been reported to exhibit activity when treating liver diseases, such as preventing ethanol-, CCl(4)- a ameliorating fatty liver, liver fibrosis, and inhibiting liver cancer cells (Ao *et al,* 2009).

2.8 Wild edible mushrooms in Malaysia

There are over 90 different groups of indigenous people in Malaysia each with their own distinct language and culture, with the majority being found in the states of Sabah and Sarawak on the island of Borneo. The indigenous minority peoples of Peninsular

Malaysia collectively called the Orang Asli, are composed of 18 subethnic groups classified under the three major tribes of the Negrito, Senoi and Proto-Malays with a population of 149,512 persons in 2004. The Orang Asli and other rural communities in Malaysia are known to utilize certain local wild mushrooms for food, medicine and spiritual purposes (Lee *et al.,* 2009). In a recent study carried out by Lee and co-workers documented at least 31 species of macrofungi collected for food but only 14 species were used for medicinal purposes. Therefore scientific validation of culinary-medicinal properties of these mushrooms and specially their products was needed to provide evidence of credibility of claims.

CHAPTER 3

MATERIALS AND METHODS

3.1 Materials and instrumentation

Evaporation of solvents was performed with a Butchi Rotavapor R-114 with an accompanying chiller B-741, waterbath B-480 and vacuum system V-700. A freeze-dryer from Christ was also used. Various chemicals and solvents from Merck were used which included acetone ($(CH_3)_2CO$), butanol (C_4H_9OH), ethanol (CH_3CH_2OH), hexane (C_6H_{14}), ethyl acetate ($CH_3COOCH_2CH_3$), methanol (CH_3OH), acetonitrile (CH_3CN), formic acid (CH_2O_2), ammonium formate (NH_4HCO_2). Silica gel beads (size: 0.063 – 0.200nm, 0.015-0.040nm) was used. The supplier of the silica gel was sigma-aldrich. A heating mantle (5 litre capacity) from topo (Germany) was also used.

- **GC – MS analysis**

The GC - MS analysis was performed on a Agilent Technologies 6890 N gas chromatography equipped with a 5979 Mass Selective Detector (70 eV direct inlet); a HP-5 ms (5% Phenyl-methylpolysiloxane) capillary column (30.0 m × 250 μm ID × 0.25 μm film thickness) initially set at 150°C, then programmed to 280°C at 5°C per min using helium as carrier gas at a flow rate of 1 ml/min was used. The total ion chromatogram obtained was auto-integrated by Chemstation and the constituents were tentatively identified by comparison with the accompanying mass spectra data (NIST Lib, 2005) whenever possible.

- **LCMS/MS analysis**

The LC-MS/MS analysis was performed on AB Sciex Liquid chromatography tandem MS-MS. The column used was phenomenex aqua C18-50mm x 2.0mmx 5µm. The mobile phase A was 100% Acetonitrile. Mobile phase B was water and acetonitrile with 0.1% formic acid and 5mM ammonium formate. The gradient run program was 10% A and 90% B initially and then was ramped up to 90% A and 10% B from 0.01min to 8.0min. This was then held for two minutes and then back to 10% A in 0.1 minutes and re-equilibrated for five minutes. Compounds were positively identified by NIST 06 Database and ACD/labs advanced chemometrics mass fragmentation predictive software.

3.2 Thin Layer Chromatography

Thin layer chromatography (TLC) was carried out using pre-coated TLC plates 60 F_{254} (20.25 mm thickness) and were visualized in UV light (254 and/or 365 nm) and /or iodine vapour.

3.3 Sample Collection

Fresh fruiting bodies of *Pleurotus giganteus* were collected from Nas Agro farm in Sepang, Selangor**,** Malaysia on the 14th of April 2011.

3.4 Extraction and fractionation of the ethyl acetate extract

The five kg fresh fruiting bodies were washed, sliced and freeze-dried for two days. The freeze-dried mushrooms were ground to a fine powder using a blender. The dried, ground sample (404.10g, 8.08%) was then soaked in a 1.5L solution methanol and water

solution (8:2) for three days at room temperature. The solvent-containing extract was then decanted and filtered. The extraction of the ground sample was further repeated (3×) with 1.5L of methanol and water (8:2). The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give a light- brown thick extract (8.95g, 2.22%).The percent yield is based upon the initial sample of 404.10g. The methanol extract was further partitioned with a mixture of ethyl acetate and water (1:1) to give an ethyl acetate-soluble fraction (PGETHYL1) and aqueous-soluble fraction. The aqueous extract was further extracted with butanol to obtain a butanol extract which was a dark yellowish extract (1.80g, 0.45%). Evaporation of solvent from the ethyl acetate layer gave an extract (2.50g, 0.62%) which was further fractionated using varying ratios of acetone: hexane as the eluting solvent. The aqueous extract was freeze dried to give a dark brown extract (6.45g, 1.60%).

The ethyl extract sample (2.50g) was suspended in chloroform and then adsorbed on to silica gel (size: 0.015-0.040nm) and silica was added until the consistency of silica with adsorbed sample reached a powdery state. The flash column was filled up with silica gel (size: 0.063-0.200nm) and the silica with absorbed sample was applied in an overlay fashion to the coarser silica. Elution began with 100% hexane and polarity of eluting solvent was gradually increased using acetone. Fractions of 200-400ml volumes were collected. The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. The separations were monitored using thin layer chromatography. The fractions were collected by gradient elution of hexane/acetone (h/A, v/v): h/A 100:0, 400 ml; h/A 99.5:0.5, 400 ml; h/A 99:1, 400 ml; h/A 98.5:1.5, 400 ml h/A 98:2, h/A 97.5:2.5, 400 ml; h/A 97:3, h/A 96.5:3.5, 400 ml; h/A 96:4, 400 ml; h/A 95:5, 400 ml; h/A 94:6, 400 ml; h/A 93:7, 400 ml; h/A 92:8, 400 ml; h/A 91:9, 400 ml; h/A 90:10, 400 ml; h/A 89:11, 400 ml; h/A 88:12, 400 ml; h/A

87:13, 400 ml; h/A 86:14, 400 ml; h/A 85:15, 400 ml; h/A 84:16, 400 ml ml; h/A 83:17, 400 ml; h/A 82:18, 400 ml; h/A 81:19, 400 ml; h/A 80:20, 400 ml; h/A 79:21, 400 ml; h/A 78:22, 400 ml h/A 77:23, 400 ml; h/A 76:24, 400 ml; h/A 75:25, 400 ml; h/A 74:26, 400 ml h/A 73:27, 400 ml ml; h/A 72:28, 400 ml; h/A 71:29, 400 ml; h/A 70:30, 400 ml h/A 65:35, 400 ml; h/A 60:40, 400 ml; h/A 55:45, 400 ml; h/A 50:50, 400 ml h/A 45:55, 400 ml; h/A 40:60, 400 ml; h/A 35:65, 400 ml h/A 30:70, 400 ml ml; h/A 25:75, 400 ml; h/A 20:80, 400 ml; h/A 15:80, 400 ml h/A 10:90, 400 ml; h/A 100:0, 400 ml. The fractions with a similar TLC profile were combined and yielded fractions PGF 9 (1.1mg), PGF10 (1.1mg), PGF12 (1.0mg), PGF31 (1.1mg), PGF37 (1.0mg), PGF39 (1.0mg), PGF41 (1.5mg), and PGF46 (1.3mg). The fractions were analysed for the identification of unknowns by GC-MS and LC-MS/MS. The extraction and fractionation of ethyl acetate extract of *P. giganteus* is shown in Figure 1.1.

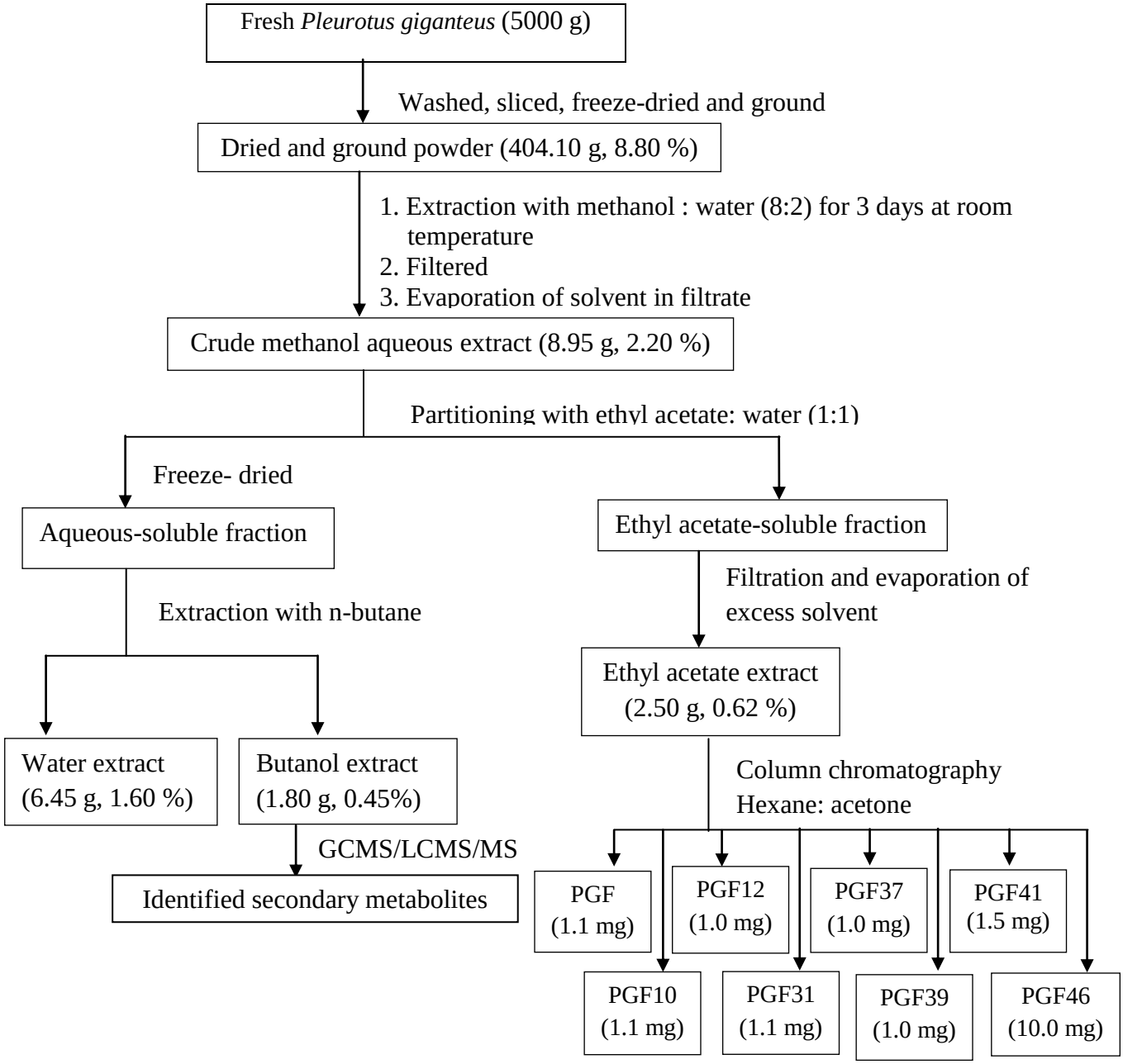

Figure 1.1

Extraction and fractionation of ethyl acetate extract of *P. giganteus*

3.5 Extraction and fractionation hexane extract

The five kg fresh fruiting bodies were washed, sliced and freeze-dried for two days. The freeze-dried mushrooms were ground to a fine powder using a blender. The dried, ground sample (403.2g) was soaked in hexane (1.5 L) for 3 days at room temperature. The solvent-containing extract was then decanted and filtered. The extraction of the ground sample was further repeated (3×) with 1.5 L each time. The filtrate from each extraction was combined and the excess solvent was evaporated under reduced pressure using a rotary evaporator to give a light- brownish thick extract (0.1g, 0.02%). The yield is based upon the initial 403.2g sample.

The hexane extract (0.1g) was dissolved in chloroform and then absorbed on to silica gel (size: 0.015-0.040nm) and silica was added until the consistency of silica with adsorbed sample reached a powdery state. The flash column was filled up with silica gel (size: 0.063-0.200nm) and the silica with adsorbed sample was applied in an overlay fashion to the coarser silica. Elution began with 100% hexane and polarity of eluting solvent was gradually increased using acetone. Fractions of 200-400ml volumes were collected. The excess solvent in the pooled fractions was evaporated under reduced pressure using a rotary evaporator. The separations were monitored using thin layer chromatography. The fractions were collected by gradient elution of hexane/acetone (h/A, v/v): h/A 100:0, 400 ml; h/A 99:1, 400 ml; h/A 98:2, , 400 ml; h/A 97:3, 400 ml; h/A 96:4, 400 ml; h/A 94:6, 400 ml; h/A 93:7, 400 ml; h/A 92:8, 400 ml; h/A 91:9, 400 ml; h/A 90:10, 400 ml; h/A 85:15, 400 ml; h/A 80:20, 400 ml; h/A 75:25, 400 ml; h/A 70:30, 400 ml; h/A 65:35, 400 ml; h/A 60:40, 400 ml ml; h/A 55:45, 400 ml; h/A 25;75, 400 ml; h/A 00:100, 400 ml. The fractions with a similar TLC profile were combined and yielded fractions PGHEX4 (1.0mg), PGHEX5 (1.1mg), PGHEX10 (1.0mg), PGHEX16 (1.1mg) and PGHEX19 (1.0mg).The fractions were analysed for the identi-

fication of unknowns by GC-MS and LC-MS/MS . The hexane extraction and fractionation of the fresh fruiting bodies of *Pleurotus giganteus* is shown in Figure 1.2.

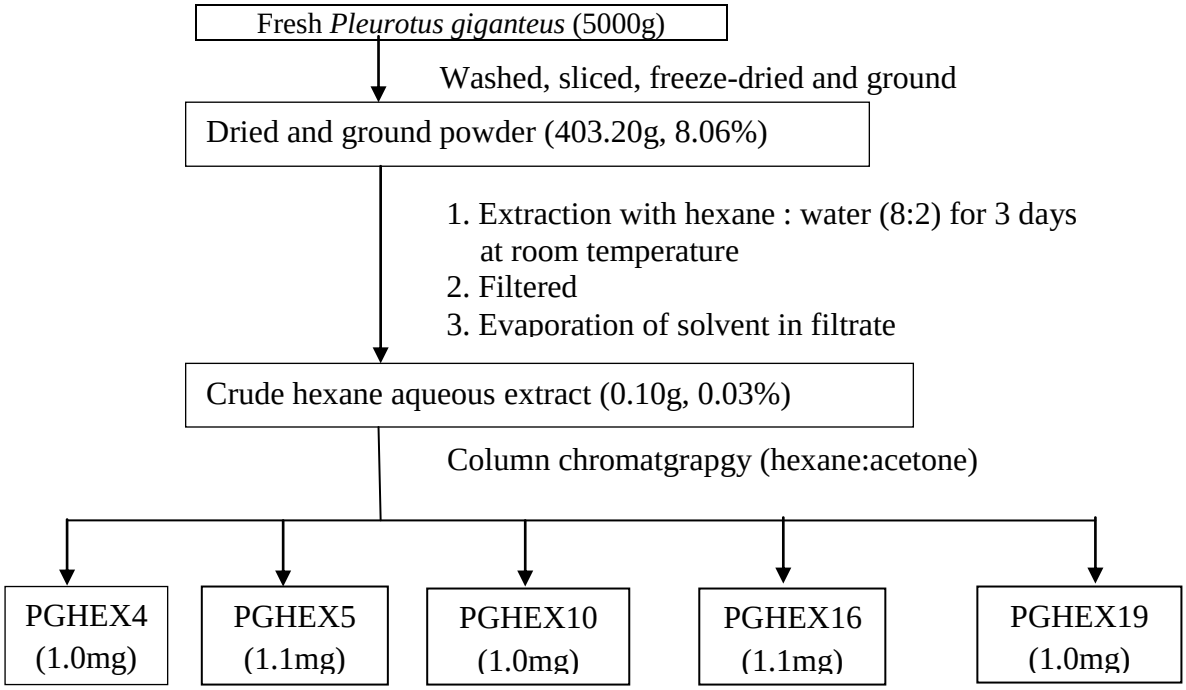

Figure 1.2

Extraction and fractionation of hexane extract of *P. giganteus*

3.6 Extraction and fractionation of aqueous extract

Components of the aqueous extract were identified using LCMS/MS. Samples were dissolved in 50:50 acetonitrile and water.

3.7 Extraction and identification of essential oils of *Pleurotus giganteus*

3.7.1 Sample collection

1 kg of the fresh fruiting bodies of *Pleurotus giganteus* was collected from a mushroom farm in Sepang, Selangor (14th April 2011).

3.7.2 Extraction of essential oils from fruiting bodies

Fresh fruiting bodies of *Pleurotus giganteus* (1kg) was collected from a mushroom farm in Sepang, Selangor (14th April 2011). The fresh fruiting bodies were washed and sliced and diced. The mushrooms were then placed in a 5L round bottom flask which was filled with water. The round bottom flask was then placed on a heating mantle. A Clevenger unit was attached to the top of the round bottom flask and the evaporated oils were collected. The oil collected was a pale yellow colour.

CHAPTER 4

RESULTS

4.1 Identification of compounds in ethyl acetate extract

The ethyl acetate extract was subjected to column chromatography and a total of forty one fractions were collected from the column chromatography. The fractions were pooled according to the spots on TLC plates (Table1.1) and several fractions were obtained. The Table 4.1 below shows the details of fractions obtained from ethyl acetate.

Table 4.1

Fractions obtained from ethyl acetate extract using column chromatography

Pooled Vials	Weight (mg)	Vials	Appearance
PGF9	1.0mg	1-9	Yellow oil
PGF10	1.1mg	10-11	Colourless crystals
PGF12	1.0mg	12-13	Colourless crystals
PGF31	1.1mg	28-31	Colourless oil
PGF37	1.0mg	34-37	Colourless oil
PGF39	1.0mg	38-39	Colourless oil
PGF41	1.5mg	37-41	Yellow oil

Table 4.1 shows the components present in the unfractionated ethyl acetate extract (PGETHYL1) as identified by GC/MS. Four components comprising of about 67.5% of the total ethyl acetate extract were identified with methyl linoleate (50.28%) forming the major constituent.

Table 4.2

Compounds in PGETHYL1 identified by GC-MS

Compounds	Retention Time (minutes)	Percentage (%)	Molecular Weight	Molecular Formula
Hexadecanoic acid, methyl ester (Methyl palmitate)	23.897	10.58	270.45	$C_{17}H_{34}O_2$
9, 12 – Octadecadienoic acid, methyl ester (Methyl linoleate)	35.965	50.28	294.47	$C_{19}H_{34}O_2$
Unidentified compound	36.484	7.80	-	-
Octadecanoic acid, methyl ester (Methyl Stearate)	38.743	2.26	298.29	$C_{20}H_{40}O_2$
Unidentified compound	64.777	1.21	-	-
Unidentified compound	70.532	9.09	-	-
Unidentified compound	79.714	2.08	-	-
Unidentified compound	94.733	1.75	-	-
Ergosterol	109.796	4.38	396.65	$C_{28}H_{44}O$
Total amount : 89.43%				

In Figure 4.1 the peak at retention time 23.867 minutes has a mass spectral data consistent with methyl palmitate. The mass spectrum exhibited a parent ion at *m/z* 270 that is consistent with the molecular formula $C_{17}H_{34}O_2$. The mass spectral data is as follows *m/z* (%): 270 ($[M]^+$,12),239 ($[M\text{-}OCH_3]^+$,10) 227($[M\text{-}CH_2COH]^+$,12), 213 ($[M\text{-}CH_2COH\text{-}CH_2]^+$,10),199 ($[M\text{-}CH_2COH\text{-}(CH_2)_2]^+$,4), 185 ($[M\text{-}CH_2COH\text{-}(CH_2)_3]^+$,5), 171 ($[M\text{-}CH_2COH\text{-}(CH_2)_4]^+$,5), 157 ($[M\text{-}CH_2COH\text{-}(CH_2)_5]^+$,3), 143 ($[M\text{-}CH_2COH\text{-}(CH_2)_6]^+$,20), 129 ($[M\text{-}CH_2COH\text{-}(CH_2)_7]^+$,10),87($[C_4H_7O_2]^+$,70), 74 ($[C_3H_6O_2]^+$,100), 55 .

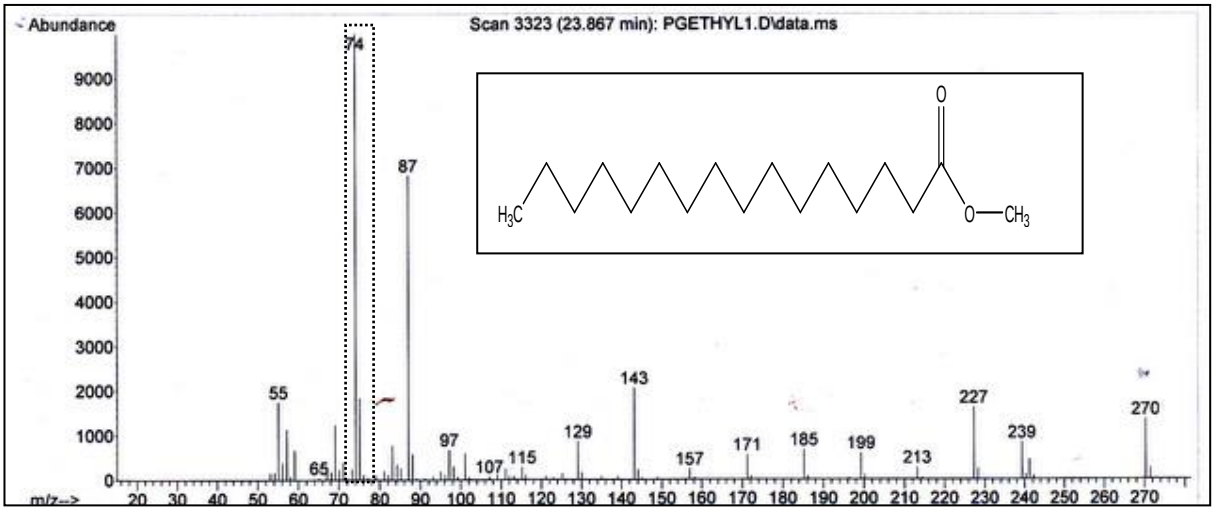

Figure 4.1

Mass spectrum of methyl palmitate

In Figure 4.2 the peak at retention time 35.965 minutes has a mass spectral data consistent with methyl linoleate. The mass spectrum exhibited a parent ion at *m/z* 294 that is consistent with the molecular formula $C_{19}H_{34}O_2$. The mass spectral data is as follows *m/z* (%): 294($[M]^+$, 20), 263($[M\text{-}CH_3O]^+$15), 245, 220($[M\text{-}CH_3O\text{-}C_2H_3O]^+$7) 150, 109, 95, 81, 67 (100), 55.

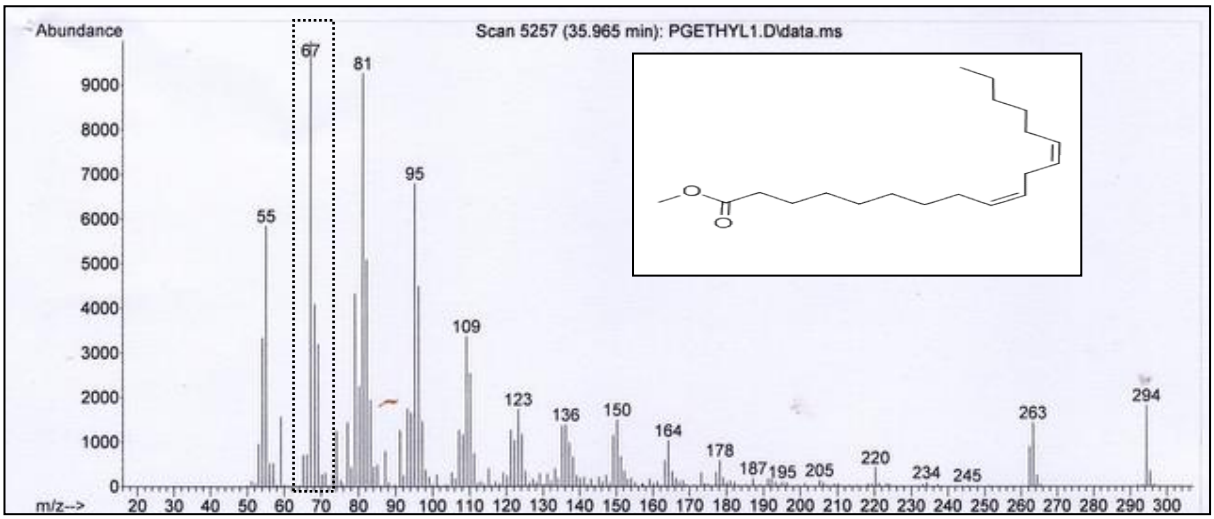

Figure 4.2

Mass spectrum of methyl linoleate

In Figure 4.3 the peak at retention time 38.742 minutes has a mass spectral data consistent with methyl stearate. The mass spectrum exhibited a parent ion at *m/z* 298 that is consistent with the molecular formula $C_{19}H_{38}O_2$. The mass spectral data is as follows *m/z* (%): 298 (M^+, 20), 267 ($[M\text{-}OCH_3]^+$, 15), 255 ($[M\text{-}OC_2H_3]^+$ 22), 241, 213, 199, 185, 171, 157, 143($[M - C_3H_7 - C_2H_4 - C_4H_8]^+$, 30), 129, 111, 97, 87 ($[M - C_3H_7 - C_2H_4 - C_4H_8 - C_4H_8]^+$, 80), 74(100), 55.

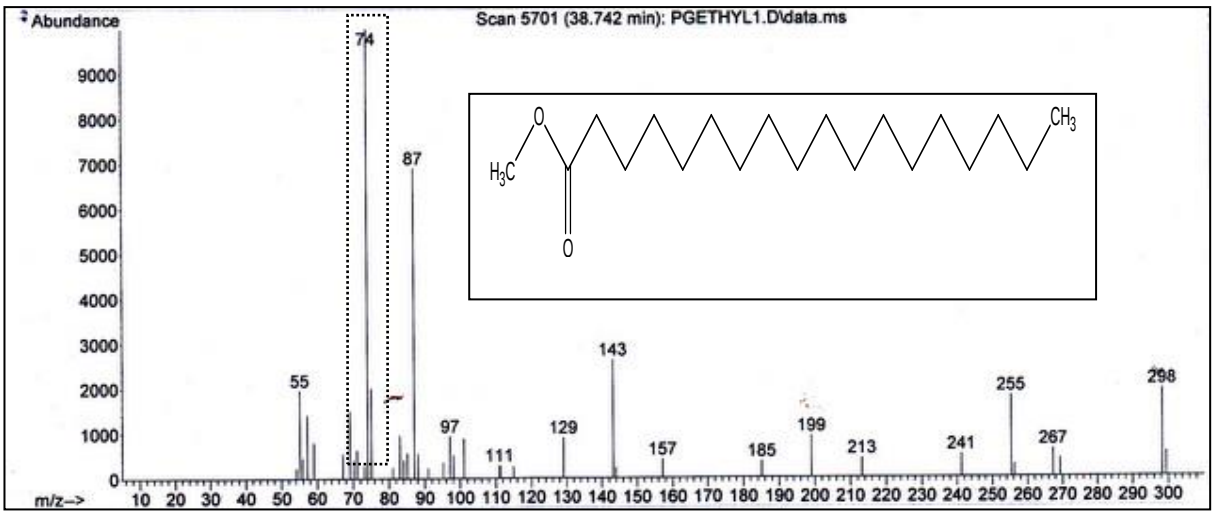

Figure 4.3

Mass spectrum of methyl stearate

Table 4.3 shows the constituents of fractionated ethyl acetate sample (PGF9), representing fraction 1-9 pooled together. Compounds in the sample were identified through GC-MS analysis. Four components comprising of 66.80% of the PGF9 fraction were identified with palmitic acid (53.59%) forming the major constituent. The next major component (31.22%) at retention time 47.524 could not be identified.

Table 4.3

Compounds in PGF9 identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Benezeneacetic acid, alpha. – hydroxyl-, methyl ester (Methyl mandelate)	38.598	11.69	166.17	$C_9H_{10}O_3$
p-tert-butyl-phenol (4-tert-Butylphenol)	39.930	0.62	150.21	$C_{10}H_{14}O$
Unidentified compound	47.524	31.22	-	-
Phenol, 2 ,5-bis (1,1-dimethylethyl) (Phenol, 2,5-di-tert-butyl)	65.909	0.90	206.32	$C_{14}H_{22}O$
n-Hexadecanoic acid (Palmitic acid)	113.768	53.59	256.22	$CH_3(CH_2)_{14}COOH$
Total amount : 98.02%				

In Figure 4.4 the peak at retention time 38.598 minutes has a mass spectral data consistent with methyl mandelate. The mass spectrum exhibited a parent ion at *m/z* 166 that is consistent with the molecular formula $C_9H_{10}O_3$. The mass spectra data is as follows *m/z* (%): 166 (M^+, 10), 150, 118, 107 ([M-CH_3COO]$^+$ 100), 91, 79, 51. The base peak at m/z 107 is consistent with the loss of CH_3COO^- moiety from the molecular ion.

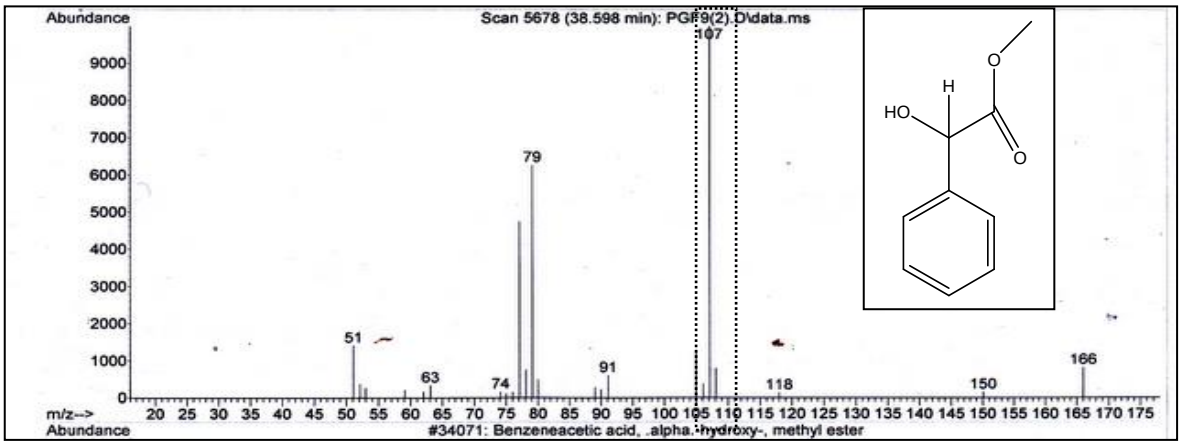

Figure 4.4

Mass spectrum of methyl mandelate.

In Figure 4.4 the peak at retention time 39.930 minutes has a mass spectral data consistent with 4-tert-butylphenol. The mass spectrum exhibited a parent ion at *m/z* 150 that is consistent with the molecular formula $C_{19}H_{34}O_2$. The mass spectra data is as follows *m/z* (%): 150 (M^+, 10), 135([M- CH_3]$^+$, 100), 107([M- CH_3-C_2H_4]$^+$, 30), 95.

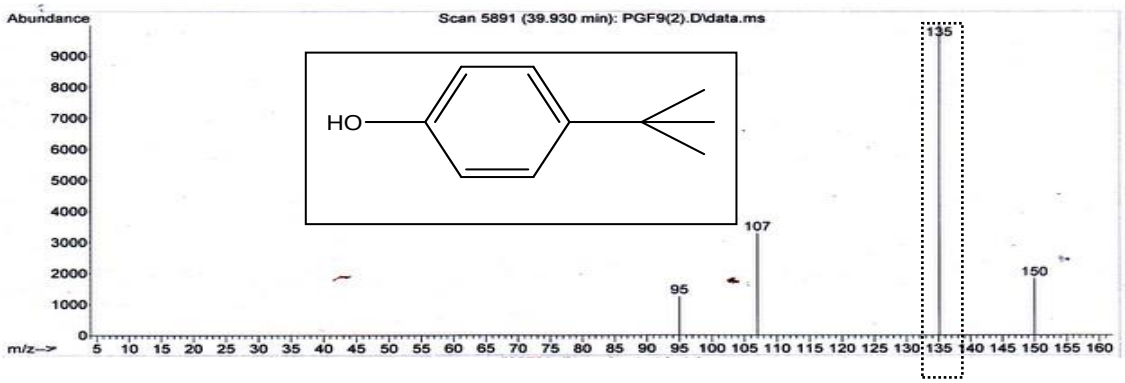

Figure 4.5

Mass spectrum of 4-tert-butylphenol

In Figure 4.6 the peak at retention time 65.909 minutes has a mass spectral data consistent with 2, 5-di-tert-butylphenol. The mass spectrum exhibited a parent ion at *m/z* 206 that is consistent with the molecular formula $C_{10}H_{14}O$ .The mass spectra data is as follows *m/z* (%): 206 (M^+, 20), 191 ($[M- CH_3]^+$,100), 163, 91, 74, 57.

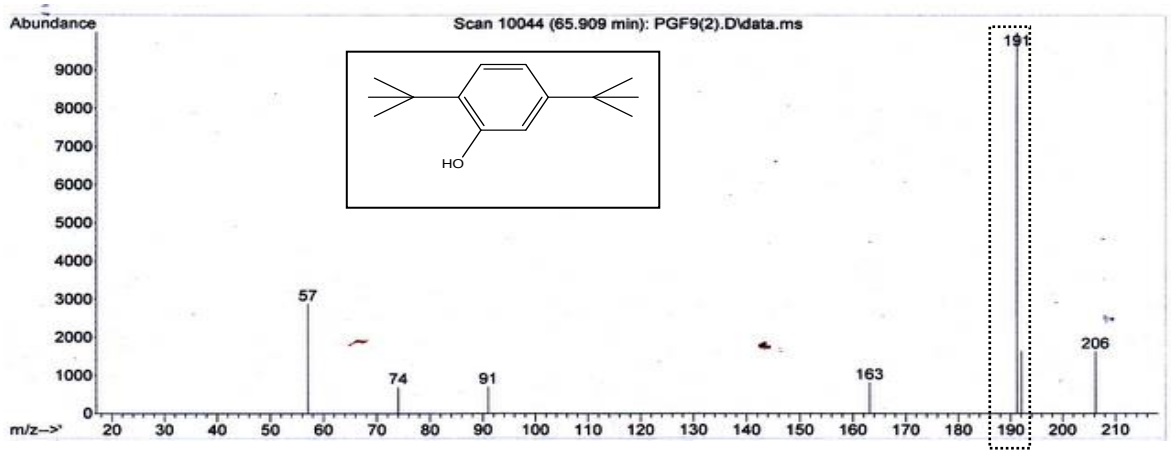

Figure 4.6

Mass spectrum of 2, 5-di-tert-butylphenol

In Figure 4.7 the peak at retention time 113.774 minutes has a mass spectral data consistent with palmitic acid. The mass spectrum exhibited a parent ion at *m/z* 256 that is consistent with the molecular formula $C_{16}H_{32}O_2$. The mass spectral data is as follows

m/z (%): 256 ($[M]^+$,12), 227($[M\text{-}CHO]^+$,2), 213 ($[M\text{-}CHO\text{-}CH_2]^+$,10),199 ($[M\text{-}CHO\text{-}(CH_2)_2]^+$,4), 185 ($[M\text{-}CHO\text{-}(CH_2)_3]^+$,5), 171 ($[M\text{-}CHO\text{-}(CH_2)_4]^+$,5), 157 ($[M\text{-}CHO\text{-}(CH_2)_5]^+$,7), 143 ($[M\text{-}CHO\text{-}(CH_2)_6]^+$,3), 129 ($[M\text{-}CHO\text{-}(CH_2)_7]^+$,50), 73 ($[(CH_2)_2COOH]^+$,100), 60 ($C_2H_4O_2^+$, 90). The base peak at m/z 60 is a characteristic peak resulting from the McLafferty rearrangement. Besides the McLafferty rearrangement peak, the spectrum also showed loss of clusters of 14 (CH_2) mass units typical of long–chain carboxylic acid.

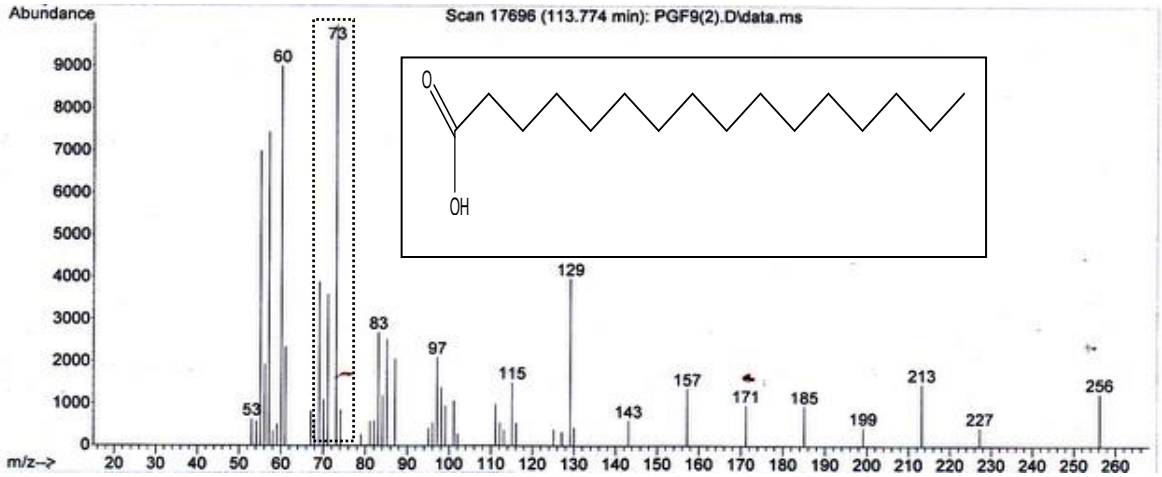

Figure 4.7

Mass spectrum of palmitic acid

Table 1.4 shows the constituents of fractionated ethyl acetate sample (PGF10). Four components comprising of 21.35% of the PGF10 fraction were identified with ergosterol (20.44%) forming the major constituent. The largest major component (41.59%) at retention time 42.245 could not be identified.

Table 4.4

Compounds in PGEF10 identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Tetradecanoic acid (Myristic acid)	13.721	0.17	228.37	$C_{14}H_{28}O_2$
Unidentified compound	28.321	20.81	228.37	$C_{14}H_{28}O_2$
Unidentified compound	42.245	41.59	-	-
Unidentified compound	43.477	8.48	-	-
Ergosterol	110.340	20.44	396.65	$C_{28}H_{44}O$
Neoergosterol	111.798	0.74	380.61	$C_{27}H_{40}O$
Total amount : 92.23%				

In Figure 4.7 the peak at retention time 13.721 minutes has a mass spectral data consistent with myristic acid. The mass spectrum exhibited a parent ion at *m/z* 228 that is consistent with the molecular formula $C_{14}H_{28}O_2$. The mass spectra data is as follows *m/z*(%): 228 (M^+, 15), 199 ($[M\text{-}CHO]^+$, 7), 185 ($[M\text{-}CHO\text{-}CH_2]^+$, 25), 171($[M\text{-}CHO\text{-}(CH_2)_2]^+$, 20), 157 ($[M\text{-}CHO\text{-}(CH_2)_3]^+$, 20), 143 ($[M\text{-}CHO\text{-}(CH_2)_4]^+$, 20), 129 ($[M\text{-}CHO\text{-}(CH_2)_5]^+$, 50), 115, 107, 97, 85, 73, 60($[CH_2COHOH]^+$,100).

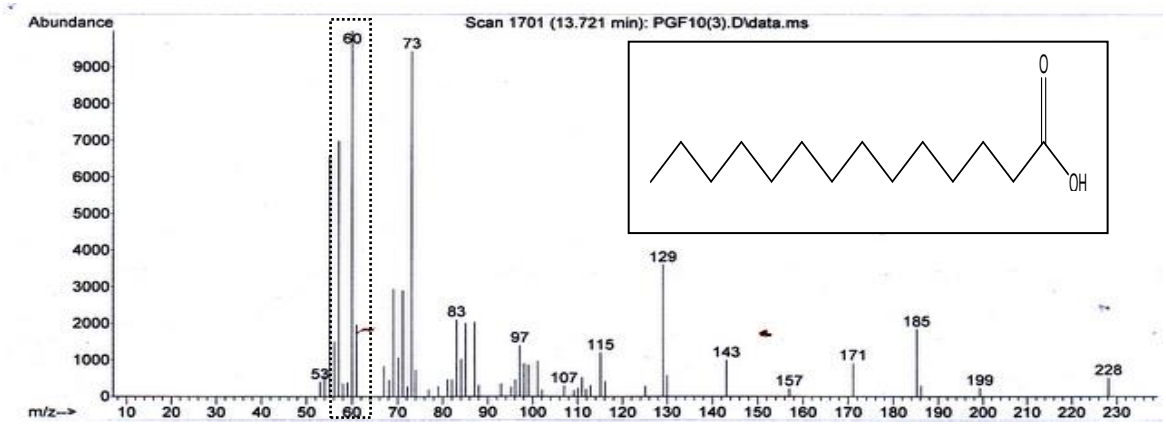

Figure 4.8

Mass spectrum of myristic acid

In Figure 4.9 the peak at retention time 110.340 minutes has a mass spectral data consistent with ergosterol. The mass spectrum exhibited a parent ion at *m/z* 396 that is consistent with the molecular formula $C_{28}H_{44}O$. The mass spectral data is a as follows *m/z* (%): 396 (M^+, 15), 378($[M-H_2O]^+$, 5)363, 337, 253, 157, 143, 81, 69([(CH) CHCH=CH]$^+$, 99), 55 ($[C_4H_7]^+$, 100).

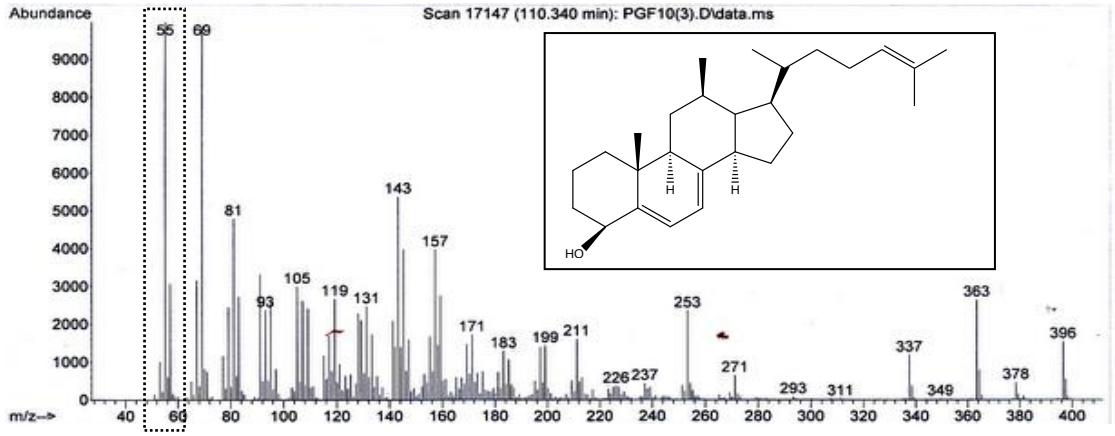

Figure 4.9

Mass spectrum of ergosterol

In Figure 4.10 the peak at retention time 111.798 minutes has a mass spectral data consistent with neoergosterol. The mass spectrum exhibited a parent ion at *m/z* 380

that is consistent with the molecular formula $C_{27}H_{40}O$. The mass spectral data is a as follows m/z(%): 380 (M^+ , 20), 362 ([M- H_20]$^+$, 15), 319 ([M- H_20- C_3H_7]$^+$, 8), 282, 267, 253, 237, 225, 213,195, 183, 165, 153, 141, 128,109,95, 81,69 ([(CH) CHCH=CH]$^+$75), 55 M^+- ([$CH(CH_3)CH(CH_3)_2$]$^+$, 100).

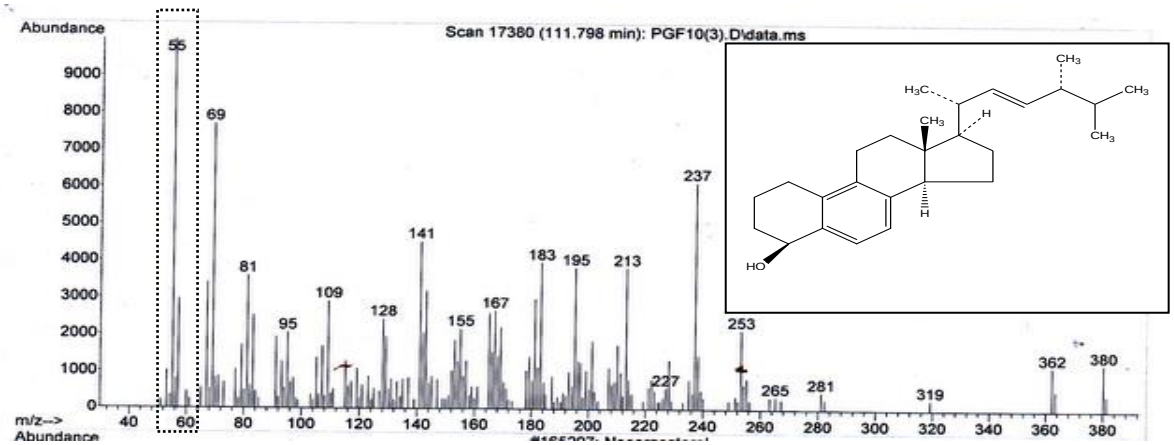

Figure 4.10

Mass spectrum of neoergosterol

Table 4.5 represents components identified in fraction PGF31. One phenolic compound, benzeneacetic acid, was identified in this fraction.

Table 4.5

Compounds in PGF31 identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Benzeneacetic acid (α-Toluic acid)	8.641	1.89	136.15	$C_8H_8O_2$
Unidentified compound	17.272	3.21	-	-
Unidentified compound	19.638	8.24	-	-
Unidentified compound	40.168	12.37	-	-
Unidentified compound	89.066	9.41	-	-
Unidentified compound	102.371	35.90	-	-
Total amount : 71.02%				

In Figure 4.11 the peak at retention time 8.635 minutes has a mass spectral data consistent with α-toluic acid. The mass spectrum exhibited a parent ion at *m/z* 136 that is consistent with the molecular formula $C_8H_8O_2$. The mass spectral data is as follows *m/z* (%): 136 (M^+, 40), 118([M- H_2O]$^+$, 2), 91([M- COOH]$^+$100), 65.

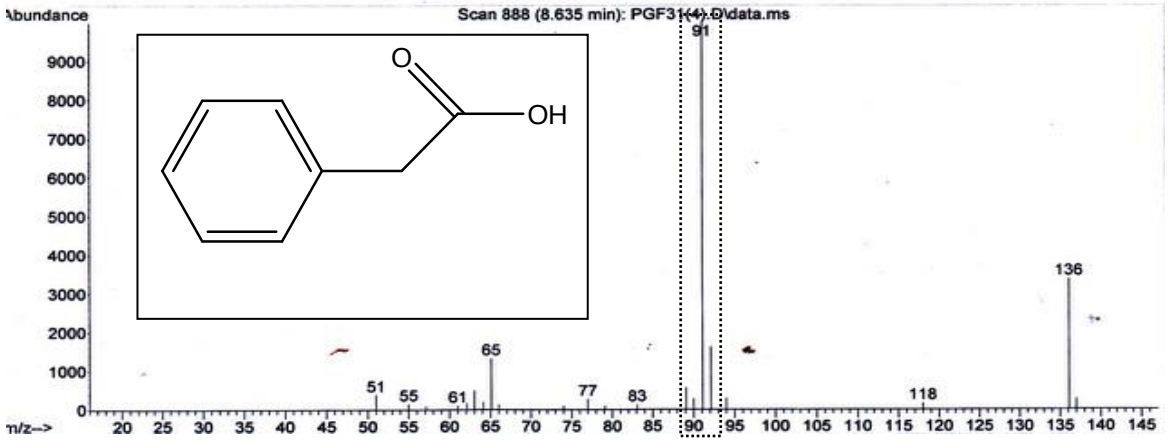

Figure 4.11

Mass spectrum of α-toluic acid

Table 4.6 shows the compounds identified in fraction PGF37, representing fraction thirty four to thirty seven pooled together. One compound, nicotinamide, was identified in this fraction.

Table 4.6

Compounds in PGF37 identified by GC-MS analysis

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
3-Pyridinecarboxamide (Nicotinamide)	51.966	1.07	122.12	$C_6H_6N_2O$
Oleic acid	114.037	95.55	282.46	$C_{18}H_{34}O_2$
Total amount : 96.62%				

In Figure 4.12 the peak at retention time 51.966 minutes has a mass spectral data consistent with nicotinamide acid. The mass spectrum exhibited a parent ion at *m/z* 136 that is consistent with the molecular formula $C_6H_6N_2O$. The mass spectral data as follows *m/z* (%): 122 ($M^{+\cdot}$ 100), 106 ($[M\text{-}NH_2]^+$, 60), 78 ([M-NH_2-CO], 80), 51.

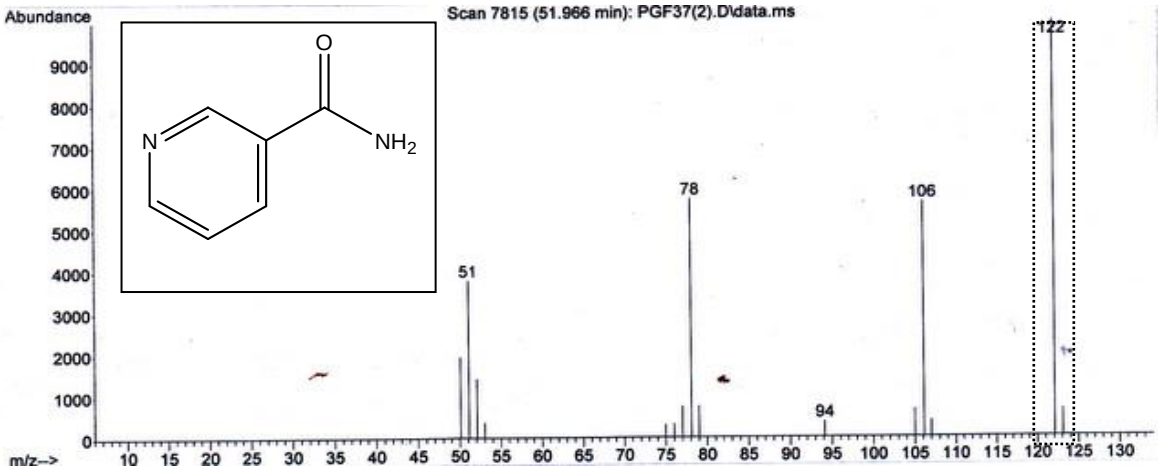

Figure 4.12

Mass spectrum of nicotinamide

Table 4.7 showed components in fraction PGF39. The peak at retention time 32.516 minutes has a mass spectral data consistent with 3, 4- dimethoxy-benzaldehyde (methyl vanillin).

Table 4.7

Compounds in PGF39 (fraction thirty nine of ethyl acetate sample) identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Unidentified compound	11.062	5.17	-	-
Unidentified compound	11.319	9.45	-	-
Unidentified compound	17.061	4.96	-	-
Unidentified compound	19.119	18.08	-	-
3,4- dimethoxy- benzalde-hyde (Methyl vanillin)	32.516	12.96	166.17	$C_9H_{10}O_3$
Unidentified compound	36.446	10.21	-	-
Total amount : 60.83%				

The mass spectrum in Figure 4.13 the exhibited a parent ion at *m/z* 166 that is consistent with the molecular formula $C_8H_8O_2$. The mass spectral data is as follows *m/z* (%): 166 (M^+, 100), 151([M- CH_3]$^+$ 11), 137, 122, 107, 95, 77, 65, 51.

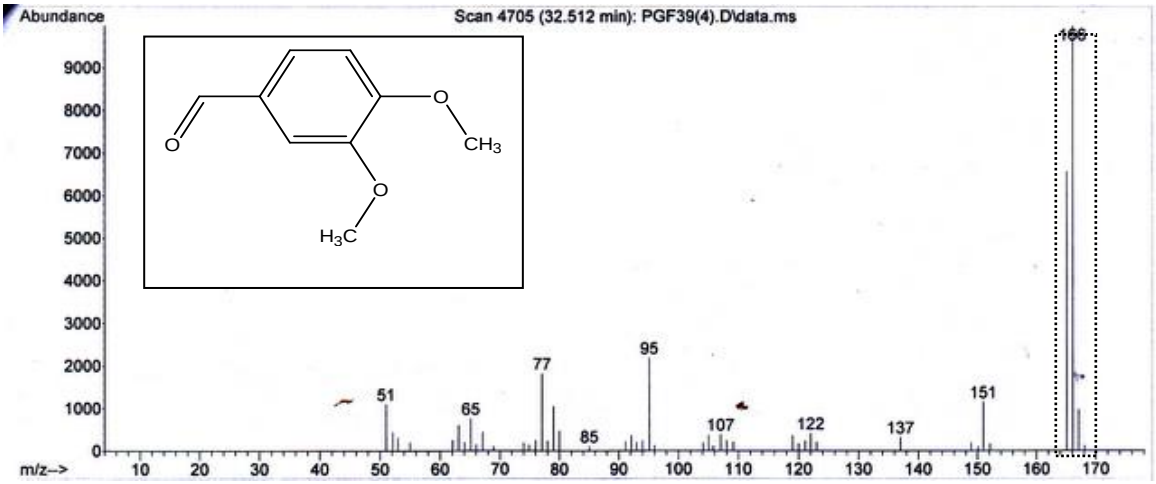

Figure 4.13

Mass spectrum of methyl vanillin

4.2 Identification of compounds in hexane extract

The hexane extract (0.1g) was subjected to column chromatography, eluting with 100 % hexane followed by hexane enriched with increasing percentages of acetone. The eluted compounds were monitored using TLC. A total of 34 fractions were collected from the column chromatography. The fractions were pooled according to the spots on TLC plates. Components in the isolated fractions were identified using GC-MS. Table 1.8 shows the physical characteristics of the isolated fractions.

Table 4.8

Fractions obtained from hexane extract using column chromatography

Pooled Vials	Weight (mg)	Vials	Appearance
PGHEX4	1.0mg	1-4	Yellow oil
PGHEX5	1.1mg	5	Colourless crystals
PGHEX10	1.0mg	6-10	Colourless crystals
PGHEX16	1.1mg	12-16	Colourless oil
PGHEX19	1.0mg	18-19	Colourless oil

Table 4.9 showed components identified in PGHEX4 fraction. Two components comprising 25.77% of the PGHEX4 subfraction were identified with methyl linoleate (15.58%) forming the major component whilst palmitic acid (10.19%) formed the next major component.

Table 4.9

Compounds in PGHEX4 fraction identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
n-Hexadecanoic acid (Palmitic acid)	13.202	10.19	256.22	$CH_3(CH_2)_{14}COOH$
9, 12 Octadecadienoic acid, methyl ester (Methyl linoleate)	16.592	15.58	294.47	$C_{19}H_{34}O_2$
Unidentified compound	18.537	4.74	-	-
Unidentified compound	29.065	46.34	-	-
Total amount : 76.85%				

In Figure 4.14 the peak at retention time 16.598 minutes has a mass spectral data consistent with methyl linoleate. The mass spectrum exhibited a parent ion at *m/z* 294 that is consistent with the molecular formula $C_8H_8O_2$. The mass spectral data is as follows *m/z* (%): 294, 263([M- $CH_3O]^+$ 5), 220, 206, 191, 178, 164, 150, 135, 123, 109, 95, 81, 67 (100), 55.

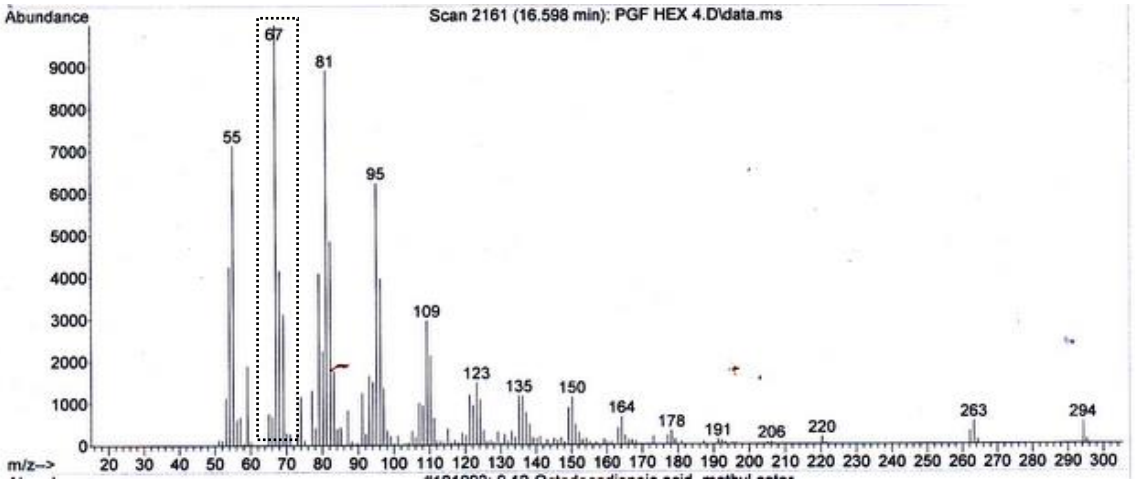

Figure 4.14

Mass spectrum of methyl linoleate

Table 4.10

Compounds in PGHEX5 fraction identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Oleic acid ((9Z)-Octadecenoic acid)	18.212	68.05	282.46	$C_{18}H_{34}O_2$
Campesterol	44.778	0.95	400.68	$C_{28}H_{48}O$
Total amount : 69.0%				

In Figure 4.14 the peak at retention time 18.212 minutes has a mass spectral data consistent with oleic acid. The mass spectrum exhibited a parent ion at *m/z* 282 that is consistent with the molecular formula $C_{18}H_{34}O_2$. The mass spectral data is as follows *m/z* (%): 282 (M^+, 3), 264([M- H_20]+, 10), 256, 235, 222, 207, 193([M - H_2O - C_2H_5 - C_3H_6]$^+$, 3),180, 165, 151, 137, 123, 111, 97, 83, 69,55([COOH]$^+$ 100).

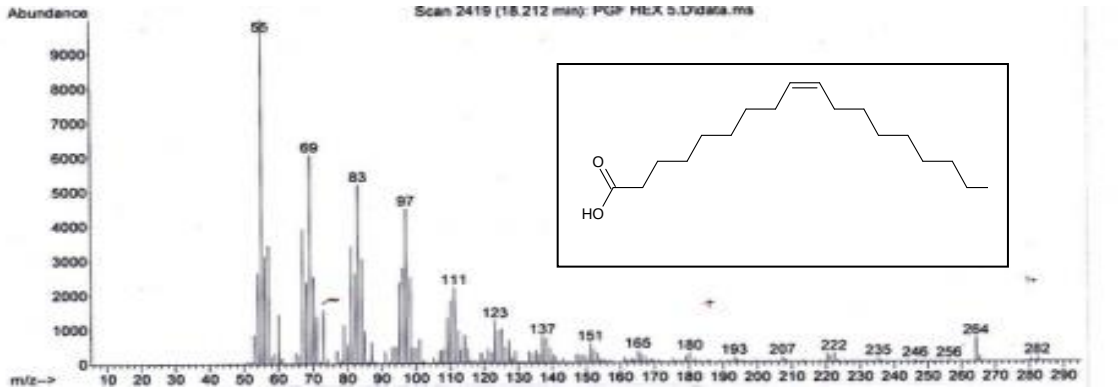

Figure 4.15

Mass spectrum of oleic acid

In Figure 4.16 the peak at retention time 44.778 minutes has a mass spectral data consistent with campesterol. The mass spectrum exhibited a parent ion at *m/z* 400 that is consistent with the molecular formula $C_8H_8O_2$. The mass spectral data is as follows *m/z* (%): 400 (M^+, 80), 385([M- CH_3]$^+$ 30), 367, 273, 255 (100), 229, 213, 161, 147, 133, 119, 107, 91, 79, 67, 55.

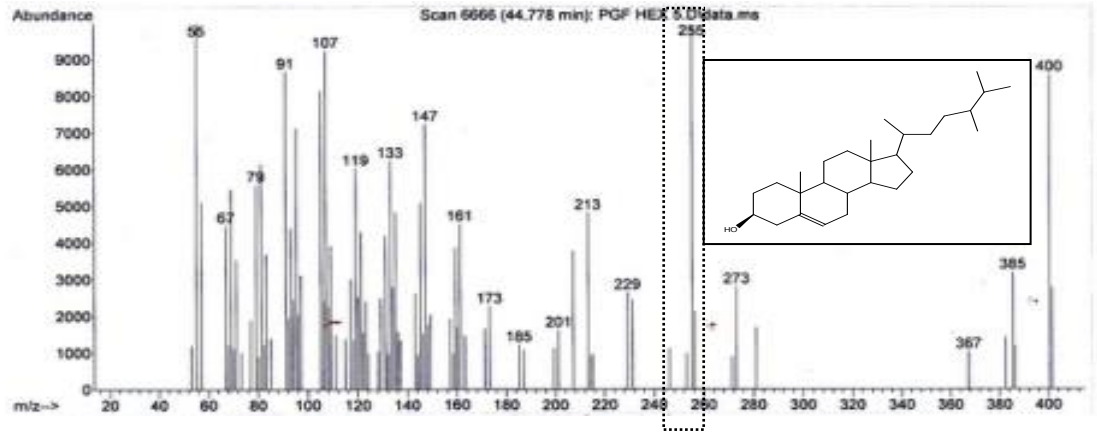

Figure 4.16

Mass spectrum of campesterol

In table 4.11 only one phenolic compound benzeneacetic acid, 4-hydroxy-, methyl ester comprising of 3.84% of the total PGHEX10 fraction was identified. Other components forming the major constituents could not be identified.

Table 4.11

Compounds in PGHEX10 fraction identified by GC-MS

Compounds Name	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Benzeneacetic acid, 4-hydroxy-, methyl ester (Acetic acid, (p-hydroxyphenyl- methyl ester	3.94	3.84	166.17	$C_9H_{10}O_3$
Unidentified compound	13.09	9.63	-	-
Unidentified compound	17.81	73.03	-	-
Unidentified compound	29.02	9.12	-	-
Total amount :95.62%				

In Figure 4.17 the peak at retention time 3.937 minutes has a mass spectral data consistent with acetic acid, p-hydroxyphenyl- methyl ester**.** The mass spectrum exhibited a parent ion at *m/z* 166 that is consistent with the molecular formula $C_9H_{10}O$. The mass spectral data is as follows consistent *m/z* (%): 166 (M^+, 25), 121, 107([M-$CH_3CO_2]^+$ 100), 83, 77, 59, 51.

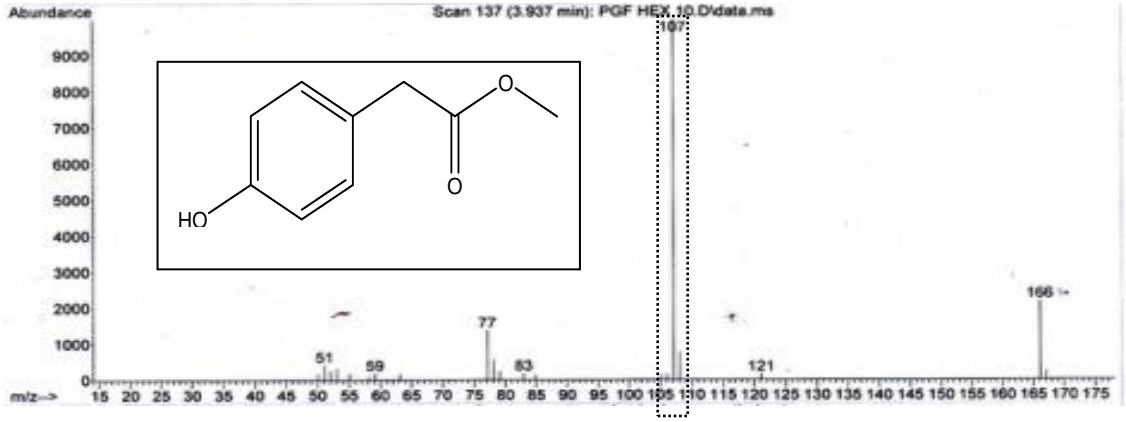

Figure 4.17

Mass spectrum of acetic acid, p-hydroxyphenyl- methyl ester

Table 4.12 represents the fractionated hexane sample, representing fraction eleven to sixteen pooled together. One alkaloid compound N-acetylphenylethylamine was present in the hexane extract.

Table 4.12

Compounds in PGHEX16 fraction identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Acetamide, N-(2-phenylethyl)- *N*-acetylphenylethylamine	4.344	5.07	163.21	$C_{10}H_{13}NO$
Unidentified compound	17.580	8.91	-	-
Unidentified compound	31.611	17.79	-	-
Unidentified compound	52.160	14.97	-	-
Unidentified compound	53.035	53.27	-	-
Total amount : 100%				

In Figure 4.17 the peak at retention time 4.344 minutes has a mass spectral data consistent with N-acetylphenylethylamine. The mass spectrum exhibited a parent ion at *m/z* 163 that is consistent with the molecular formula $C_{10}H_{13}NO$. The mass spectral data is as follows *m/z* (%): 163 (M^+, 20), 118, 104([M- $CH_3CONH]^+$, 100), 91, 85, 77, 72, 65, 51.

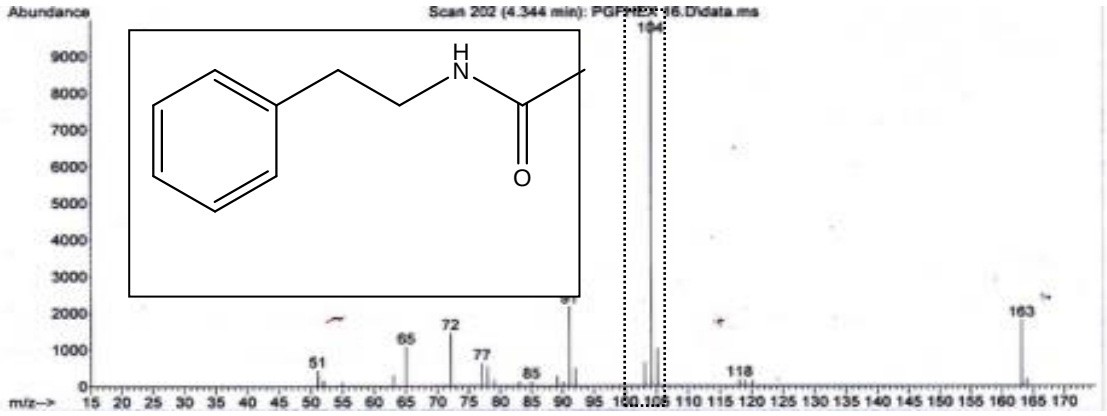

Figure 4.18

Mass spectrum of *N*-acetylphenylethylamine

Table 4.13 represents the fractionated hexane sample, representing fraction seventeen to nineteen pooled together. One phenolic compound tyrosol was identified.

Table 4.13

Compounds in PGHEX19 Fraction identified by GC-MS

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Benzeneethanol, 4 – hydroxyl (Tyrosol)	3.343	0.84	138.16	$C_{10}H_{13}NO$
Unidentified compound	37.435	7.17	-	-
Unidentified compound	41.857	5.50	-	-
Unidentified compound	59.241	86.49	-	-
Total amount : 100%				

In Figure 4.19 the peak at retention time 3.343 minutes has a mass spectral data consistent tyrosol. The mass spectrum exhibited a parent ion at *m/z* 138 that is consistent with the molecular formula $C_8H_8O_2$. The mass spectral data is as follows *m/z* (%): 138 (M^+, 30), 107([M- $CH_2OH]^+$, 100), 91, 83, 77.

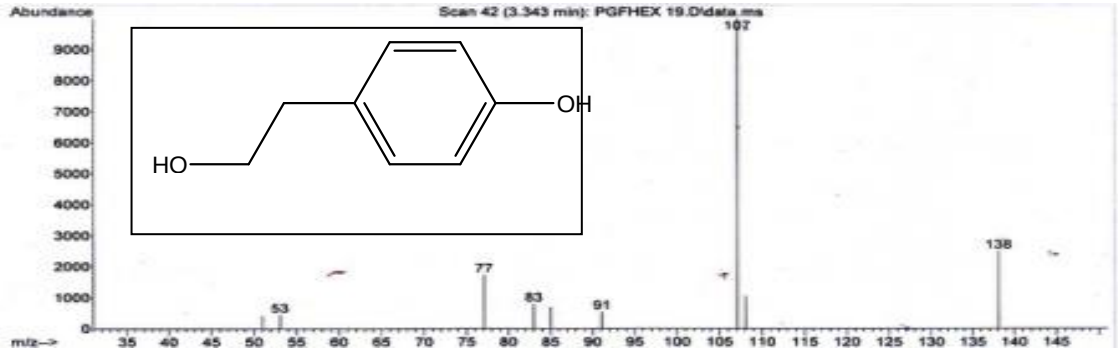

Figure 4.19

Mass spectrum of tyrosol

4.3 Identification of compounds in aqueous extract

Table 4.14 represents components identified in the aqueous extract. Succinic acid and 4 phenolic compounds comprising of 16.19 % of the aqueous extract were identified. Major compounds at retention times 5.82, 5.66, 6.94, 8.92 and 11.18 minutes representing 13.72%, 26.44%, 12.56%, and 22.63% respectively, could not be identified.

Table 4.14

Components identified in the aqueous extract

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Succinic Acid (Amber Acid)	0.97	12.57	118.08	$C_4H_6O_4$
Caffeic Acid	1.29	1.31	180.16	$C_9H_8O_4$
Benzoic Acid (Benzenecarboxylic acid)	2.24	2.14	122.12	$C_7H_6O_2$
Hydroxylated Cinnamic acid (Coumaric Acid)	2.58	0.07	164.16	$C_9H_8O_3$
Cinnamic Acid (trans-Cinnamic Acid)	2.75	0.10	148.16	$C_9H_8O_2$
Unidentified compound	5.66	0.22	-	-
Unidentified compound	5.82	13.72	-	-
Uridine (Uracil riboside)	6.79	0.55	244.2	$C_9H_{12}N_2O_6$
Unidentified compound	6.94	26.44	-	-
Unidentified compound	7.27	0.64	-	-
Unidentified compound	8.42	12.56	-	-
Unidentified compound	11.18	22.63	-	-
Total amount : 93.59%				

In Figure 4.20 the peak at retention time 0.965 minutes has a mass spectral data consistent amber acid. The mass spectrum exhibited a parent ion at *m/z* 117 that is consistent with the molecular formula $C_4H_6O_4$. The mass spectral data is as follows *m/z* (%): 117 (M-H, 75), 99, 74, 73 ([M-H–COO]$^+$, 100), 72, 70.

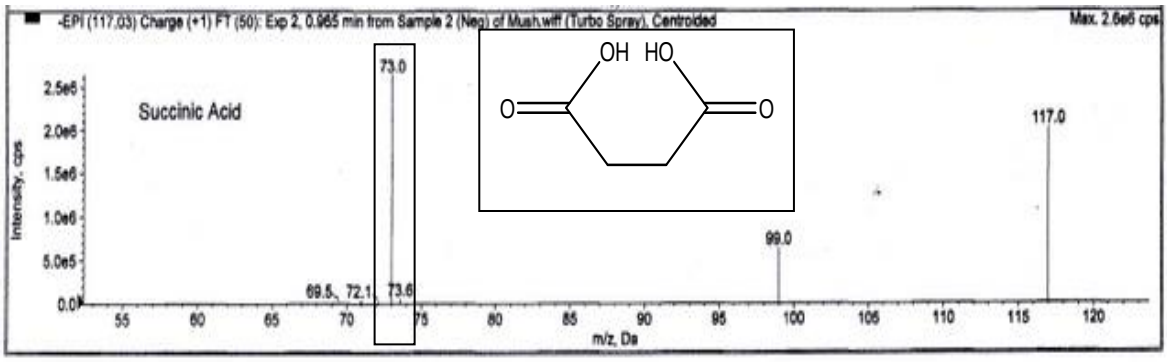

Figure 4.20

Mass spectrum of amber acid

In Figure 4.21 the peak at retention time 2.261 minutes has a mass spectral data consistent benzenecarboxylic acid. The mass spectrum exhibited a parent ion at *m/z* 121 that is consistent with the molecular formula $C_7H_6O_2$. The mass spectral data is as follows *m/z* (%): 121 (M-H, 100), 120, 108, 93, 92, 91, 55.

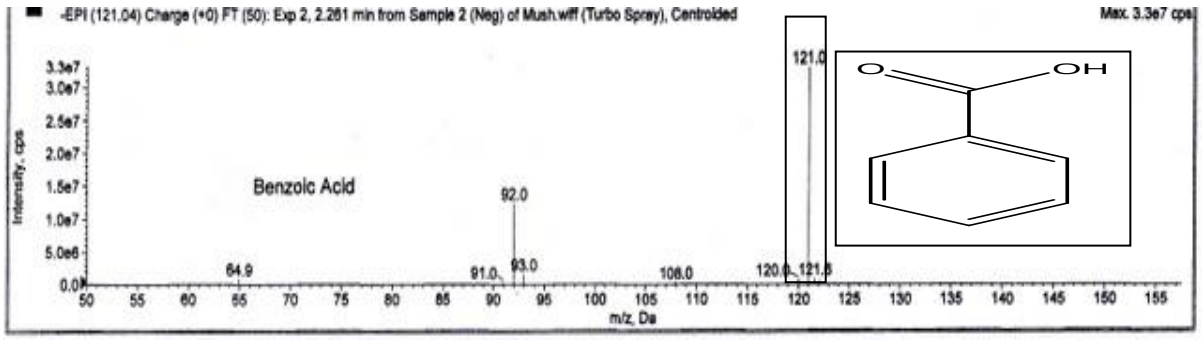

Figure 4.21

Mass spectrum of benzenecarboxylic acid

In Figure 4.22 the peak at retention time 2.584 minutes has a mass spectral data consistent coumaric acid. The mass spectrum exhibited a parent ion at *m/z* 165 that is consistent with the molecular formula $C_9H_8O_3$. The mass spectral data is as follows *m/z* 165 (M-H, 80), 147 ($[M-H-H_2O]^+$, 100), 121, 119($[M-H-H_2O-CO]^+$, 60), 118, 117, 103, 102, 101, 91, 89, 73.

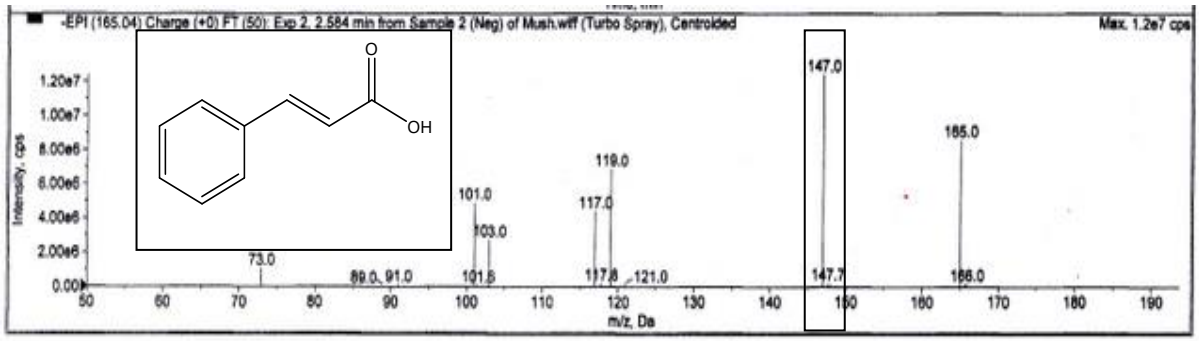

Figure 4.22

Mass spectrum of coumaric acid

In Figure 4.23 the peak at retention time 2.745 minutes has a mass spectral data consistent *trans*-cinnamic acid. The mass spectrum exhibited a parent ion at *m/z* 147 that is consistent with the molecular formula $C_9H_8O_2$. The mass spectral data is as follows *m/z* (%): 147(M-H, 100), 119($[M-H-CO]^+$40), 103($[M-H-CO-OH]^+$40), 77.

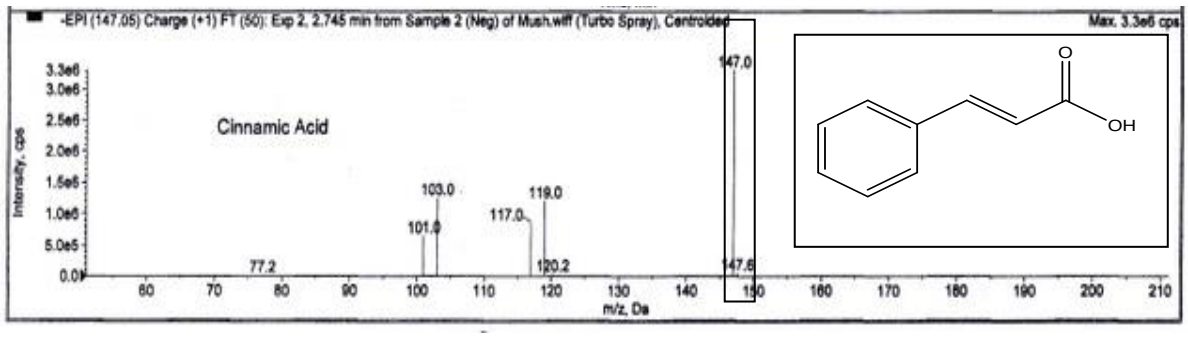

Figure 4.23

Mass spectrum of *trans*-cinnamic acid

In Figure 4.24 the peak at retention time 1.289 minutes has a mass spectral data consistent with caffeic acid. The mass spectrum exhibited a parent ion at *m/z* 181 that is consistent with the molecular formula $C_9H_8O_4$. The mass spectral data is as follows *m/z* (%): 181(M-H, 100) 163([M-H $-H_2O]^+$,70), 135([M-H $-H_2O$-CO]$^+$,72), 134, 119, 117, 107, 101, 93, 73.

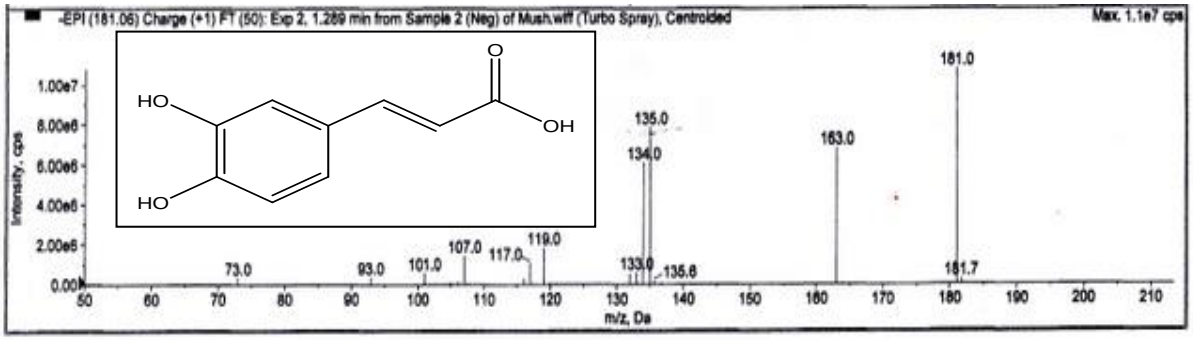

Figure 4.24

Mass spectrum of caffeic acid

In Figure 4.25 the peak at retention time 6.79 minutes has a mass spectral data consistent with uracil riboside. The mass spectrum exhibited a parent ion at *m/z* 243 that is consistent with the molecular formula $C_9H_{12}N_2O_6$. The mass spectra (%): 243 (M-H, 100), 226, 225, 207, 199, 181, 153, 136, 123.

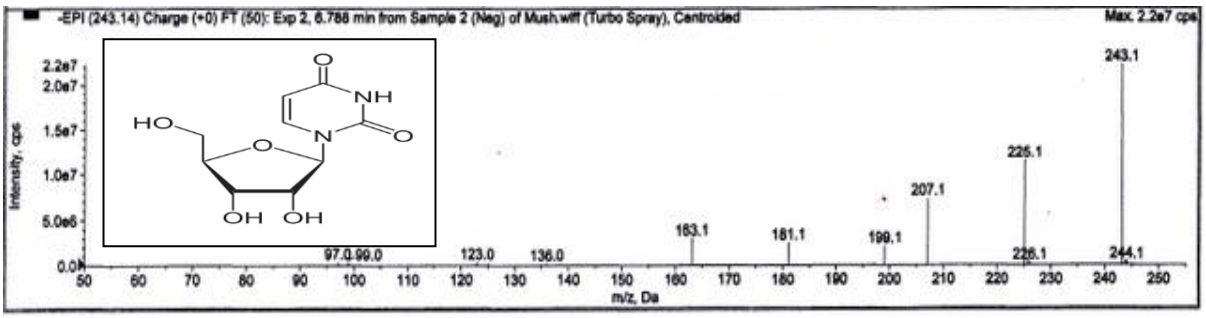

Figure 4.25

Mass spectrum of uracil riboside

4.4 Identification of compounds in essential oil

Table 2.5 shows the results for the essential oil extract. Two compounds were identified in the essential oil extraction. They were benzyl alcohol and 2-phenyl-2-butenal. Both compounds together represented only 2.27% of the total essential oil content. Other major peaks at retention times 30.354 and 70.071 minutes could not be identified.

Table 4.15

Components identified in essential oil

Compounds	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Unidentified compound	7.071	14.28	-	-
Benzyl Alcohol (Benzenemethanol)	11.644	1.01	108.14	C_7H_8O
Unidentified compound	8.091	4.61	-	-
Benzeneacetaldehyde, alpha. –ethylidene (2-Phenyl-2-Butenal)	25.626	1.26	146.19	$C_{10}H_{10}O$
Unidentified compound	30.053	15.40	-	-
Unidentified compound	30.354	44.54	-	-
Unidentified compound	33.950	4.58	-	-
Total amount : 85.68%				

In Figure 4.26 the peak at retention time 11.644 minutes has a mass spectral data consistent benzenemethanol. The mass spectrum exhibited a parent ion at *m/z* 108 that

is consistent with the molecular formula C_7H_8O. The mass spectral data is as follows: *m/z* (%): 108(M^+), 107([M-H]$^+$ 65), 105, 91([M-OH]$^+$ 20), 79([C_6H_5)]$^+$, 100) 65, 57, 51.

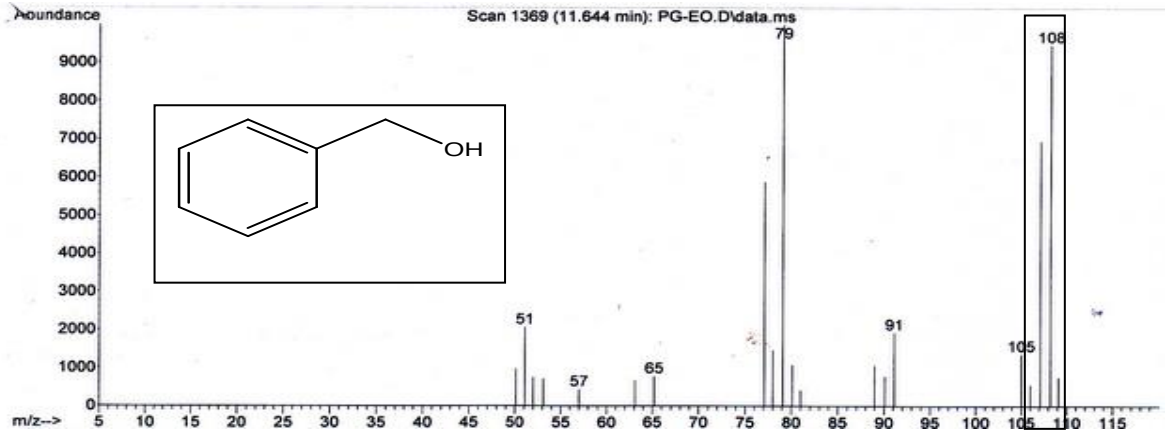

Figure 4.26

Mass spectrum of benzenemethanol

In Figure 4.27 the peak at retention time 25.262 minutes has a mass spectral data consistent with 2-phenyl-2-butenal. The mass spectrum exhibited a parent ion at *m/z* 146 that is consistent with the molecular formula C_7H_8O.The mass spectral data is as follows *m/z* (%): 146(M^+, 80), 131([M-CH_3]$^+$, 10), 115([M-CH_3-CHO]$^+$, 100), 102, 97, 91, 78, 74.

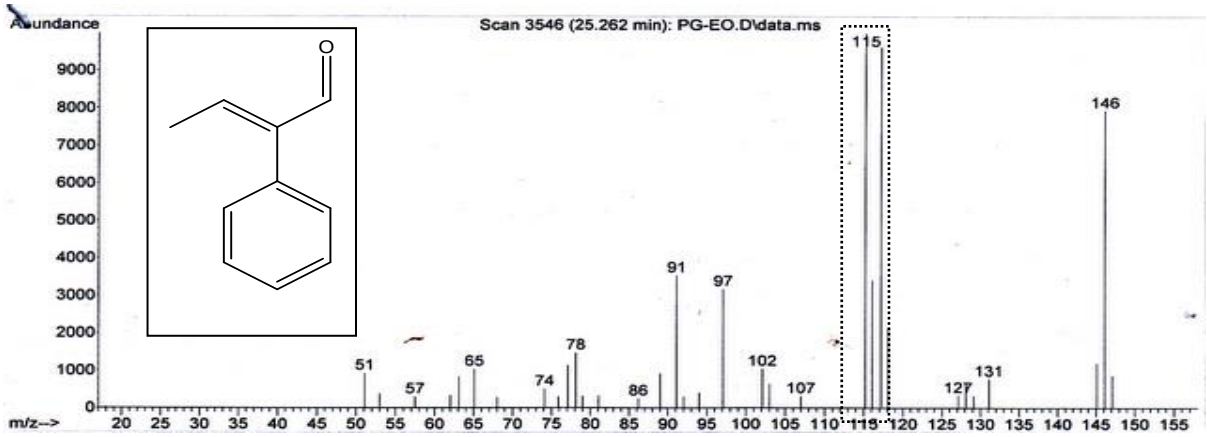

Figure 4.27

Mass spectrum of 2-phenyl-2-butenal

4.5 Identification of compounds in butanol extract

The butanol extract was run on GC-MS and LC-MS/MS. Only hydroxybenzoic acid amounting to 0.67% of the extract was identified in this fraction. No other peaks in the spectrum could be identified.

Table 4.16

Components identified in butanol extract

Compounds Name	**Retention Time (minutes)**	**Percentage (%)**	**Molecular Weight**	**Molecular Formula**
Unidentified compound	0.84	1.56	-	-
Hydroxybenzoic acid	1.29	0.67	138.12	$C_7H_6O_3$
Unidentified compound	2.58	0.54	-	-
Unidentified compound	2.91	0.78	-	-
Unidentified compound	5.82	3.78	-	-
Unidentified compound	6.15	3.12	-	-
Unidentified compound	7.57	5.45	-	-
Unidentified compound	7.95	2.78	-	-
Unidentified compound	9.43	4.89	-	-
Unidentified compound	9.88	10.23	-	-
Unidentified compound	10.25	21.67	-	-
Unidentified compound	11.54	20.45	-	-
Unidentified compound	12.69	10.56	-	-
Unidentified compound	13.47	9.23	-	-
Total: 95.71%				

In Figure 4.28 the peak at retention time 1.287 minutes has a mass spectral data consistent with hydroxybenzoic acid. The mass spectrum exhibited a parent ion at *m/z* 137 that is consistent with the molecular formula $C_9H_8O_4$. The mass spectral data is as follows *m/z* (%): 137 (M+H, 75), 81(100), 79, 63.

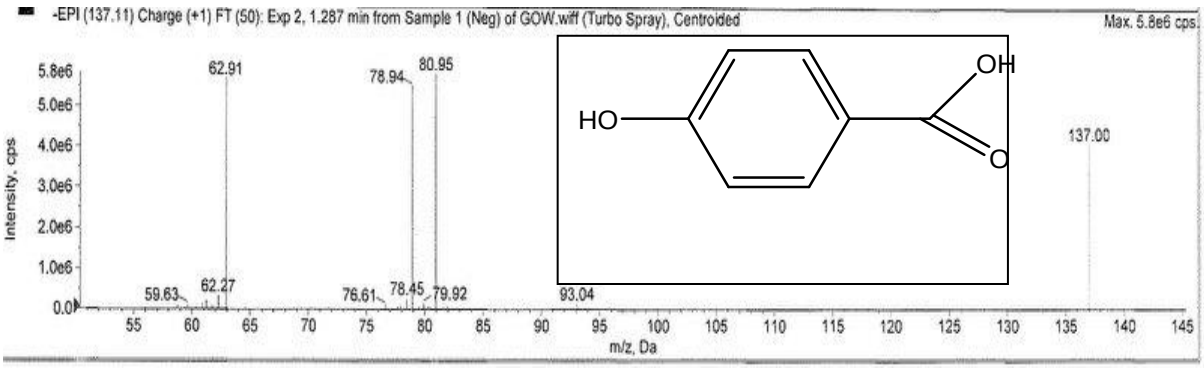

Figure 4.28

Mass spectrum of hydroxybenzoic acid

CHAPTER 5

DISCUSSION

5.1 Fatty acids and their methyl esters

The fruiting bodies of *Pleurotus giganteus* is composed of several fatty acids and esters of fatty acids. Palmitic acid, methyl palmitate, methyl linoleate, methyl stearate, methyl mandelate, myristic acid are some of the fatty acids and fatty esters identified in *Pleurotus giganteus*. The composition of methyl stearate, methyl palmitate acid and methyl linoleate in the total ethyl acetate extract of *Pleurotus giganteus* was 2.26%, 10.58% and 50.28%, respectively. Amongst the unsaturated fatty esters, methyl linoleate was the predominant fatty ester in *Pleurotus giganteus* forming 50.28% of the total ethyl acetate extract. Further fractionation of the crude ethyl acetate extract allowed identification of methyl mandelate and palmitic acid from the PGF9 subfraction. The composition of methyl mandelate and palmitic acid contributed 11.69% and 53.59% respectively of the PGF9 subfraction. In subfraction PGHEX4 palmitic acid was also identified and represented 10.19% of the subfraction. Palmitic acid is the first fatty acid produced during lipogenesis (fatty acid synthesis) and from which longer fatty acids can be produced. In PGF10 subfraction myristic acid was identified and its composition was 20.98%.In the hexane extract of *Pleurotus giganteus,* oleic acid was identified in the PGHEX5 and PGF37 subfractions. The composition of this fatty acid in the subfractions was 68.05% and 95.55% and is as a monounsaturated omega-9 fatty acid. Oleic acid has been reported to reduce coronary heart disease risk by 20–40% mainly via LDL-cholesterol reduction (Lopez-Huertas, 2010). As intakes of saturated fat in many countries are higher that the recommended levels, an increase in the intake of oleic acid may be beneficial as it limits the intake of saturated fat (Lopez-Huertas, 2010).

5.2 Phenolic Compounds

Natural phenolics are compounds possessing one or more aromatic rings with one or more hydroxyl groups and can range from simple molecules (phenolic acids, phenylpropanoids, flavonoids) to highly polymerised compounds (lignins, melanins, tannins). Particularly, phenolic acids can be subdivided into two major groups, hydroxybenzoic acids and hydroxycinnamic acids. Hydroxybenzoic acids include p-hydroxybenzoic, protocatechuic, vanillic, syringic, and gallic acids. They are commonly present in the bound form and are typically a component of a complex structure like lignins and hydrolyzable tannins. They can also be found linked to sugar derivatives and organic acids in plant foods. Hydroxycinnamic acids include p-coumaric, caffeic, ferulic, and sinapic acids. In natural sources they are mainly found esterified with small molecules like, e.g., quinic or tartaric acids, as well as bound to cell-wall structural components such as cellulose, lignin, and proteins through ester bonds (Barros *et al.,* 2009).

In this study, nine phenolic compounds were identified in *Pleurotus giganteus*. In PGF9 subfraction of the ethyl acetate extract p-tert-butylphenol and 2, 5-bis (1, 1-dimethylethyl) phenol were identified and amounted to 0.62% and 0.90% of the subfraction. Methyl vanillin was also identified in the ethyl acetate subfraction (PGF39) and amounted to 12.96% of the fraction. In the hexane extract subfraction (PGHEX19) 4 –hydroxyl benzeneethanol was identified and amounted to 0.84% of the fraction. Coumaric acid, cinnamic acid, benzoic acid and caffeic acid comprising of 0.07%, 0.10%, 2.14% and 1.31% respectively.They have been previously identified in fungi (Ribeiro *et al.,* 2007, Kanagasabapathy *et al.,* 2011, Ferreira *et al.,* 2009). Hydroxybenzoicacid was identified in the butanol extract and represented 0.67% of the extract.

Cinnamic acid and its amides, esters and other derivatives are important but under utilized class of compounds. As anticancer agents they are cytotoxic, cytostatic, antiproliferative, antiangeogenetic, antileukemic, active against solid tumors, inhibit different enzymes e.g. transglutaminase, aminopeptidase N, histone deacetylase etc, cause DNA-damage and many more. In spite of all these multi-activities of cinnamic acid derivatives their mode of action and understanding of molecular mechanisms remains unclear (Del *et al.,* 2010). In animal experiment eugenol and cinnamic acid inhibited HCL/ethanol induced gastric lesions and increased the mucus content. It is suggested they are useful neutraceuticals for gastritis (Jung *et al.,* 2011).

Coumaric acid and caffeic acid have also exhibited antioxidant activity (Cuvelier *et al.,* 1992). Tyrosol was for the first time identified in fungi during this study. As an antioxidant, tyrosol (4 –hydroxyl, benzeneethanol) can protect cells against injury due to oxidation. Although it is not as potent as other antioxidants present in olive oil, its higher concentration and good bioavailability indicate that it may have an important overall effect. This effect may contribute significantly to the health benefits of olive oil and, more generally, the diet. Tyrosol present in white wine is also shown to be cardioprotective (Giovannini *et al.,* 1999). Samuel *et al.,* (2008) have shown that tyrosol-treated animals showed significant increase in the phosphorylation of Akt, eNOS and FOXO3a. In addition, tyrosol also induced the expression of longevity protein SIRT1 in the heart after myocardial infarction in a rat MI model (Samuel *et al.,* 2008). In conclusion these findings suggest that tyrosol induces myocardial protection against ischemia related stress by inducing survival and longevity proteins that may be considered as anti-aging therapy for the heart.

5.3 Sterols

Even though mushrooms are deficient in vitamin D_2, earlier researchers have found them to be rich source of ergosterol. In this study the percentage of ergosterol present in the ethyl acetate subfraction (PGF10) of *Pleurotus giganteus* was 20.44 %. Many reports have revealed that ergosterol acts as a biological precursor to vitamin D_2. It is turned into viosterol by ultraviolet light, temperature of irradiation and moisture content and is then converted into ergocalciferol, which is a form of Vitamin D_2 (Jasinghe & Perera, 2007) used for pharmaceutical applications and food supplements. Ergosterol is a component of fungal cell membranes, serving the same function as cholesterol in animal cells. Ergosterol is the principal sterol and is present in two forms, as free ergosterol and esterified ergosterol. Ergosterol and one of its isomers were reported in this investigation. The isomer of ergosterol that was identified was neoergosterol, also found in fraction PGF10. These compounds present the characteristic template of tetracyclic triterpenes, mostly sterols, with structural characteristics that vary in number and position of the unsaturation's in the rings and in the side chains, and in the keto and hydroxyl groups located in different positions of the triterpenic template. Kuo *et al*. (2011) reported that ergosterol was able to inhibit LPS-induced inflammation in RAW 264.7 marophages by suppressing the NF-κB signaling pathway. Some other mushrooms in which ergosterol has been identified are Japanese edible mushroom 'Buna-shimeji', (*Hypsizigus marmoreus)*, 'white button (*Agaricus bisporus)*, enoki (*Flammulina veluptipes)*, shiitake (*Lentinus edodes)*, maitake (*Grifola frondosa*), oyster (*Pleurotus ostreatus)*, crimini (*Agaricus bisporus)* and portabella (*Agaricus bisporus)* (Yasukawa *et al.*, 1994 & Phillips *et al.*, 2011).

Campesterol is a phytosterol which the chemical structure is similar to that of cholesterol. Campesterol was the minor component in *Pleurotus giganteus.* In this study the percentage of campesterol present in the hexane subfraction (PGHEX9) of *Pleurotus*

giganteus was 0.95 %. Many vegetables, fruits, nuts and seeds contain campesterol, but in low concentrations. Banana, pomegranate, pepper, coffee, grapefruit, cucumber, onion, oat, potato and lemon grass (citronella) are few examples of common sources containing campesterol at 1-7 mg/100 mg of the edible portion. In contrast, canola and corn contain 50-200 mg of campsterol (Seguraa *et al.,* 2006*)*. It is so named because it was first isolated from the rapeseed (*Brassica campestris*). It is known to have cholesterol-lowering activity (Seguraa *et al.,* 2006*)* and anti-inflammatory effect (Gabay *et al.,* 2010). It was reported to inhibit several pro-inflammatory and matrix degradation mediators typically involved in osteoarthritis-induced cartilage degradation (Gabay *et al.,* 2010). Campesterol has been previously identified in 'morel' *Morchella spp* of mushroom (Phillips *et al.,* 2011).

5.4 Succinic Acid

Succinic acid was identified in the aqueous extract and amounted to 12.57% of the extract. Succinic acid is a dicarboxylic acid and plays a biochemical role in the citric acid cycle. The name derives from Latin *succinum*, meaning amber, from which the acid may be obtained. The carboxylate anion is called succinate and esters of succinic acid are called alkyl succinates. Succinic acid has been previously identified in mushrooms (Valentao *et al.,* 2005). In the study conducted by Valentao *et al*. (2005), six species of wild edible mushrooms were studied, namely *Amanita caesarea, Boletus edulis, Gyroporus castaneus, Lactarius deliciosus, Suillus collinitus,* and *Xerocomus chrysenteron.* Succinic acid was present in very small amounts, below 0.2% of the total quantified compounds in all the tested species (Valentao *et al.,* 2005).

The inhibition of lipid peroxidation by idebenone in brain mitochondria in the presence of succinate is the process whereby free radicals "steal" electrons from the

lipids in our cell membranes, resulting in cell damage and increased production of free radicals. Lipid peroxidation in brain mitochondria was induced by NADH in the presence of ADP and $FeCl_3$. A novel quinone compound, idebenone, inhibited this peroxidation and the inhibition was markedly enhanced by succinate, a substrate of mitochondrial respiration (Suno & Nagaoka, 1989). The concentration of succinate required to exert the maximal effect was 1.5 mM. The concentration of idebenone giving 50% inhibition was 0.5 and 84mM in the presence and absence of succinate, respectively, indicating that succinate enhances the inhibition by 170-fold. These results indicate that idebenone is changed to its reduced form, which protects mitochondria against lipid peroxidation (Suno & Nagaoka, 1989).

The life-extending gene Indy encodes an exchanger for Krebs-cycle intermediates. A longevity gene called Indy, with homology to mammalian genes encoding Na-dicarboxylate cotransporters, was identified in *Drosophila melanogaster*. Efflux of [14C]-citrate from INDY-expressing oocytes was greatly accelerated by the addition of succinate to the external medium, indicating citrate-succinate exchange. From the study it concluded that INDY functions as an exchanger of dicarboxylic and tricarboxylic Krebs cycle intermediates. The effect of decreasing INDY activity, as in the long- lived Indy mutants, may be to alter energy metabolism in a manner that favours life span extension (Knauf *et al.,* 2006).

Cardioprotective effect of succinate is protective against ischemia/reperfusion injury (Sakamoto *et al.,* 1998). In another study, it was investigated the protective effects of succinate, which is a respiratory substrate and a potential antioxidant, on myocardial ischemia/reperfusion injury with the whole heart. Isolated rat hearts were loaded with 25-min normothermic global ischemia followed by 30- min reperfusion in a working heart model. Succinate administered either before reperfusion or added to the cardioplegic solution improved the postischemic cardiac function significantly. The hearts

arrested with succinate-supplemented cardioplegic solution replenished high-energy phosphates and maintained the total adenine nucleotides during the reperfusion period, whereas those arrested with succinate-nonsupplemented cardioplegic solution replenished the high-energy phosphates less, and also lost total adenine nucleotides during that period. It was concluded that succinate administered before reperfusion may decrease the degree of mitochondrial damage during reperfusion and thereby reduce the amount of myocardial ischemia/reperfusion injury. Postischemic administration of succinate reverses the impairment of oxidative phosphorylation after cardiac ischemia and reperfusion injury (Sakamoto *et al.,* 1998).

5.5 Nicotinamide

Nicotinamide which was identified in the subfraction (PGF37) represented 1.07% of the extract. In a previous study on *Pleurotus sajor caju* mushroom, nicotinamide was identified (Kanagasabapathy *et al.,* 2011). Nicotinamide, also known as niacinamide and nicotinic acid amide, is the amide of nicotinic acid (vitamin B_3 / niacin). Nicotinic acid is converted to nicotinamide and the two are identical in their vitamin functions. Investigations on the medicinal properties of nicotinamide have been studied for more than a decade. Nicotinamide was reported to inhibit lipid peroxidation in rat brain mitochondria. It is stated that, the percentage of lipid peroxidation inhibition displayed by nicotinamide is comparable to tryptophan and isonicotinic acid and higher than ascorbic acid. This is due to the amide group in nicotinamide that plays an important role as O_2 and other reactive species quenchers (Kamat & Devasagayam, 1999). Hence nicotinamide can be considered as a potent antioxidant capable of protecting the cellular membranes in brain, which is highly susceptible to pro-oxidants, against oxidative damage induced by ROS.

5.6 Uridine

Uridine which was identified in the aqueous fraction represented 0.55% of the extract. Uridine is one of the four basic components of ribonucleic acid (RNA); the other three are adenosine, guanosine, and cytidine. The bioavailability of uridine is particularly crucial to the synthesis of RNA and biomembranes *via* the formation of pyrimidine nucleotideÐlipid conjugates, and thus is essential for cellular function and growth. Clinical trials involving therapeutic doses of uridine and its nucleotides are being investigated to treat dieses such hereditary orotic aciduria, cystic fibrosis, liver dysfunction, cancer, schizophrenia and epilepsy (Connolly & Duley, 1999).

5.7 Alkaloid

Phenylethylamine alkaloids are aromatic amine made up of a benzene ring to which an ethylamine side chain is attached . The phenylethylamine alkaloid group is a precursor of many natural and synthetic compounds. Several substituted phenylethylamine alkaloid group are pharmacologically active compounds found in plants and animals. This group includes simple phenylamine (tyramine, hordenine) and catecholamine (dopamine). The latter was found in animals and terrestrial plants (Barroso & Rodriguez., 1996). The structure of phenylethylamine alkaloid group allows substitutions on the aromatic ring, the α and β carbons and terminal amino group. N-acetylphenylethylamine which is a phenylethylamine alkaloid, was identified in the hexane extract (PGHEX16). It represented 5.07% of the fraction. This is the first time N-acetylphenylethylamine was identified in mushrooms. In a previous study *N*-acetylphenylethylamine was isolated from the red algae *Phyllophora crispa* and *Gelidium crinale*. They exhibited pharmacological activity on the nigrostriatal dopamine neurotransmission (Barroso & Rodriguez., 1996).

5.8 Benzyl alcohol

Benzyl alcohol was identified in the essential oil extract and it represented 1.01% of the fraction. It was also identified in other mushrooms for example *Agaricus bisporus* (Espín & Wichers., 1999). Benzyl alcohol is a fragrance ingredient used in many compounds. It may be found in fragrances used in decorative cosmetics, fine fragrances, shampoos, toilet soaps and other toiletries as well as in non-cosmetic products such as household cleaners and detergents. It is a colourless liquid with a faint, nondescript odour, rather sweet, but varies considerably with the quality of the alcohol. This material has been reported to occur in nature, with highest quantities observed in species of allium plants (Scognamiglio *et al.*, 2011).

5.9 Recommendations for further studies

As a large number of compounds were detected but unidentified, further work can performed on the characterization/identifaction of these compounds which could lead to new interesting and potentially previously undiscovered compounds. These compounds can open up new avenues of research.

CHAPTER 6

CONCLUSION

In this study, the compounds isolated from *Pleurotus giganteus* identified by GC-MS or LC/MS/MS. Various types of extraction methods were applied in this investigation because different compounds are extracted by different solvents based on the solvent's polarity. The solvents employed in the present study were methanol, hexane, ethyl acetate, distilled water and butanol. The ethyl acetate and hexane extracts were then subjected to flash column chromatography with stepwise addition of hexane and acetone. Finally the identification of compounds in the sub-fractions from ethyl acetate and hexane were done using GC-MS and LC-MS/MS analyses. Extraction of the flavour components (essential oil) was performed. Compounds such as benzyl alcohol were identified in this extract.

Twenty six compounds were identified from the fruiting bodies of *Pleurotus giganteus*. The secondary metabolites were fatty acids such as palmitic acid and oleic acid; fatty acids methyl esters such as methyl linoleate; phenolics such as caffeic acid and cinnamic acid; sterols such as ergosterol and neoergosterol; organic acids such as succinic acid and alkaloids such as *N*-acetylphenylethylamine.

Phenolic compounds were the most abundant compounds identified. Phenolic compounds such as benzoic acid, coumaric acid, cinnamic acid and caffeic were identified in the aqueous extract. This was due to phenolic compounds tend to be water-soluble. Other phenolic compounds were also identified in the hexane and the ethyl acetate such as 4-tert-butylphenol. Fatty acids such oleic acid were the predominant fatty acids identified in *Pleurotus giganteus.*

The compounds identified in *Pleurotus giganteus* possess medicinal properties and have been reported in many scientific literatures. Individually and through syner-

gism these compounds can potentially provide health benefits. *Pleurotus giganteus* has a high potential to be produced economically on a large scale in Malaysia. The nutraceutical value of this mushroom should be emphasized to increase the mushroom intake in daily diet. By doing so, the popularity of *Pleurotus giganteus* could eventually grow and be comparable to other popular edible or medicinal mushrooms such as *Tuber sp.* (truffles) and *G. Lucidum* (ling-zhi). However further study validating the finding of this thesis need to be performed. This would include isolating the identified compounds so characterization of potentially bioactive activity could be compared to previous work carried out in research literature.

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