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Research Article

GC-MS Analysis of Methanolic And N-Hexane Extracts of Freshly Harvested Sclerotia of King Tuber Mushroom (*Pleurotus tuber-regium* (Fr.) Sing.)

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ABSTRACT

King tuber mushroom (*Pleurotus tuber-regium* (Fr.) Sing.) is a macro-fungus, found in the tropics with edible mycelia that serve as underground storage tubers known as sclerotia. The sclerotia grows on decaying wood in the forest, collected by farmers used food, food additives and medicine by trado-medical practitioners in the treatment of stomach ailments, fever, asthma, smallpox, high blood pressure, and cancer. The aim of this research was to identify and quantify compounds with nutritional and health promoting benefits that are present in the methanolic and n-hexane extracts of freshly harvested sclerotia of King tuber mushroom, in order to determine its health benefit claims by trado-medical practitioners. Methanolic and n-hexane extracts of freshly harvested sclerotia were used for chromatography – mass spectrophotometric (GC-MS) analysis. Standard GC-MS conditions for analysis were employed and compounds found in extracts were matched with that of the National Institute of Standards and Technology (NIST) reference library database. GC-MS analysis of methanolic extract revealed the presence of six (6) compounds, which included hexadecanoic acid methyl ester (24.57%) a derivative of palmitic acid, with antioxidant and hypocholesterolemic properties; 9, 12-octadecadienoic acid [-z-z] methyl ester (8.48%) that is a derivative of the essential fatty acid linoleic acid; α-tocospiro A and B (9.40%), with anti-cancer activity. The GC-MS results n-hexane extract showed the presence of eight (8) compounds, which also included hexadecanoic acid methyl ester (12.41%); 9, 12-octadecadienoic acid [-z-z] methyl ester (8.42%); 9-octadecenoic acid methyl ester, (33.33%) a derivative of oleic acid, with LDL lowering effect and anti-hypertensive property; heptadecanoic acid-16- methyl- methyl ester (10.12%) and 2-methyl-7-phenylindole (8.75%), with anti-cancer activity. Freshly harvested sclerotia of King tuber mushrooms *(Pleurotus tuber-regium)* contain health beneficial compounds, which may explain its health benefit claims by trado-medical practitioners. These health promoting compounds could be used in the discovery and development of new drugs.

INTRODUCTION

The King tuber mushroom (*Pleurotus tuber-regium* (Fr.) Sing) belongs to the family basidiomycetes. It is a macro-fungus commonly found in the tropics on decaying woods in the forest. This mushroom like other mushrooms has a fruit body that grows above the soil and mycelia that grows underneath the soil. It is however, unique to other mushrooms because its mycelia form an underground storage tuber known as sclerotia. The sclerotia can be used as food and herbal medicine for humans (Huang *et al.,* 2012). The king tuber mushroom also aids in transforming agricultural wastes to edible biomass due to its ability to

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grow on them. The mushroom derives nutrients for growth through the degradation of lignocelullosic material in the wood, using its inherent ligninolytic enzymes that break down lignin to form new fruiting bodies and sclerotia. Their carnivory mechanism helps to trap and digest nematodes producing nitrogen needed for their growth, thus reducing environmental waste pollution (Zhang, 2007; Ferreira *et al*., 2014).

Sclerotia of King Tuber mushroom are usually irregularly shaped, dark-brown on the outside, whitish on the inside. It has been considered for decades as a profound health promoting mushroom based on its great nutritional qualities and inherent medicinal properties (Ikewuchi *et al*., 2013). In Nigeria, the sclerotia of King tuber mushrooms are collected from the forest by farmers, especially those in rural communities and taken to the market for sale, thereby serving as source of income. It is locally called Uhenru in Bini, ero usu in Igbo, Ohu in Yoruba and katala in Hausa. As food, these sclerotia are ground, mixed with some water, molded into round shape, cooked and eaten with different soups. They are also widely used as a food additive especially by local restaurants when ground and added to various soups such as Egusi or melon, Oha, Okro, Achi, Ofe akwu or banga soups. As food additives they serve as thickener, bulking and flavoring agents, increasing the overall quantity and enhancing the taste of the food. The thickening and bulking effects of the sclerotia have been attributed to their ability to absorb fluid and swell up to three times their volume and also due to the presence of high level of dietary fibre (Okoye and Onyekweli, 2016). As Flavoring agents their taste and aroma could be attributed to the presence of aromatic compounds such as phenolics, fatty acids derivatives such as methyl esters and amino acids such as valine, leucine and isoleucine (Moliszewska, 2014). The sclerotia contain vitamin C and B (Wani *et al*., 2010), they are rich sources of protein, fiber and polysaccharides. They contain essential unsaturated fatty-acids and are rich in minerals such as iron, zinc, potassium, calcium, phosphorus, sodium and magnesium (Ijioma *et al*., 2015).

As herbal medicine, African trado-medical practitioners have for long used the sclerotia of King tuber mushroom for the treatment of stomach ailments, fever, asthma, smallpox, high blood pressure and cancer including skin diseases, obesity, inflammation, headache, cough, cold, fever, childhood anemia, malnutrition and the management of diabetes (Huang, 2004; Zhang *et al.,* 2007; Ferreira *et al.,* 2014). Their trado-medicinal could be attributed to their biologically properties such as immuno-stimulatory, hypo-cholesterolaemic, anti-inflammatory, anti-hyperglycemic, anti-tumor, anti-hypertensive, antioxidant, anti-microbial and antiviral activities (Patel *et al*., 2012).

The aim of this research was to identify and quantify compounds with nutritional and health promoting benefits that are present in the methanolic and n-hexane extracts of freshly harvested sclerotia of King tuber mushroom, in order to determine its health benefit claims by trado-medical practitioners.

MATERIALS AND METHODS

Sample Collection and Identification

Freshly harvested sclerotia of King tuber regium mushroom was obtained at Ugbojobo village, Ovia North-East LGA in Edo State, and identified and authenticated in Pharmacognosy Department, Faculty of Pharmacy University of Benin, Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State and African Centre for Mushroom Research and Technology Innovation (ACMRTI) University of Benin, where a herbarium voucher specimen number ACMRTI/ S.0011 was deposited in its reference.

Preparation of Pulverized Sample

The dark-brown skin of the sclerotia of King tuber regium was carefully peeled with a clean knife, cut into small bits and ground with mortar and pestle and analyzed by Gas chromatographic – mass spectrophotometer (GC-MS).

GC-MS Conditions and Analysis

GC-MS analysis was carried out on an Agilent technologies 7890A extractor made by Denville in Kolterman Denville, USA. Auto-injector model of Agilent technologies 7683B series injector was used. Column type HP5MX with dimension measuring 30m × 0.32mm × 0.25mm. Nitrogen was used as carrier gas.

Two grammes (2 g) of the pulverized sample was weighed into a thimble and placed in a soxhlet was connected to a round bottom flask containing 30ml each of 100% methanol or n-hexane as extracting solvent. It was then reflux for 2 h with the aid of a reflux condenser connected to constant running tap. The process was repeated twice, and the extract was concentrated with nitrogen and later treated with silica gel and anhydrous sodium sulphate to remove impurities and water. The extract was used for GC-MS analysis.

An aliquot of 1µl of extract was injected in to the GC-MS. Inlet temperature was maintained as 250°C. Initial temperature of oven was programmed at 40 °C for 2 min and total run time of 90 min was used for analysis. GC-MS was analyzed using electron impact ionization at 70eV and data evaluated using total ion count (TIC) for compound identification and quantification. Component spectrums were compared with the database of spectrum of known components stored in the GC-MS library. Measurement of peak areas and data processing was carried out by Turbo-Mass OCPTVS-Demo SPL software.

RESULTS AND DISCUSSION

A golden yellow extract was obtained after methanolic extraction and Fig. 1 shows the Total Ion Chromatogram

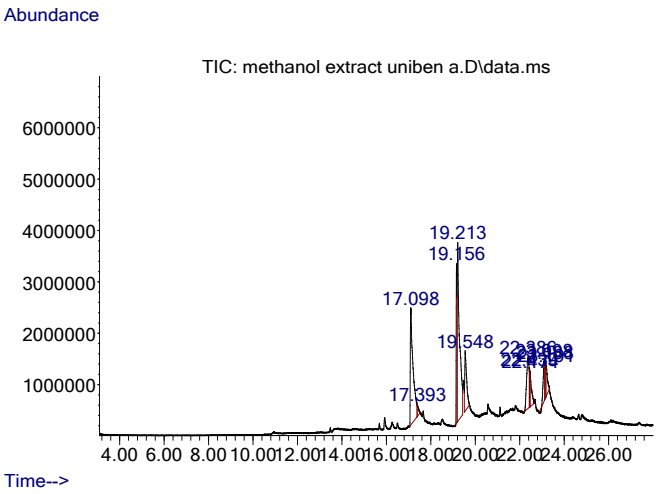
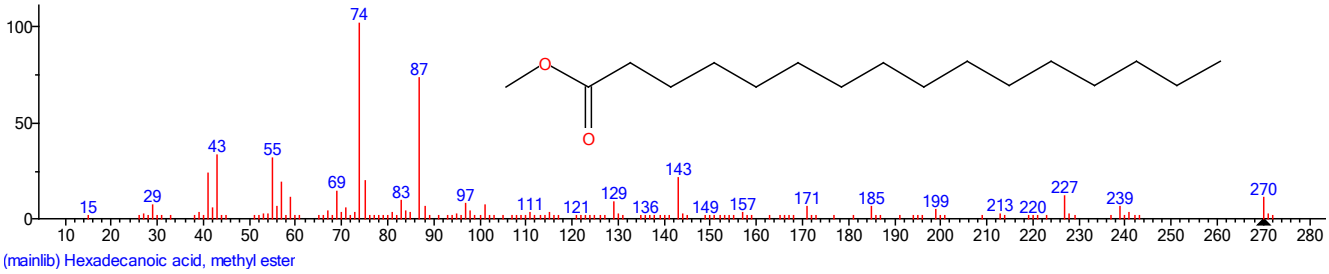


Fig. 1: Total Ion Chromatogram (TIC) of methanolic extract of sclerotia of king tuber mushroom.

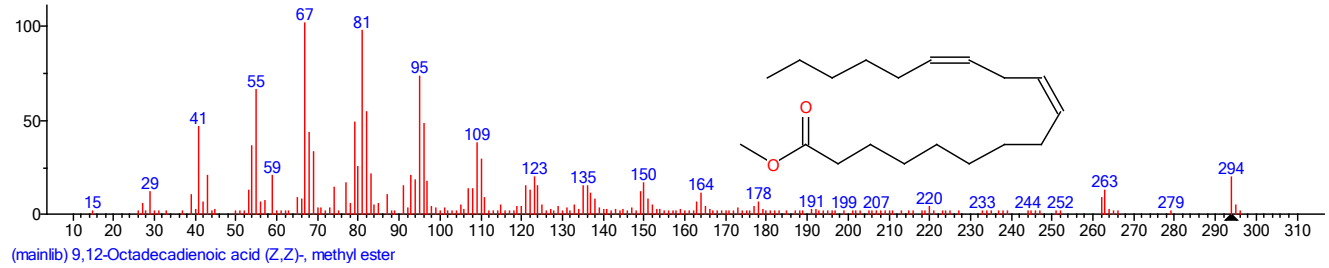
(TIC) of methanolic extract of sclerotia of King tuber mushroom obtained from the GC-MS analysis. The analysis revealed the presence of six (6) compounds, which were hexadecanoic acid methyl ester, 9, 12-octadecadienoic acid [-z-z] methyl ester, 11-octadecenoic acid methyl ester, methyl stearate, α-tocospiro A and α-tocospiro B as shown in Fig 2.

A golden yellow extract was also obtained after n-hexane extraction and Fig. 3 shows the Total Ion Chromatogram (TIC) of n-hexane extract of sclerotia of King tuber mushroom obtained from the GC-MS analysis. The analysis showed eight (8) compounds; hexadecanoic acid methyl ester, 9, 12-octadecadienoic acid [-z-z] methyl ester, 9-octadecenoic acid methyl ester, cyclooctene -3-ethenyl-, heptadecanoic acid -16- methyl- methyl ester, 2,3-diphenylcycopropyl methyl- phenyl sulfoxide trans-, 1H-indole-5-methyl-2- phenyl and 2-methyl-7-phenylindole as shown in Fig. 4.

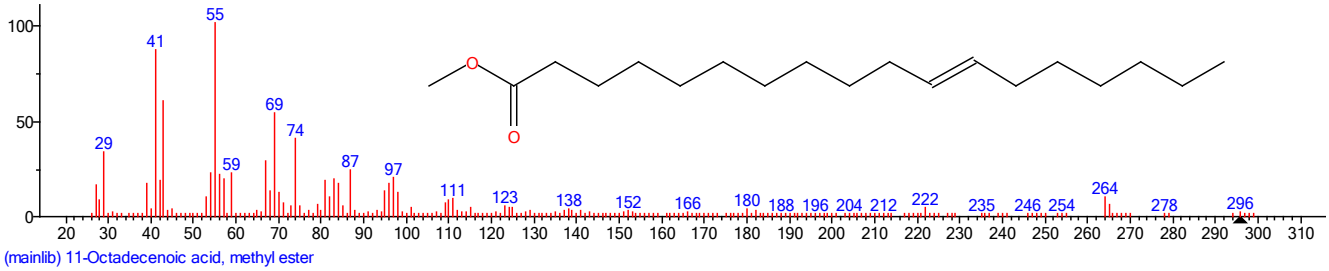
(A) Compound 1: Hexadecanoic acid methyl ester



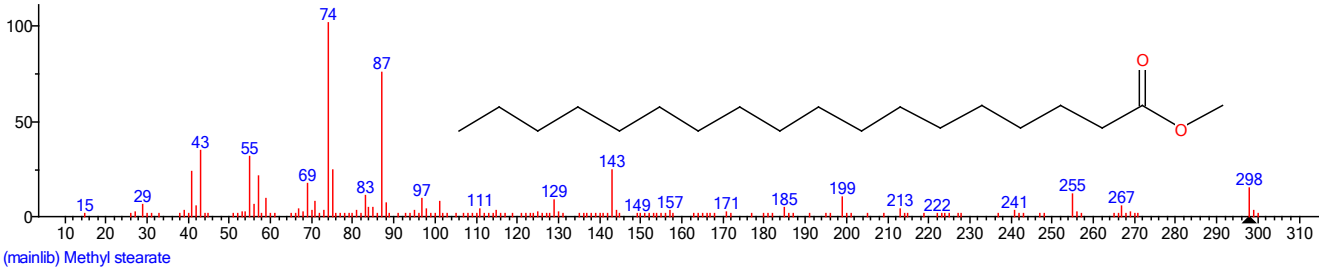
(B) Compond 2: 9, 12- Octadecadienoic acid methylesters



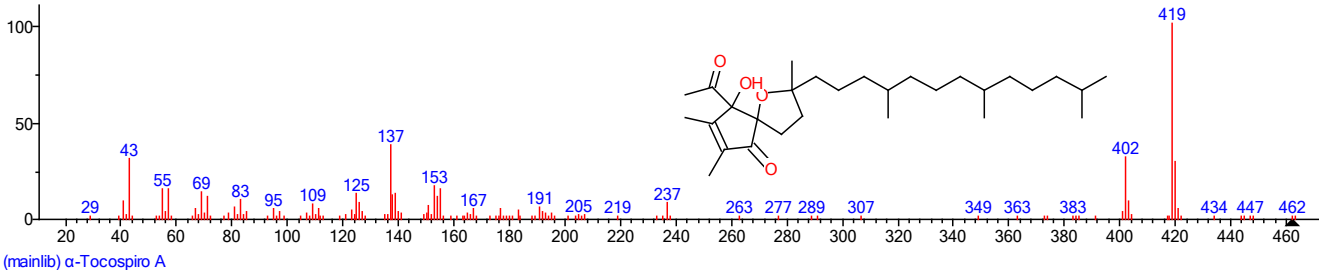
(C) Compound 3: 11- Octadecenoic acid methyl ester



(D) Compound 4: Methylstearate



(E) Compound 5: Alpha – Tocospiro A



(F) Compound 6: Alpha – Tocospiro B

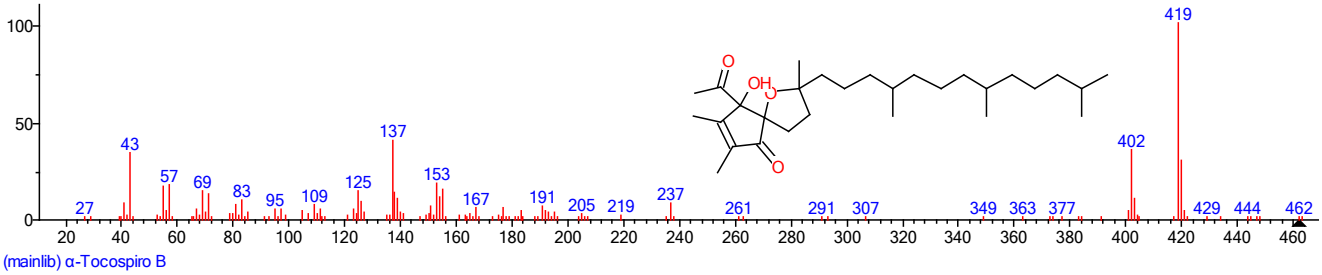
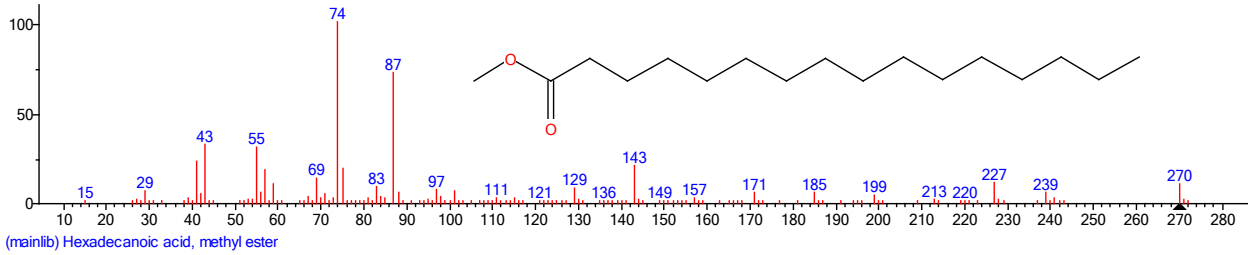
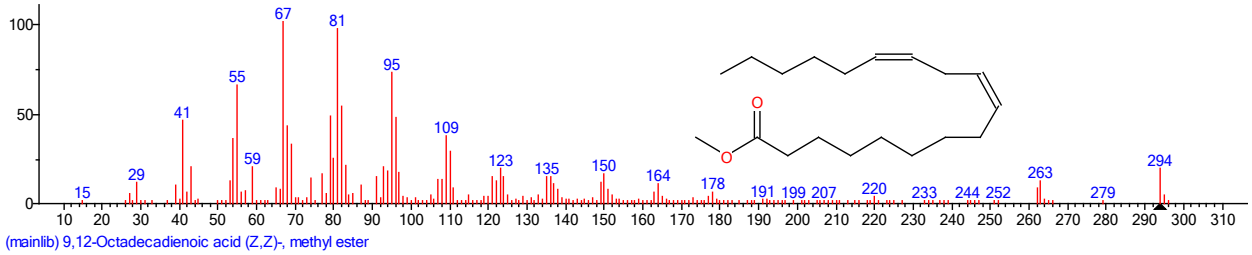


Fig. 2a: The various compounds present in the methanolic extract of sclerotia of the king tuber mushroom.

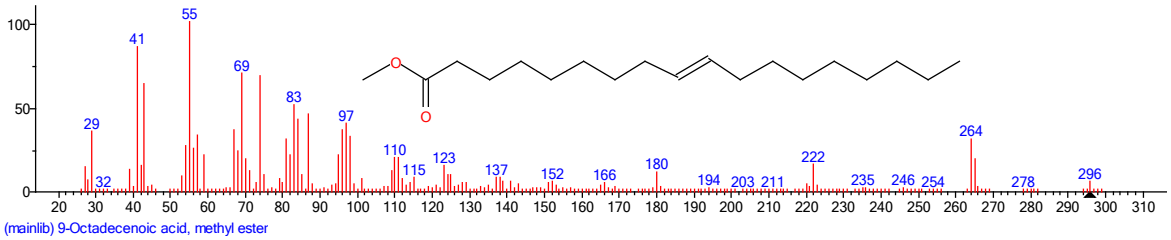
(A) Compound 1: Hexadecanoic acid, methyl ester



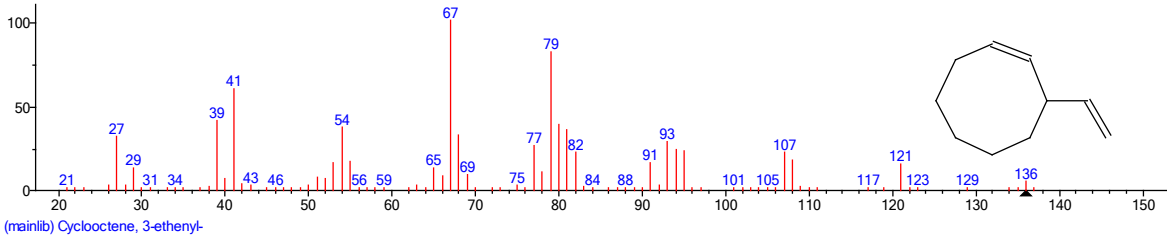
(B) Compound 2: 9, 12- Octadecadienoic acid (Z, Z)- methyl ester



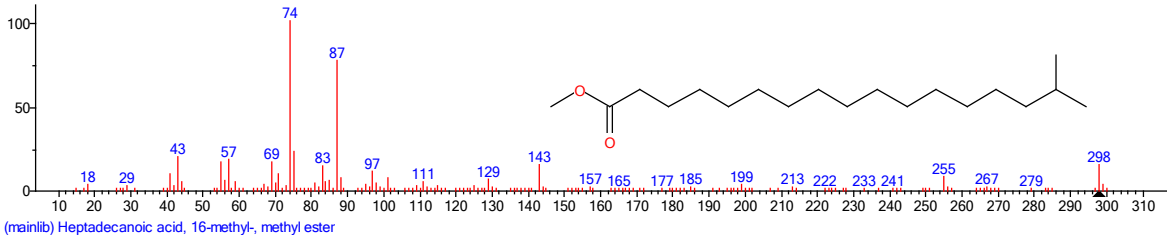
(C) Compound 3: 9- Octadecenoic acid methyl ester



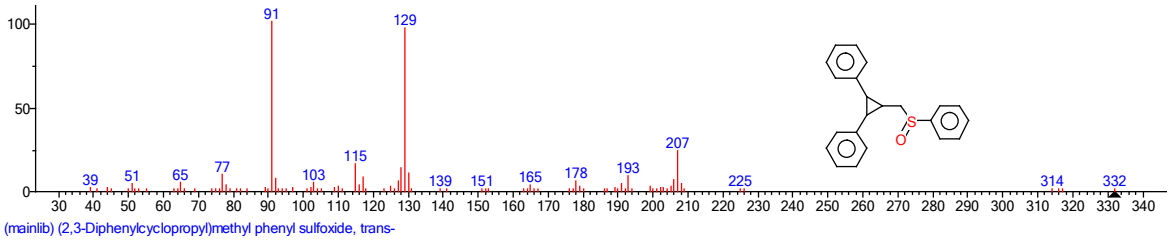
(D) Compound 4: Cyclooctene- 3-ethenyl



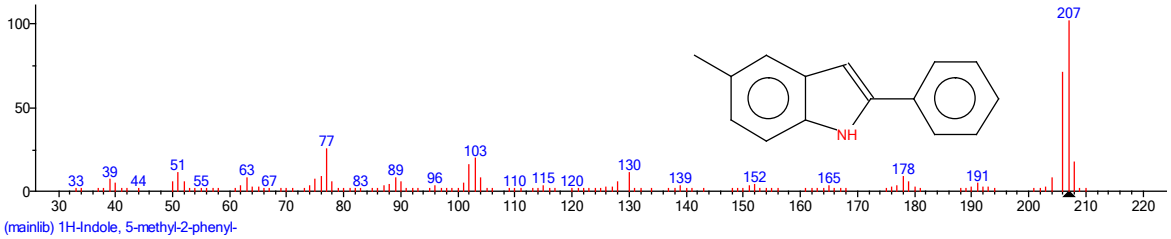
(E) Compound 5: Heptadecanoic acid -16-methyl- methyl ester



(F) Compound 6: (2,3- diphenylcyclopropyl) methylenyl sulfoxide trans-



(G) Compound 7: 1H- indole-5-methyl-2-phenyl-



(H) Compound 8: 2-methyl-7-phenylindole

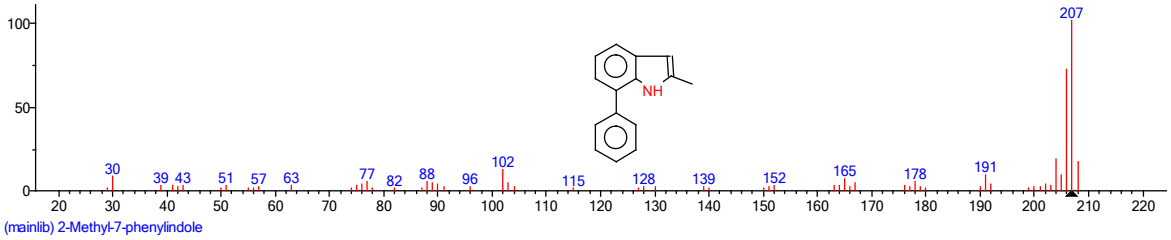


Fig. 2b: The various compounds present in the n-hexane extract of sclerotia of the king tuber mushroom.

Table 1: GC-MS components of methanolic extract of freshly harvested sclerotia of king tuber mushroom and their biological activities.

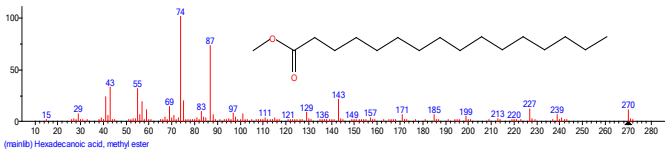
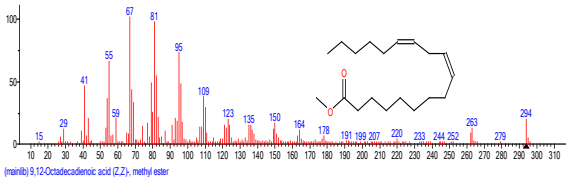
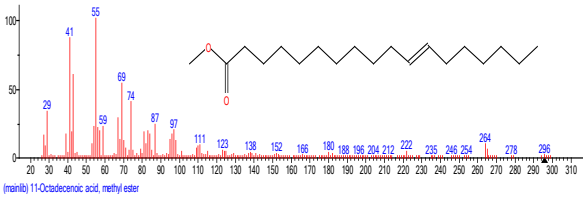
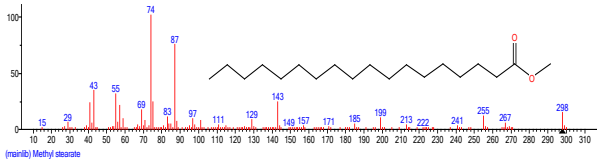
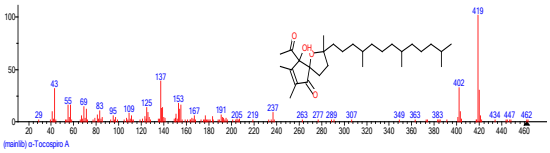
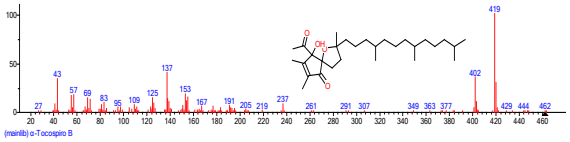
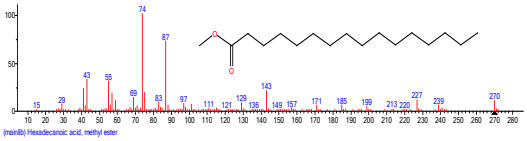
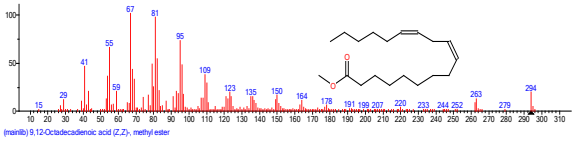
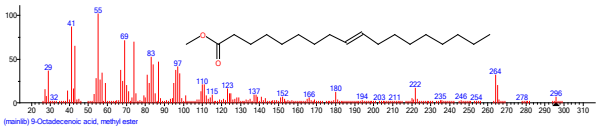
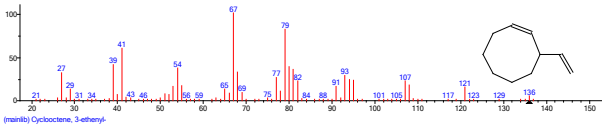
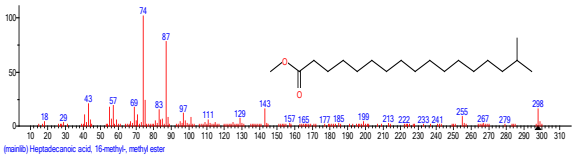
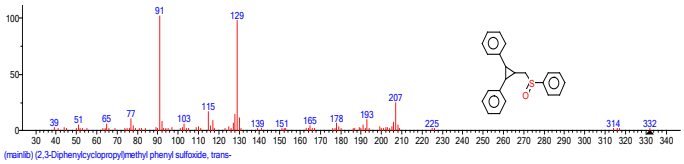
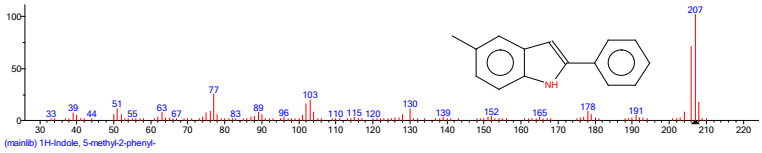
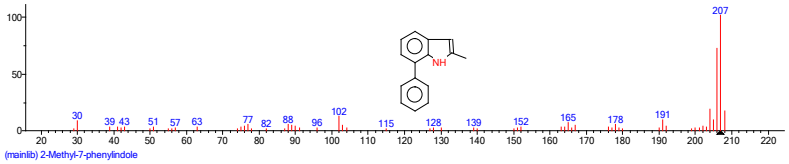
Compound name; chemical formula	*Retension time (min)*	*Area*	*Common names; Molecular weight; Chemical structure; Biological activities.*
Hexadecanoic acid, methyl ester $C_{17}H_{34}O_2$	17.098	24.57%	Methyl palmitate; 270.459g/mol  Flavouring agent, anti- inflammatory, anti-bacteria and antifungal (Chandrasekaran *et al.*, 2011).
9,12- octadecadienoic acid, methyl ester $C_{19}H_{34}O_2$	19.156	8.48%	Methyl linoleate; 294.479g/mol  Anti-hypertensive, antioxidant and anti-cancer (Yu *et al.*, 2005).
11- octadecenoic acid, methyl ester $C_{19}H_{36}O_2$	19.213	31.71%	Methyloctadecenoate; 296.495g/mol  Anti-cholesterolaemic and anti-carcinogenic (Asghar *et al.*, 2011).
Methylstearate $C_{19}H_{38}O_2$	19.548	8.75%	Stearic acid; 298.511g/mol  White crystal semi-solid ester; flavor component in food; Lubricant; used in the manufacture of pharmaceuticals, cosmetics and soaps; Surfactant and softening agent (Enas and Duha, 2014).
α -Tocospiro A $C_{29}H_{50}O_4$	22.387	9.30%	462.715g/mol  Cytotoxicity against human A549 and HL60 Lung cancer cell lines. Anti- mycobacterial activity against mycobacterium tuberculosis H37RV (Chen *et al.*, 2010).
α- Tocospiro B $C_{29}H_{50}O_4$	23.097	4.18%	462.715g/mol  Cytotoxic activity against P-388 (leukamia) and HT-29 (human colon adenocarcinoma) cell lines invitro (Chen *et al.*, 2006).

Table 2: GC-MS components of n-hexane extract of freshly harvested sclerotia of king tuber mushroom and their biological activities.

Compound name; chemical formula	*Retention time (min)*	*Area*	*Common names; Molecular weight; Chemical structure; Biological activities.*
Hexadecanoic acid, methyl ester; $C_{17}H_{34}O_2$	17.146	12.41%	Methyl palmitate; 270.459g/mol;  Anti- inflammatory, hemolytic-5-α reductase inhibitor activity, flavouring agent, anti-bacteria and antifungal activity. (Chandrasekaran *et al.*, 2011; Ojekale *et al.*, 2016).
9,12- octadecadienoic acid, methyl ester; $C_{19}H_{34}O_2$	19.160	8.42%	Methyllinoleate; 294.479g/mol  Anti-hypertensive, antioxidant and anti-cancer (Yu *et al.*, 2005). Hypo-cholesterolemic, Hepato-protective, Anti-histaminic, Antiacne, Anti-arthritic, Anti-eczemic (Manju *et al.,* 2015).
9-octadecenoic acid methyl ester; $C_{19}H_{36}O_2$	19.217	33.33%	Methyl oleate; 296.495g/mol  LDL lowering effect, anti-hypertensive and anti-carcinogenic properties (Vinay and Sarashetti, 2017; Asghar *et al*., 2011).
Cyclooctene-3- ethenyl- $C_{10}H_{16}$	19.372	5.20%	3-Ethenylcyclooctene; 136.238g/mol  No activity reported.
Heptadecanoic acid -16-methylester; $C_{19}H_{38}O_2$	19.566	10.12%	Methyl isostearate; 298.511g/mol  Long chain saturated fatty acid methyl ester. Nutrient stabilizer, surfactant, emulsifier, for the manufacture of oral cavity products, cosmetics, soaps, perfumes and drugs for dermatological disorder (Ojekale *et al.*, 2016).
(2,3-diphenylcyclopropyl) methyl phenyl sulfoxide trans- $C_{22}H_{20}OS$	23.417	1.41%	332.461g/mol;  A sulfoxide metabolite observed in cancer metabolism; a promising therapeutic target considered in the design and early target of cancer treatment (Vermeersch *et al*., 2014).

1H-indole-5-methyl-2-phenyl $C_{15}H_{13}N$	24.647	5.12%	207.276g/mol  Antitumor, anticancer and antioxidant activity (Laxmi *et al*., 2016).
2-methyl-7-phenylindole $C_{15}H_{13}N$	24.882	8.75%	207.276g/mol  Cytotoxicity effects on cancer cell lines (Yantao *et al.,* 2016).

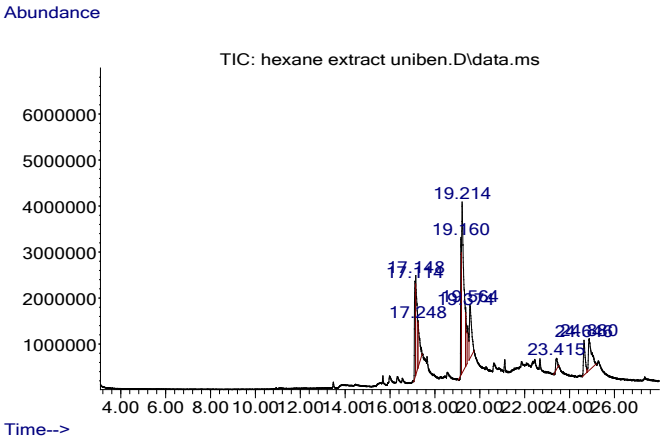


Fig. 3: Total Ion Chromatogram (TIC) of n-hexane extract of sclerotia of king tuber mushroom.

In this study, the comparison of the mass spectra obtained from the GC-MS analysis of methanolic and n-hexane extract of the King tuber mushroom (Figs. 1 and 3) with the NIST database library gave more than 90% match as well as a confirmatory compound structure match. Compounds present and their various biological activities are shown in Figs. 2 and 4 including Tables 1 and 2 as shown above.

The highest percentage of compound in extracts were 9-octadecenoic acid methyl ester (33.33%) of the n-hexane extract which possesses LDL lowering effect, anti-hypertensive and anti-carcinogenic properties (Vinay and Sarashetti, 2017). This was closely followed by 11-octadecenoic acid, methyl ester (31.71%) of the methanolic extract with anti-cholesterolaemic and anti-carcinogenic (Asghar *et al*., 2011).

Hexadecanoic acid, methyl ester and 9, 12- octadecadienoic acid (z, z)- methyl ester were found predominant in both methanolic and n-hexane extracts of the sclerotia of king tuber regium, with the methanolic extract showing higher percentages of the compounds as shown in Tables 1 and 2 exhibiting anticancer, anti-inflammatory, antihypertensive activities amongst others (Ojekale *et al*., 2016). The above was in correlation with the results obtained by Afieroho and Ugoeze, 2014; Gas Chromatography-Mass Spectroscopic (GC-MS) Analysis of n-hexane Extract of *Lentinus tuber-regium* (Fr.) Fr. (*Polyporaceae*) Syn. *Pleurotus tuber regium* Fr. sclerotia.

Identified, characterized and quantified compounds present were generally characteristics of anticancer, antioxidant, anti-cholesterolaemic, anti- diabetic, anti-inflammatory, nematicide, pesticide, anti-hypertensive, antimicrobial and hemolytic-5-α reductase inhibitor properties amongst others which is similar to the research findings of Gregori *et al*., 2007 and which showed similar medicinal properties in some *Pleurotus* species and Huang *et al.,* 2012 which shows how *Pleurotus tuber-regium* polysaccharides is used in the treatment of hyperglycaemia and oxidative stress in experimental diabetic rats, thereby exhibiting similar biological properties possessed by identified compounds in this research study.

These identified secondary metabolites and their observed natural biological activities has given credence to possible reasons for its trado-medical use in the treatment of high blood pressure, high blood sugar levels, fever, headache, cough and catarrh, skin diseases, small pox, anemia, stomach and digestive problems amongst others, and thus could be employed in the manufacture of products of pharmaceutical and therapeutic value (Huang *et al*., 2012; Zhang *et al.,* 2007; Ferreira *et al.,* 2014).

CONCLUSION

Freshly harvested sclerotia of King tuber mushrooms *(Pleurotus tuber-regium)* contains health beneficial compounds, which may explain its health benefit claims by trado-medical practitioners. It can be concluded that the diverse benefits of mushrooms towards human by the words of the father of medicine that is, Hippocrates "Let food be your medicine and medicine be your food". This saying aptly suits mushrooms, as they have tremendous medicinal food, drugs and mineral values; hence they are valuable asset for the welfare of human.

This is usually the first step towards the understanding of the nature of bioactive compounds in this edible Sing's sclerotia, however to scientifically establish their potentials on medical dysfunctions, further extensively detailed study as seen in the isolation and purification of these secondary metabolites to derive their pure forms as well as evaluating their safety or toxicity for human and other animal use will be extremely beneficial as to proceed in the possible discovery and development of new drugs.

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