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Identification of Bioactive Compounds in Sclerotia Extracts from *Pleurotus tuber-regium* (Fr.) Sing. using Gas Chromatograph–Mass Spectrometer (GC-MS)

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Abstract

King tuber mushroom (*Pleurotus tuber-regium*) is a tropical mushroom commonly seen in Australia, Africa and Asia. Trado-medical practitioners use the sclerotia for the treatment of various health disorders. This research aimed at identifying the biologically active compounds present in the sclerotia of this mushroom. In this study, the fungus was first extracted with methanol and re-extracted with ethyl acetate (EA) and dichloromethane (DCM) separately to obtain EA and DCM extracts. Gas chromatography–mass spectrometric (GC-MS) technique was used for the identification of compounds present in EA and DCM extracts. Results revealed the presence of a total of at least twenty-six (26) compounds with 14 and 12 from EA and DCM extracts respectively. The bioactives include n-Hexadecanoic acid, Oleic acid, 10-Octadecenal, Palmitoleic acid, 9, 17-octadecadienal-(z), 11-octadecenoic and methyl ester-(z). In EA extract, n-Hexadecanoic acid had highest concentration (37.67%) followed by 20.65% of 9,12 Octadecadienoic acid and the least was 9,17 Octadecadienal with 0.539%. In DCM extract, 11-Octadecenoic acid methyl ester was most abundant (18.344%) followed by Pentadecanoic acid 14 methyl-methyl ester (14.105%) and the least (4.058%) was 9,12-Octadecadienoic acid. These compounds possess various reported medicinal properties (such as the treatment of high blood pressure, diabetes, asthma, fever and cancer) that could be harnessed for health benefits. Other identified compounds with unknown functions are Trans-2-Dodecen-1-ol trifluoroacetate, cis-11-Hexadecenal, Methyl-18-methylnonadecanoate and Methyl-18-methyl-tetracosanoate. This identification is useful for the establishment of the medicinal properties of the mushroom and isolation of the bioactive compounds, which production can further be enhanced by cost-effective biotechnology techniques.

Keywords: King tuber mushroom, *Pleurotus tuber-regium*, GC-MS, medicinal properties

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Introduction

Mushroom is a fungus that grows above or under the ground. Mushrooms, being fungi, are heterotrophs, lacking chlorophyll, hence do not photosynthesize but obtain nutrients from other sources (Christopher et al., 2012). Over 10, 000 species of mushrooms are known today; some species are generally known to be edible; others are poisonous (Wasser, 2002). The major way one can reliably discern the poisonous species is by accurate identification of the species. Over twenty-five (25) species of mushroom are generally considered as safe foods worldwide and are commercially cultivated (Cho, 2004; Maria et al., 2014). These edible species of mushroom are considered delicacies and are

consumed for their nutritional values. Because of their low level of fat and high levels of protein, fibre, vitamin and mineral they are valued for their nutritional purpose (Mattila et al., 2001; Barros et al., 2007). For a vegetarian's diet, mushrooms are useful because of their capability of providing essential amino acids and that their protein contents are higher than many vegetables (Bilal et al., 2010).

Furthermore, mushrooms are utilized either for their organoleptic merits or their medicinal values (Agoreyo and Oseghale, 2019). Compounds with a lot of health benefits are contained in edible mushrooms. Apart from the fact that they are valued for their nutritional purpose, some species of these mushrooms are explored for their anticancer, anti-inflammatory, anti-diabetic, antimicrobial and antihypertensive properties (Huang et al., 2012) because of the health-promoting compounds contained in them (Agoreyo and Oseghale, 2019; Zeb and Lee, 2021).

The rate of the cultivation of mushrooms in the world is at a spontaneous due to their medical value (Ergonul et al., 2013). The genus *Pleurotus* spp. are fast growing fungus which belongs to the Basidiomycota group. They also are comprised of most popular edible mushrooms which are known for their medicinal properties, dynamic growth and undemanding cultivation conditions (Andrej and Jure, 2007). They have been used as part of human diets throughout the world due to their rich nutrients such as protein, fibre, carbohydrate, minerals, vitamins and lipids. An increasing number of studies from different centres confirm the fact that mushroom species of the *Pleurotus* genus exhibit multidirectional health-promoting effects (Correa et al., 2016) which can be extracted from different parts such as fruiting body, sclerotia, mycelia and culture broth.

Pleurotus tuber-regium is a mushroom that has a mycelium known as a sclerotium that can be ellipsoid in shape, white on the inside and dark-brown on the outside (Isikhuemhen and Lebauer, 2004). This mushroom is of great economic importance in all parts of Nigeria and its sclerotia are usually harvested from the forest for eating (Ohiri et al., 2018). In Nigeria, it is known among different tribes as "osu" or "eronsu" in Ibo, "orlu" in Yoruba and "katala" or "rumbagada" in Hausa Languages. The *P. tuber-regium* is the only specie that produces true sclerotia (Isikhuemhen et al., 2000). The outer portion of the sclerotium which is brown is usually peeled off and the inner portion which is white is cut into small pieces, ground and used in making soup. This mushroom may be used as a substitute or an addiction to melon in soup (Agoreyo and Oseghale, 2019). The fruiting bodies and sclerotia are rich in proteins while the sclerotia contain considerable amounts of carbohydrates, fats and fibre. The sclerotia also contain non-starch polysaccharides that are responsible for some of pharmacological actions (Tao et al., 2006).

Extracts of *P. tuber-regium* sclerotia have been reportedly used traditionally in preparations for cures for various ailments such as headache, stomach diseases, asthma, high blood pressure (Guillam´on et al., 2010). The extracts have also been reported to serve as good antitumor (Abdullah et al., 2022), anti-hyperglycemic and anti-hypertensive agents (Zhang et al., 2007). This study was carried out to assess the bioactive compounds present in the ethyl acetate and dichloromethane extracts of the sclerotia, in order to establish the medicinal properties of this mushroom. Moreover, these bioactive compounds, when identified, could be used for drug development.

Materials and Methods

Collection of Samples

Freshly harvested sclerotia of the mushroom were procured from the forest at Ugbojobo, Ovia North-East LGA, Edo State, identified and authenticated at the Forest Research Institute of Nigeria (FRIN), Ibadan, Oyo State and African Centre for Mushroom Research and Technology Innovation, University of Benin, Nigeria.

Preparation of Sample Extract

The outer dark-brown layer (Plate 1a) of the freshly harvested sclerotia of *Pleurotus tuber-regium* were carefully peeled using a clean knife. The white sclerotia (Plate 1b) were chopped into bits and pulverized. A 700g of the pulverized sample was placed in an extraction jar to which 2.5 litres of absolute methanol was added and covered for proper interaction of the pulverized sample with the solvent so as to

obtain the crude extract. Maceration lasted for 72 hours during which the sample was regularly agitated by stirring with a glass rod to allow proper solute-solvent interaction between the sample and the methanol. After 72 hours, the mixture was filtered through 2 layers of cheesecloth and squeezed properly. The residue was re-extracted twice by soaking in absolute methanol for 72 hours each time, to allow exhaustive extraction. The filtrate from each round of extraction was collected and concentrated using a rotary evaporator at 50^{o}C. The concentrate was transferred into a crucible and evaporated to dryness using a water bath at 50^{o}C. The concentrate was carefully scraped off from the crucible and transferred into another crucible which was kept in a desiccator to properly remove moisture. The powdered form of the concentrate (crude extract) obtained was transferred to a glass container and covered for further analysis.

(a) **(b)**

Plate 1: (a) Unpeeled sclerotia **(b)** Peeled sclerotia of *Pleurotus tuber-regium*

Gas Chromatography– Mass Spectrometry (Gc-Ms) Analysis

Gas Chromatography-Mass Spectrometry (GC-MS) analysis was done on dichloromethane (DCM) and ethyl acetate (EA) extracts of *P. tuberregium.* To obtain the DCM extract, 1g of the crude (methanolic) extract was dissolved in 10 mL of DCM, thereafter a solid phase extraction process was used to filter to remove impurities. For the ethyl acetate extract, 1.0 g of the crude (methanolic) extract was dissolved in 10 mL of ethyl acetate and filtration was also carried out using a solid phase extraction process. The filtrate was then used for the GC-MS analysis.

The analysis was carried out using Gas Chromatograph (Agilent technologies, 7890 GC system) coupled with a Mass Spectrometry detector (Agilent technologies 5975). The column used was Agilent H5MS column measuring 30 m in length, 0.320 mm in diameter and 0.25 µm in thickness. Helium gas was used as carrier gas at a flow rate of 0.5 ml/min. A sample volume of 1 µL was injected into the gas chromatograph. The oven temperature was programmed initially at 80˚C for 2 minutes with a gradual increase of 10˚C per minute until a final temperature of 240˚C for 6 minutes. The total time for running was 90 minutes. The GC-MS analysis was done by using electron impact ionization at 70 eV and total ion count (TIC) was used to evaluate the data for a proper compound identification and quantification. A comparison was done between the spectrum of the components and the database of spectrum of known compounds stored in the data system of the National Institute of Standards and Technology (NIST) library.

Results

The GC-MS analysis of the ethyl acetate and dichloromethane extracts of the sclerotia of *P. tuber-regium* carried out showed a total of twenty-six (26) compounds that are present in both the ethyl acetate and dichloromethane extracts. The GC-MS analysis of ethyl acetate and dichloromethane extracts of the sclerotia of *P. tuber-regium* yielded many peaks with well identified 16 and 12 peaks respectively. The GC-MS Chromatogram and the compounds of ethyl acetate extract of the sclerotia of *P. tuber-regium* are shown in Figure 1 and Table 1. The compound n-Hexadecanoic acid was revealed to be the highest in concentration with 37.67% and a retention time of 15.020, followed by 9,12 octadecadienoic acid with a 20.65% in concentration and a retention time of 16.425. The least was found to be 9,17 Octadecadienal with 0.539% in concentration and retention time of 18.598. The peaks with the retention times of 18.642 and 19.701 were identified to be 9, 12-Octadecadienoic acid-(z,z) and 9, 17-octadecadienal-(z) respectively. These compounds were not repeated in the Table 1 since they have been listed for other retention

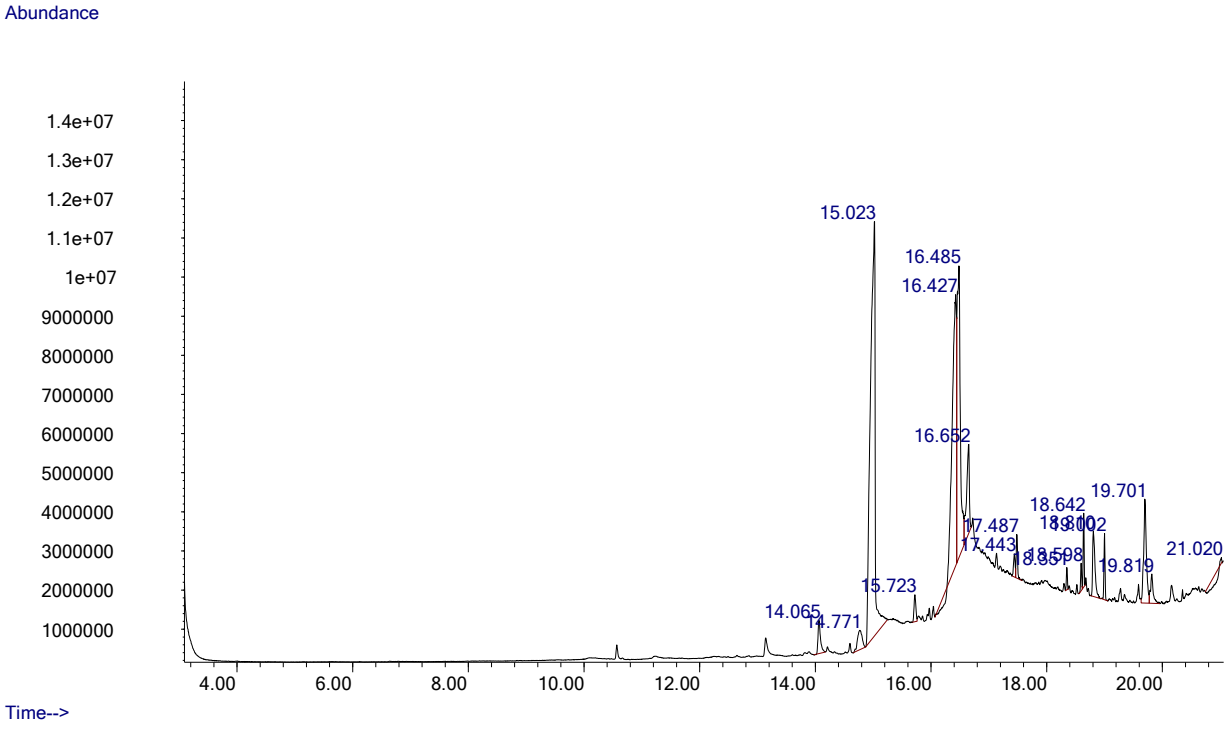
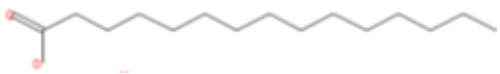
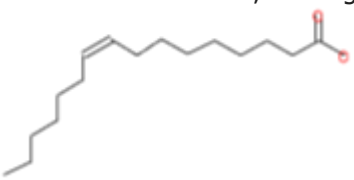
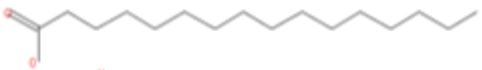
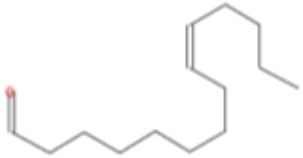
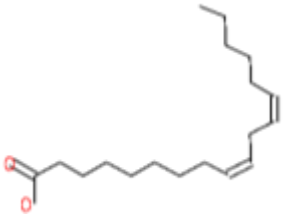
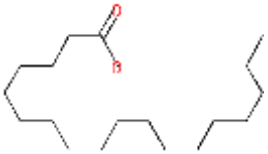
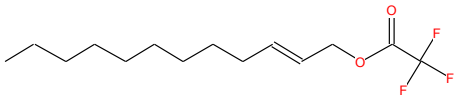
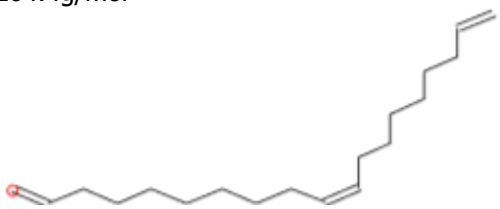
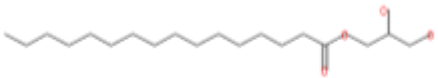
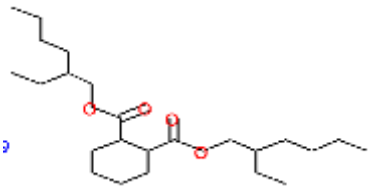
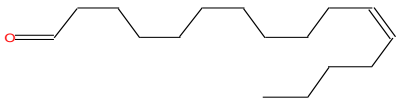


Figure 1: Total ion chromatogram (TIC) of ethyl acetate extract of the sclerotia of King Tuber mushroom (*Pleurotus tuber- regium*).

Table 1: GC-MS compounds of ethyl acetate extract of the sclerotia of King Tuber mushroom (*Pleurotus tuber-regium)*

Compound name; Chemical formula	Retention time (min)	Area (%)	Common names; Molecular Weight; Chemical structure; Biological activities.
Pentadecanoic acid $C_{15}H_{30}O_2$	14.065	1.549	Pentadecylic acid; 242.403g/mol  Anti-inflammatory, antifibrotic, red blood cell-stabilizing and mitochondrial-reparative activities (Venn-Watson et al., 2020).
Palmitoleic acid $C_{16}H_{30}O_2$	14.771	1.696	cis-9-Hexadecenoic acid;254.41g/mol  **Anti-inflammatory.
n-Hexadecanoic acid $C_{16}H_{32}O_2$	15.023	37.67	Palmitic Acid; 256.42g/mol  Anti-inflammatory (Aparna et al., 2012). **Antioxidant, hypocholesterolemic, nematicide, pesticide, anti-androgenic flavour, hemolytic, 5-alpha reductase inhibitor and potent mosquito larvicide.
9- Tetradecenal-(z) $C_{14}H_{26}O$	15.723	0.94	210.36g/mol  **Sex pheromone.
9, 12-Octadecadienoic acid-(z,z) $C_{18}H_{32}O_2$	16.427	20.65	Linoleic acid; 280.4455g/mol  **Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge(cide), antihistaminic and antieczemic.

Oleic acid $C_{18}H_{34}O_2$	16.485	18.57	cis-9-Octadecenoic acid; 282.5g/mol  Cancer preventive, anemiagenic, insectifuge, antiandrogenic, dermatitigenic and antibacterial (Awa et al., 2012).
Octadecanoic acid $C_{18}H_{36}O_2$	16.652	4.89	Stearic acid; 284.5g/mol  Anti-microbial activity (Rahuman et al., 2000).
Trans-2-Dodecen-1-ol, trifluoroacetate $C_{12}H_{23}F_3O_2$	17.443	0.67%	280.33g/mol  *BANF
6-Octadecenoic acid-(z) $C_{18}H_{34}O_2$	17.487	1.14	Petrosenilic acid; 282.5g/mol  **Cancer preventive and insectifuge.
9, 17-Octadecadienal-(z) $C_{18}H_{32}O$	18.598	0.539	264.4g/mol  **Anti-inflammatory, antioxidant and antimicrobial.
Glycerol 1-palmitate $C_{19}H_{38}O_4$	18.811	2.93	Monopalmitin; 330.5g/mol  **Cytotoxicity and anti-viral.
Bis (2-ethylhexyl) phthalate $C_{24}H_{38}O_4$	19.002	1.34	390.564g/mol  **Anticancer agent.
10-Octadecenal $C_{18}H_{34}O$	19.819	5.45	266.5g/mol  **Antibacterial.

cis-11-Hexadecenal $C_{16}H_{30}O$	21.020	1.38%	Hexadec-11-enal; 238.41g/mol  *BANF

**Duke (2016); *BANF: Biological Activity Not Found

times of 16.427 and 18.598. The GC-MS Chromatogram and compounds of dichloromethane extract of the sclerotia of *P. tuber-regium* are shown below in Figure 2 and Table 2. The compound: 11-Octadecenoic acid methyl ester was revealed to be the highest in concentration with 18.344% and a retention time of 16.044, followed by Pentadecanoic acid 14 methyl-methyl ester with a 14.105% in concentration and a retention time of 14.602. The least was found to be 9,12-Octadecadienoic acid with 4.058% in concentration and retention time of 16.336.

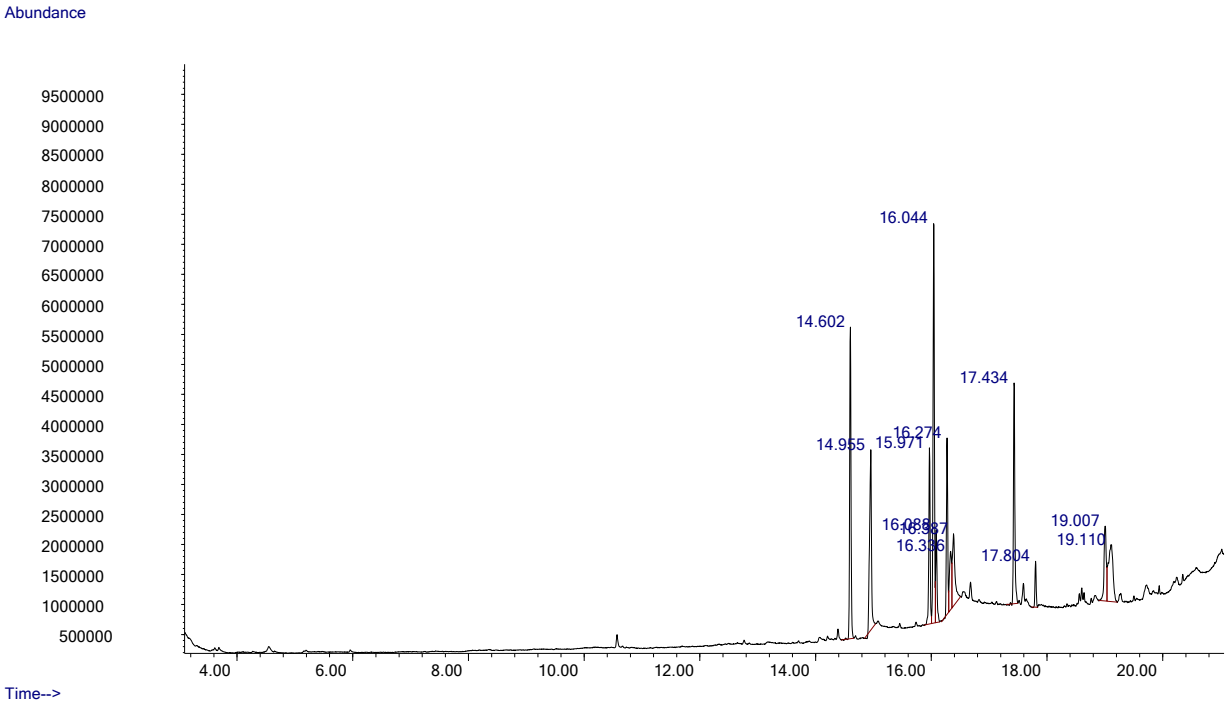
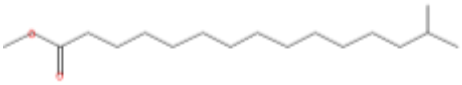
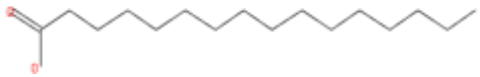
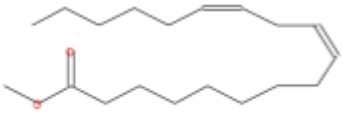
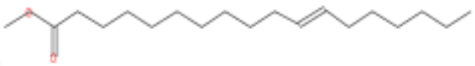
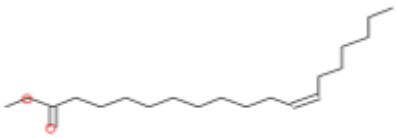
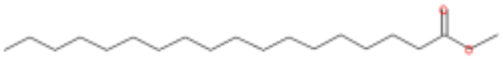
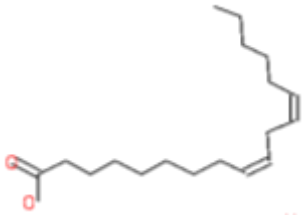
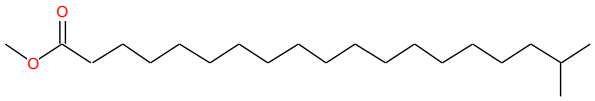
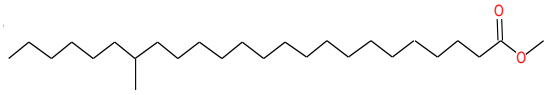
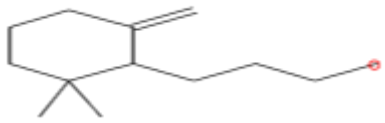


Figure 2: Total ion chromatogram (TIC) of dichloromethane extract of the sclerotia of King Tuber mushroom (*Pleurotus tuber-regium*).

Table 2: GC-MS compounds of dichloromethane extract of the sclerotia of King Tuber mushroom (*Pleurotus tuber-regium)*

Compound name; Chemical formula	Retention time (min)	Area (%)	Common names; Molecular Weight; Chemical structure; Biological activities.
Pentadecanoic acid-14-methyl-methyl ester $C_{17}H_{34}O_2$	14.602	14.105	Palmitic acid methyl ester; 270.4507g/mol  Antioxidant, antifungi and antimicrobial (Arumugam and Vijisaral., 2014).
n-hexadecanoic acid $C_{16}H_{32}O_2$	14.955	11.276	Palmitic acid; 256.42g/mol  **Anti-inflammatory, antioxidant, hypocholesterolemic, nematicide, pesticide, antiandrogenic flavour, hemolytic, 5-alpha reductase inhibitor and potent mosquito larvicide.
9, 12- octadecadienoic acid-(z-z)- methyl ester $C_{19}H_{34}O_2$	15.971	7.871	Methyl linoleate; 294.479g/mol  **Anti-hypertensive, antioxidant and anticancer.
11- octadecenoic acid, methyl ester $C_{19}H_{36}O_2$	16.044	18.344	Methyloctadecenoate; 296.495g/mol  Anti-cholesterolemic and anti-carcinogenic (Asghar et al., 2011).
11-Octadecenoic, methyl ester-(z) $C_{19}H_{36}O_2$	16.088	4.122	Methyl cis-Vaccenate; 296.4879g/mol  **Antibacterial and hypolipidemia effect.
Methyl stearate $C_{19}H_{38}O_2$	16.274	7.438	Stearic acid methyl ester; 298.511g/mol  White crystal semi-solid ester; flavour component in food; lubricant, used in manufacture of pharmaceuticals, cosmetics and soaps; surfactant and softening agents (Enas and Duha, 2014).

9, 12-Octadecadienoic acid-(z-z) $C_{18}H_{32}O_2$	16.336	4.058	Linoleic acid; 280.4455g/mol  **Anti-inflammatory, hypocholesterolemic, cancer preventive, hepatoprotective, nematicide, insectifuge(cide), antihistaminic and antieczemic.
Oleic acid $C_{18}H_{34}O_2$	16.387	6.456	cis-9-Octadecenoic acid; 282.5g/mol  Cancer preventive, anemiagenic, insectifuge, antiandrogenic, dermatitigenic and antibacterial (Awa et al., 2012).
9-Octadecenoic acid, 12-hydroxy-methyl ester, (R-(Z)) $C_{19}H_{36}O_3$	17.434	10.081	Methyl ricinoleate; 312.4673g/mol  **Lubricant, cutting oil additive, wetting agent, corrosive inhibitors and anti-scaling agent.
Methyl-18-methylnonadecanoate $C_{21}H_{42}O_2$	17.804	1.802	326.557g/mol  *BANF
Methyl-18-methyl-tetracosanoate $C_{26}H_{52}O_2$	19.007	5.498	396.7g/mol  *BANF
Cyclohexanepropanol-2, 2-dimethyl-6-methylene $C_{12}H_{22}O$	19.110	8.949	3-(2',2'-dimethyl-6'-methylidenecyclohexyl)-1-propanol; 182.3g/mol  **Raw material for active drugs.

**Duke (2016); *BANF: Biological Activity Not Found

Discussion

In the extracts, the compounds identified are known to have varying biological activities. Among them n-Hexadecanoic acid revealed to be the highest in concentration as seen in the results of ethyl acetate extract is an anti-inflammatory compound (Beschi et al., 2021) which is found to bind at the active site of phospholipase A2 competitively inhibiting this enzyme thereby preventing inflammation (Vasudevan et al., 2012). Other compounds in the extracts that have anti-inflammatory properties include palmitoleic acid, linoleic acid, and 9,17-Octadecadienal. Oleic acid and 6-octadecanoic acid also found in the ethyl acetate extracts are known to have cancer preventive properties, they are believed to inhibit the enzymes involved in DNA synthesis in tumour cells. Oleic acid is known to suppress the over-expression of HER (erbB-2) gene, a well characterized oncogene which plays a key role in several human cancers (Menendez et al., 2005; Ozge Ozsen et al., 2019). The following detected compounds in the extracts: Octadecenoic acid and 10-Octadecenal have antimicrobial property and may play this role by inhibiting enzyme activities, impair nutrient uptake and also causes the generation of peroxidation and auto-oxidation degradation products or initiate direct lysis of microbial cells (Guillermo et al., 2012).

The 9, 12-Octadecadienoic acid-(z,z) and 9, 17-Octadecadienal-(z) that were associated with two different peaks respectively may each exist in two forms in the mushroom sclerotia. Also, the compounds without traced or known biological activity may be novel that need to be further investigated to reveal their functions. The varying biological activities of the bioactive compounds found in the extracts of the sclerotia of *Pleurotus tuber-regium* may account for the trado-medicinal use of the sclerotia for the treatment of health disorders such as high blood pressure, diabetes, asthma, fever and cancer (Agoreyo and Oseghale, 2019; Oni et al., 2020).

Conclusion

The GC-MS analysis of the dichloromethane and ethyl acetate extracts of the sclerotia of *P. tuber-regium* revealed a number of bioactive compounds thereby giving credence to its application traditionally in the treatments of various ailments. The identified compounds can be developed into useful drugs.

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