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Gas Chromatography Mass Spectrometry Analysis of Volatile Compounds from *Lignosus rhinocerus* (Tiger milk mushroom).

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ABSTRACT

Lignosus rhinocerus "Tiger milk mushroom" is used by the indigenous communities in Malaysia as an antipruritic agent and general tonic for treating fever, cough and other diseases. Currently, no studies have reported the constituents of *L. rhinocerus*, including the volatile constituents. The analysis of the composition of *L. rhinocerus* would provide a basis for further research on the potential of this mushroom, not only for its nutritional value but also for its medicinal properties. In this study, we aim to identify the volatile components present in *L. rhinocerus* using gas chromatography mass spectrometry (GCMS). Cultivated dried mushroom powder (1 g) of *L. rhinocerus* was continuously extracted using five types of solvents with different polarities, such as non-polar solvents petroleum ether and hexane and the polar solvents diethyl ether, ethyl acetate and methanol. Each extraction was individually injected into the GCMS system in duplicate. Overall, forty-four constituents were identified from various groups. Compounds from the fatty acid group were the most predominant (68.58%) followed by fatty acid esters (10.18%), sterols (6.26%), amides (5.76%), carboxylic acids (3.01%), alcohols (1.64%), alkanes (1.26%) and ketones (1.3%). The most common constituent was linoleic acid (49.39%), followed by palmitic acid (11.29%) and linolelaidic acid methyl ester (7.4%) indicating that the eight-carbon atoms components typical in many mushroom species seem to play a minor role in the volatiles of *L. rhinocerus*. Methanol extracted the greatest number of volatile compounds from *L. rhinocerus*. Our study is the first to report on the volatile constituents of *L. rhinocerus*, which is found to be rich in linoleic acid (octadecadienoic acid) and palmitic acid (hexadecanoic acid).

Keywords: *L. rhinocerus*, volatile composition, GCMS

List of non-standard abbreviations: GCMS: gas chromatography-mass spectrometry; PE: petroleum ether; DE: diethyl ether; Hx: hexane; EA: ethyl acetate; MTL: methanol; Amu: atomic mass unit; TIC: total ion current.

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INTRODUCTION

Plants have been used as medicine and immune enhancers for centuries [1]. In fact, plants have varying unique roles as sedatives, analgesics, cardiotonic agents, anti-inflammatories, oxytoxics and immune modulators [2]. The chemicals present in plants make them remarkable agents to be utilized against diseases, such as cancer, jaundice, typhoid and hypertension [3]. Plant chemicals consist of both primary metabolites (sugars, amino acids, proteins, purines and pyrimidines of nucleic acids and chlorophyll) and secondary metabolites, which are the remaining plant chemicals, produced from m the primary metabolites. These secondary metabolites include alkaloids (which are derived from amino acids), terpenoids, phenolics (which are derived from carbohydrates), tannins, steroids and volatiles [4]. Research to explore and understand plant chemicals and their properties is crucial. For example, mushrooms, which are one of the oldest and most valuable sources of nutrition and immunomodulators, are being extensively studied for their chemical properties. Compounds such as proteins, peptides and polysaccharides, which are commonly identified in mushrooms, have significant effects on the immune system and are essential in regulating specific aspects of the host response [5].

The medicinal properties of mushrooms have been widely beneficial to mankind for centuries, although scientific evidence of their efficacy remains to be found. *Lignosus rhinocerus* (*L. rhinocerus*) is one of the most crucial mushrooms used by native peoples in Southeast Asia for its various medicinal properties. *L. rhinocerus* belongs to the *Polyporaceae* family. It is locally known as the "Tiger Milk Mushroom" due to the belief that it vegetates from the ground where the tigress leaked its milk while feeding her cubs. It is believed that the tuber or sclerotia of *L. rhinocerus* can remain in the ground for periods ranging from several months to decades, and the mushroom will only grow solitarily when nature calls. The underground tuber is the most treasured portion of *L. rhinocerus* because of its medicinal value (**Figure I**). *L. rhinocerus* has been widely used by the indigenous community as an anti-pruritic, general tonic, cancer, food poisoning, fever, cough and wound healing medicine as well as for the treatment of various other types of ailments [6].

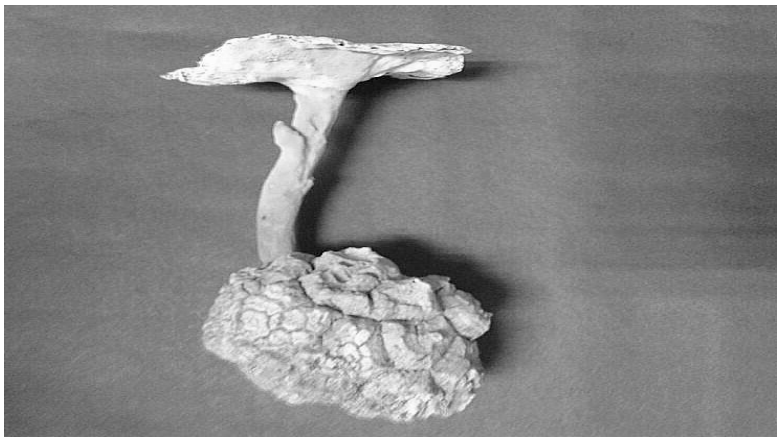

Figure I: The whole mushroom (left) and the tuber (right), which is important for the medicinal properties of the mushroom.

To date, many organic compounds have been identified in several types of mushrooms by using gas chromatography mass spectrometry (GCMS), and these include lipids, sterols and ketones [7,8,9,10]. GCMS is a very powerful technique for the separation and detection of volatile organic compounds. Therefore, GCMS has proved suitable for the interpretation of the volatile compositional characteristics of mushrooms [7].

Conventional sampling methods for volatiles present in mushrooms include solvent extraction [11] and simultaneous distillation extraction. However, these methods require long extraction times and large amounts of solvents and involve multiple steps. Furthermore, many unstable volatile compounds may be heat-degraded during thermal extraction or distillation, which may affect the activity of enzymes that might be involved in the metabolism of some compounds. Therefore, a more friendly method, such as liquid-liquid extraction, may preserve the amount of volatiles present while detecting the largest number of compounds. In addition, many studies have utilized only a single solvent in their extraction methods. Our study involved a continuous extraction method and took advantage of the properties of five different solvents.

To date, volatile compounds have been used as antibiotics because of their immunosuppressant, phytotoxic and mycotoxic activities. Volatile compounds tend to be the intermediate or end product of diverse metabolic pathways and have been reported to belong to various classes, such as mono- and sesquiterpenes, alcohols, ketones, lactones, esters or C8 compounds [12]. Mushroom volatiles are not only an important factor in the flavor but also contain many antioxidant compounds [7]. Although approximately 150 different volatile constituents from different chemical classes have been reported in several mushroom species[13,14,15], the information related to the compounds and constituents of *L. rhinocerus* has not been reported, thus hindering a better understanding of its potential mechanism or confirmation of its traditional claims. Therefore, in the current study, the volatile constituents present in *L. rhinocerus* were investigated to elucidate its composition, which may contribute to its medicinal roles.

MATERIALS AND METHODS

Preparation of L. rhinocerus

Cultivated dried *L. rhinocerus* mushroom powder was obtained from Ligno™ Biotech Sdn. Bhd, (Selangor, Malaysia). The powder was stored at 28°C in dry conditions.

Liquid-liquid extraction

A continuous liquid-liquid extraction starting from 1) petroleum ether, 2) diethyl ether 3) hexane, 4) ethyl acetate and 5) methanol was conducted. The solvents selected consisted of different types, including non-polar (petroleum ether and hexane) and polar (diethyl ether, ethyl acetate and methanol) solvents to take advantage of each of their properties. Following extraction by each solvent type, the samples were individually injected into the GCMS system in duplicate.

Briefly, 1 ml of petroleum ether was added into the capped glass tube containing 1 g of *L. rhinocerus* powder. The mixture was then vortexed for 1 min, followed by centrifugation at 2500 rpm for 5 min. The top organic layer was carefully aspirated before being transferred (100 µl) into a new auto-sampler vial for GCMS injection. The residue remaining at the bottom was then used for subsequent extraction using diethyl ether followed by hexane, ethyl acetate and, finally, methanol using similar GCMS parameters. Each sample was analyzed against a blank organic solvent.

GCMS analysis

GCMS analyses were performed on a HP6890 GC coupled with a HP5973 mass spectrometer. The column was a HP-5MS fused-silica capillary column (30 m x 0.25 mm i.d.; 0.25 µm film thickness) with helium as the carrier gas and was run at a constant pressure of 9.78 psi. Injection was conducted using a splitless mode at an injector temperature of 250°C. The oven temperature was ramped from 35 to 280°C (1 min hold) at a rate of 25°C/min. The oven temperature was held at 310°C for 6 min following each analysis. The total run time for each sample was approximately 25 min. The GCMS interface temperature was set to 280°C. MS mode was used during analytical scanning from 20-650 atomic mass units (amu). The ion source temperature was set to 250°C. The blank was injected first, followed by the sample injection. The chromatograms obtained from the total ion current (TIC) were integrated without any correction for co-eluting peaks, and the results were expressed as total abundance. TIC peaks and chromatograms were analyzed using Agilent MSD ChemStation G1701DA software (version D 02.00, CA, USA). All peaks were identified based on mass spectral matching (≥ 90%) from both the National Institute of Standards and Technology (NIST) and Wiley libraries. Only compounds with 90% or greater spectral matching accuracy were reported.

RESULTS

Twelve groups of compounds were identified, namely fatty acids, fatty acid esters, sterols, amides, carboxylic acids, alcohols, alkanes, ketones, furans, alkaloids, aldehydes and alkenes. The majority of the constituents were fatty acids (**Figure II)**. A total of 44 constituents were extracted using the five different solvents with differing polarities, i.e., petroleum ether, diethyl ether, hexane, ethyl acetate and methanol. Methanol extracted the highest number of constituents (22 compounds) followed by petroleum ether (20

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compounds), diethyl ether (14 compounds), hexane (10 compounds) and ethyl acetate (nine compounds) **(Table I)**.

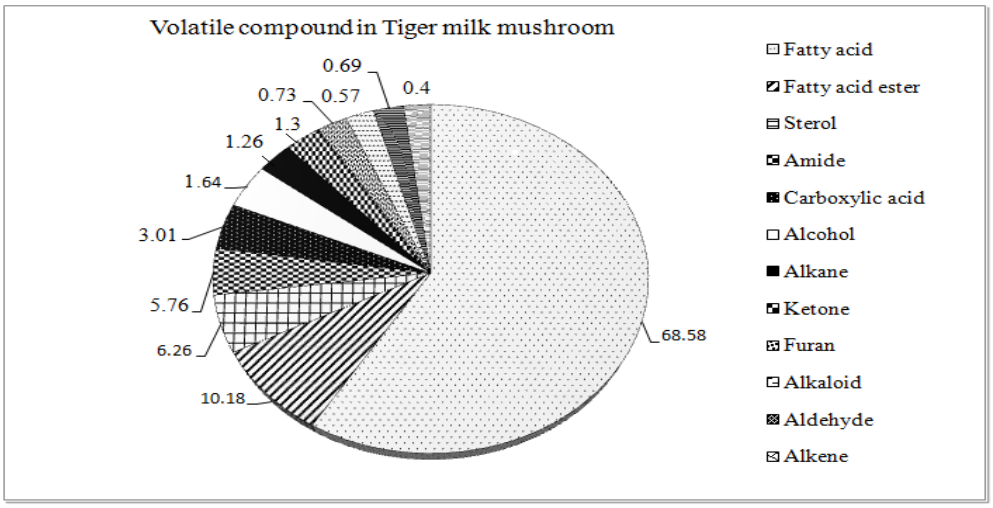

Figure II: Groups of different compounds present in *L. rhinocerus.*

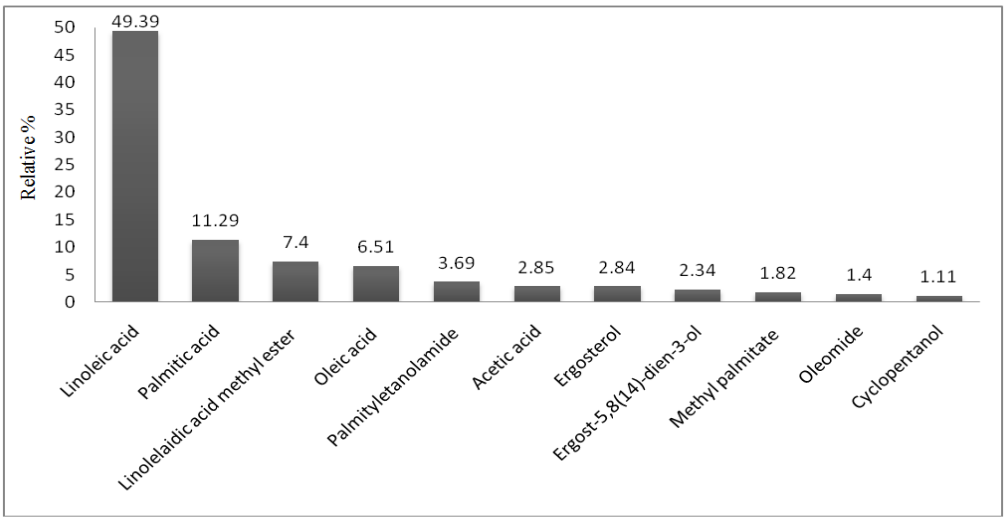

Figure III: Volatile compounds (compounds >1%) detected in *L. rhinocerus* and their relative percentages.

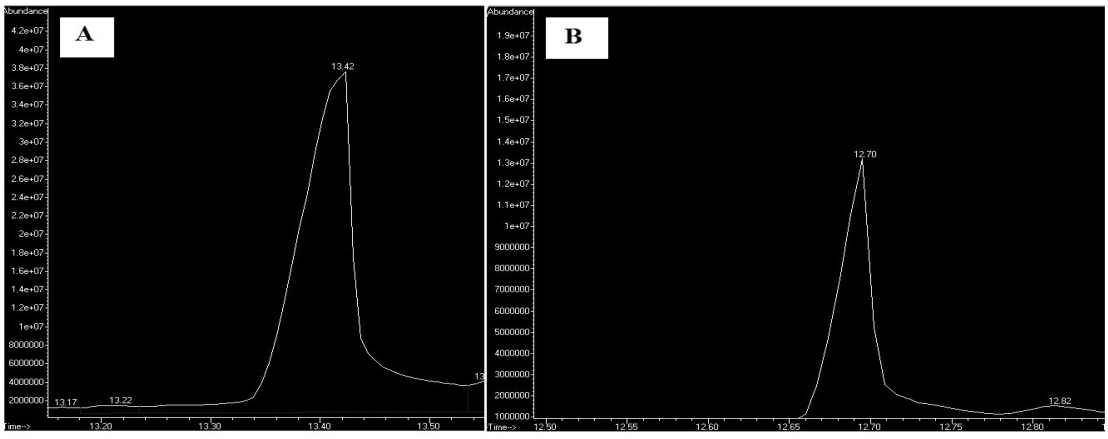

Figure IV: (A) TIC of hexane extract of *L. rhinocerus* showing a linoleic acid peak (RT 13.42 min) and (B) TIC of hexane extract of *L. rhinocerus* showing a palmitic acid peak (RT 12.70 min).

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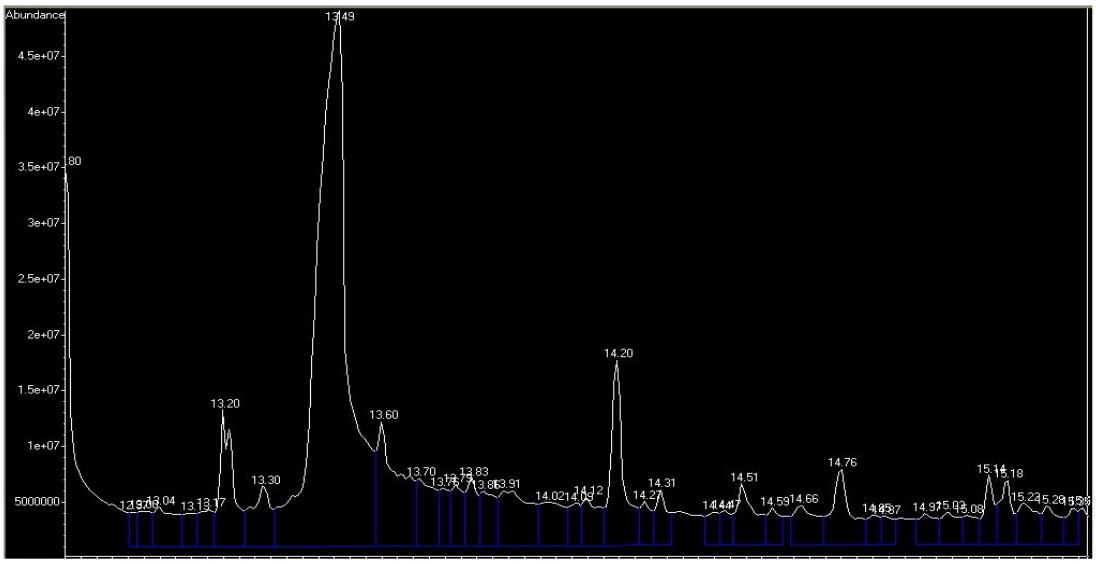

Figure V: TIC of diethyl ether extract of *L. rhinocerus*.

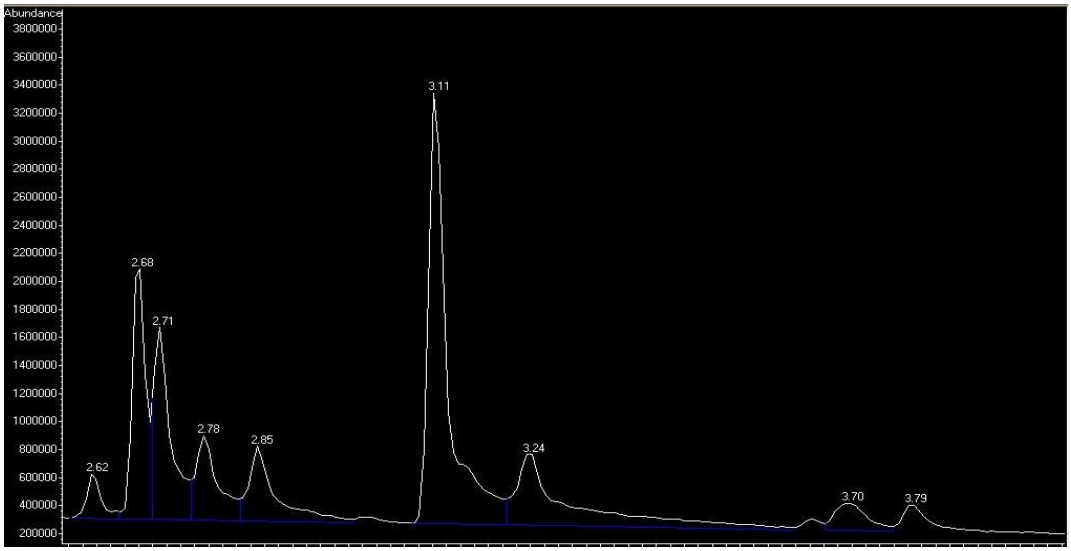

Figure VI: TIC of ethyl acetate extract of *L. rhinocerus*.

Of the compounds present, the majority were fatty acids (68.58%, estimated weight fraction of 685.8 mg), followed by esters (10.18%, estimated weight fraction of 101.8 mg), sterols (6.26%, estimated weight fraction of 62.6 mg), amides (5.76%, estimated weight fraction of 57.6 mg), carboxylic acids (3.01%, estimated weight fraction of 30.1 mg), alcohols (1.64%, estimated weight fraction of 16.4 mg), alkanes (1.26%, estimated weight fraction of 12.6 mg) and ketones (1.30%, estimated weight fraction of 13.0 mg). Other groups were furans, alkaloids and aldehydes, which each composed less than 1% of the total. The highest percentage constituent detected was linoleic acid (49.39%), followed by palmitic acid (11.29%) (**Figure III**). The TIC of linoleic and palmitic acid peaks from hexane extract are shown in **Figure IV.** Other TIC of compounds extracted from diethyl ether and ethyl acetate are shown in **Figures V** and **VI**.

Additional compounds detected in *L. rhinocerus* were linolelaidic acid methyl ester (7.4%), oleic acid (6.51%), palmitoylethanolamide (3.69%), acetic acid (2.85%), ergosterol (2.84%), ergost-5,8(14)-dien-3-ol (2.34%), methyl palmitate (1.82%), oleamide (1.40%) and cyclopentanol (1.11%). Overall, these compounds comprised >90% of the constituents present in *L. rhinocerus* **(Table II**). Interestingly, *L. rhinocerus* was found to contain an ester called lignoceric acid methyl ester.

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Table 1: Volatile composition of *L. rhinocerus* following extraction using various solvents and their respective retention times, RT (min).

	Compounds	P.E	R.T	D.E	R.T	HX	R.T	E.A	R.T	MTL	R.T	Relative %
1	Palmitic acid	✓	12.41	✓	12.8	✓	12.69	✓	12.68	✓	12.7	**11.29**
2	3,5-Dimethoxybenzaldehyde	✓	10.36	✓	10.35	✓	10.36			✓	10.35	**0.43**
3	Tetradecanoic acid	✓	11.84	✓	12	✓	11.83			✓	11.85	**0.87**
4	Linoleic acid	✓	12.35			✓	13.42	✓	13.38	✓	13.39	**49.39**
5	Ergosterol	✓	17.71	✓	17.74			✓	17.7			**2.84**
6	Ergost-5,8(14)-dien-3-ol	✓	17.81	✓	17.84			✓	17.81	✓	17.83	**2.34**
7	Methyl linolelaidate	✓	13.21	✓	13.2					✓	13.21	**7.4**
8	Methyl palmitate	✓	12.53							✓	12.53	**1.82**
9	Methyl dodecanoate	✓	10.74							✓	10.74	**0.11**
10	Caffeine	✓	12.32							✓	12.33	**0.5**
11	Oleamide	✓	14.17					✓	14.17			**1.4**
12	Pentadecanoic acid	✓	12.26									**0.02**
13	Palmitoleic acid	✓	12.46									**0.05**
14	Methyl heptadecanoate	✓	12.93									**0.22**
15	Methyl octadecanoate	✓	13.3									**0.35**
16	Pentadecane	✓	10.62									**0.02**
17	3,5-Cyclo-6,8(14),22-ergostatriene	✓	15.95									**0.2**
18	Stigmast-4-en-3-one	✓	19.73									**0.1**
19	Alphacadinol	✓	11.48	✓	11.47							**0.08**
20	Palmityletanolamide	✓	11.44	✓	13.6							**3.69**
21	Oleic acid			✓	13.5							**6.51**
22	Methyl lignocerate			✓	15.28							**0.23**
23	Nonadecane			✓	15.87							**0.31**

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24	(9Z,12Z)-9,12-Octadecadien-1-ol	□	20.34								**0.38**
25	Myristyl aldehyde	□	11.64						□	11.65	**0.26**
26	2,5-Bis(4-bromophenyl)furan	□	16.58	□	16.57	□	16.58				**0.73**
27	Cyclopentanol					□	2.71				**1.11**
28	Acetic acid					□	3.1				**2.55**
29	(3R)-3-Methylcyclopentanone					□	3.79				**0.28**
30	3-Hexanone			□	2.59			□	2.96		**0.4**
31	Beta-sitosterol			□	18.5			□	18.53		**0.95**
32	(22E)-Ergosta-4,6,8(14),22-tetraen-3-one			□	19.08						**0.1**
33	Squalene			□	15.65						**0.13**
34	Octadecane			□	15.86						**0.07**
35	Lauric acid							□	10.94		**0.5**
36	Cyclohexadecane							□	13.15		**0.58**
37	4-Hexadecanyl heptafluorobutanoate							□	11.08		**0.28**
38	(Z)-tetradec-2-ene							□	10.06		**0.2**
39	(Z)-docos-13-enamide							□	15.49		**0.67**
40	(4-methylphenyl)-phenylmethanone							□	11.94		**0.2**
41	2-hydroxy-3-methylcyclopent-2-en-1-one							□	7.49		**0.05**
42	Di(phenyl)methanone							□	11.36		**0.16**
43	Furan-2-ylmethanol							□	4.5		**0.07**
44	Phenol							□	7		**0.16**
		Total percentage									**100**

PE: Petroleum ether, **DE**: Diethyl ether, **HX**: Hexane, **EA**: Ethyl acetate, **MTL**: Methanol

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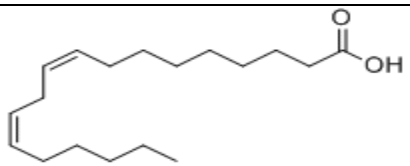
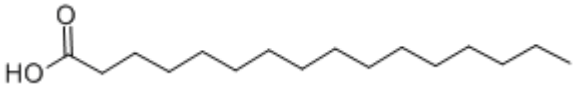
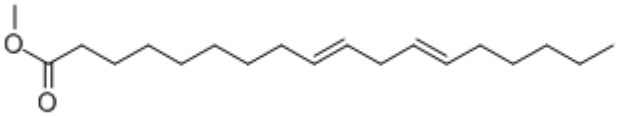
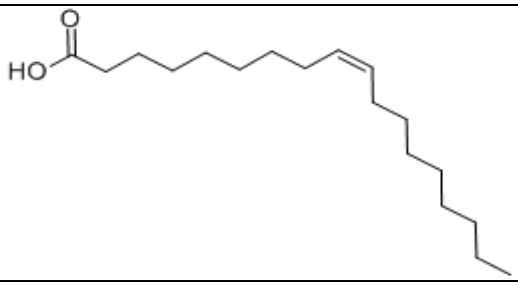
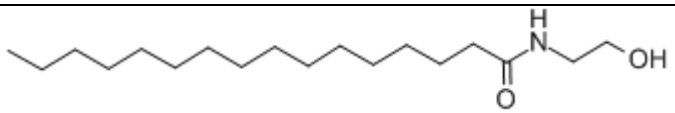
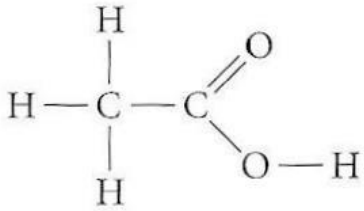
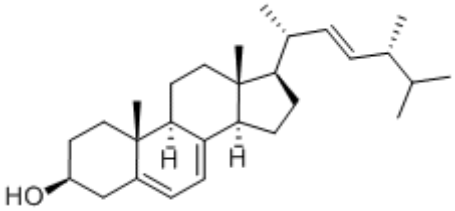
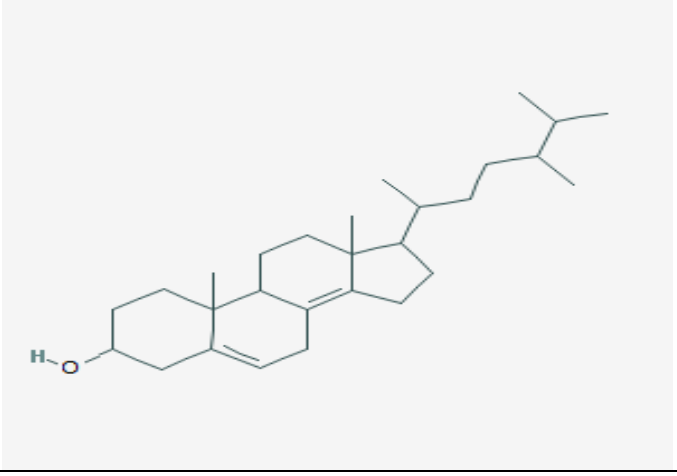
Table II: Chemical structures of the major volatiles present in *L. rhinocerus*.

Compounds	Molecular formula	Chemical structures
Linoleic acid	C18H32O2	
Palmitic acid	C16H32O2	
Methyl linolelaidate	C19H34O2	
Oleic acid	C18H34O2	
Palmitoylethanolamide	C18H37NO2	
Acetic acid	C2H4O2	
Ergosterol	C28H44O	
Ergost-5,8(14)-dien-3-ol	C28H46O	

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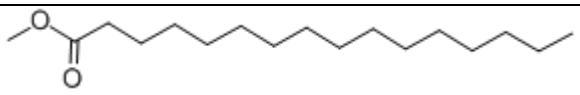
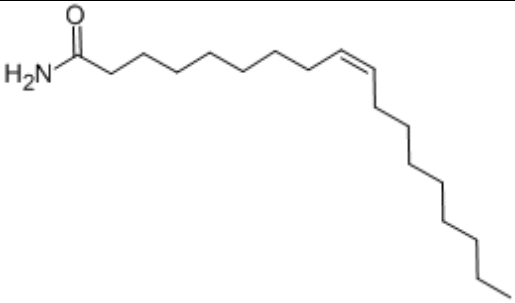
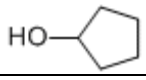
Methyl palmitate	C17H34O2	
Oleamide	C18H35NO	
Cyclopentanol	C5H10O	

DISCUSSION

Our study is the first to identify volatile constituents of *L. rhinocerus* which consists mainly of fatty acids. Besides that, fatty acid esters, sterols, amides, carboxylic acids, alcohols, alkanes, ketones, furans, alkaloids, aldehydes and alkenes were also present. A total of 44 volatile constituents were detected of which linoleic and palmitic acids were present in high amounts**.** There were also linolelaidic acid methyl ester, oleic acid, palmitoylethanolamide, acetic acid, ergosterol, ergost-5,8(14)-dien-3-ol, methyl palmitate, oleamide, cyclopentanol and lignoceric acid methyl ester.

Methanol extracted the highest number of constituents. Continuous extraction method used in our study takes advantage of both the polar and non-polar properties of the solvents, where polar solvents will tend to attract polar compounds, while non-polar solvents tend to attract non-polar compounds. In fact, this method is advantageous since heating process which may lead to loss of volatiles is eliminated. The sequential extraction not only preserves the amount of volatiles, but also improves the process selectivity and recovery of different types of extracts from similar materials. The compounds were selected based on spectral library matching and achieved matches of greater than 90%.

The volatile constituents of many mushrooms, such as *Agaricus bisporus, Agaricus campestris, Lentinus edodes Sing, Boletus edulis, Cantharellus cibarius, Gyromitra esculenta, Lactarius trivialis, Lactarius torminosus, Lactarius rufus* and *Tricholomam atsutake Sing*, are reported to consist of mainly eight-carbon atom components (1-octen-3-ol, 2-octen-l-ol, 3-octanol, 3-octanone and 1-octanol), which are known as "mushroom alcohol"[16]. The formation of the eight-carbon atom components is believed to be a result of an enzymatic reaction(s) where linoleic acid is used as the substrate [17]. However, in the current study, the eight-carbon atom components seem to play a minor role in the volatiles of *L. rhinocerus* as reported for *Agaricus subrufecens* [16]. It is plausible that the enzymatic systems responsible for building the eight-carbon atom components in other mushrooms are either depleted or nonfunctional in *L. rhinocerus.* The possible reason for this phenomenon remains to be investigated.

Many studies have reported the presence of fatty acid compounds, such as linoleic and palmitic acids in different mushroom species. Suseem *et al.* (2013) has reported similar fatty acid esters, such as methyl octadecanoate (40.3%), along with other compounds, such as methyl heptadecanoate (13.5%), 9-octadecenoic acid [Z], and 2-hydroxyl-1-(hydroxymethyl) ethyl ester (4.9%), from the GCMS analysis of the dried fruiting body of *Pleurotus eous*[8]. Likewise, a study of the chemical composition of two inedible mushrooms from the *Agaricus* species demonstrated the presence of linolelaidic acid methyl ester (2.2% and 9%) in both *A. placomyces* and *A. pseudopratensis* with oleic acid detected in only *A. placomyces*[18]. In addition, palmitic acid was detected in the *A. bisporus* mushroom species [19]. Therefore, these results indicate that the variations seen in mushroom species are unique to the type of mushroom.

The sterol composition in *L. rhinocerus* comprises approximately 6.26% of the overall compounds. The predominant sterol recorded in this study was ergosterol (2.84%), followed by ergost-5,8(14)-dien-3-ol

(2.34%). In addition, Petrova *et al*. (2007) reported that ergosterol was the predominant sterol in mushrooms of the genus *Agaricus* [18]. In an additional study, high levels of ergosterol (83%) was detected in *A. bisporus* [19]. The fungal sterol, ergosterol, is known to be the pro-vitamin D2, and has been shown to contribute to the prevention of prostate and colon cancer [20]. Many types of ergosterol peroxides from mushrooms have been shown to inhibit cancer cell growth, induce apoptosis of HL60 human leukemia, and exhibit antioxidative and immunosuppressive characteristics [21]. Therefore, the clinical effects of *L. rhinocerus* with regard to these properties should be further investigated.

Alkanes and alkenes, such as octadecane and tetradecene, were also detected in smaller proportions in *L. rhinocerus*. A previous study by Siddiquee *et al*. (2011) demonstrated that octadecane and tetradecene were present in *Trichoderma harzianum* using GCMS analysis [22]. In contrast to our study, Zhang *et al*. (2008) reported that the predominant volatile compounds from straw and oyster mushrooms were alcohols, aldehydes and ketones. However, straw mushrooms tend to possess comparatively higher amounts of aldehydes and ketones but fewer alcohols when compared with oyster mushrooms [7].

Our GCMS result indicated the presence of phenol, which was only detected following methanol extraction. As recently described by Yap *et al*. (2013), high phenolic contents can be yielded when methanol is used as the solvent for the extraction of cultivated strains compared with the wild type strain of *L. rhinocerus* [23] indicating that cultivated and wild type strains contain different volatile compounds. Therefore, the volatile compounds in the wild type strain of *L. rhinocerus* should be investigated.

Other compounds, which were not present in *L. rhinocerus*, but have been previously reported to be present in other mushroom species at >1% include tartronic acid [8], 2-norbornanone [24], ergosteryl peroxide and neoergosterol [9], hydroquinone monopropyl ether[18], indolizine, 2-(4-methylphenyl), ethyl aspartate and piperidin-4-carboxylic acid [25], alantoic acid and pidolic acid [19] and ethanoic acid [22].

The absence/presence of certain compounds may influence the nutritional and medicinal properties of mushrooms [7]. For instance, essential fatty acids, such as linoleic and palmitic acids, are important and must be provided in the diet, as humans have no ability to desaturate fatty acid at the 3 or 6 positions from the methyl end [1]. Conversely, a dietary study proved that the hypercholesterolemic effect caused by high palmitic acid levels can be balanced with its high intake [26]. In addition, Chen *et al*. (2006), through their GCMS analysis on *A. bisporus*, claimed that linoleic and linolenic acids are active agents against breast cancer cell proliferation when compared with other acids, such as myristic, palmitic and stearic acid [27]. Previous studies have shown that essential fatty acids in *A. bisporus* inhibit the aromatase enzyme that is important for estrogen production. Due to the fact that high estrogen levels are responsible for the development of breast cancer, studies have indicated that women who consumed more than 10 g of fresh *A. bisporus* daily had a 64% lower chance of developing breast cancer [27,28]. An additional GCMS study conducted by Okwu and Ighodaro (2010) reported the presence of octadecanoic acid in *Alstonia boonei*, which has been traditionally used for the treatment of cough, asthma, sores and wounds [10]. Interestingly, *L. rhinocerus* is also traditionally utilized in treating similar ailments [29].

In a previous study conducted by Cho et al. (2006), 35 volatiles compounds were found in raw mushroom and 37 volatile compounds in cooked pine mushrooms (*Tricholoma matsutake Sing*.)[30]. In another study, Zhang *et al.* (2008), reported the presence of only 17 volatile compounds present in straw and oyster mushrooms. Variations in both the number and type of volatile compounds among plant species are caused by many factors [7]. This variation is key to distinguish varying unique roles, such as aromatic and other functional medicinal metabolites properties. Apart from this, the varying composition of similar types of plants or species can be contributed to different pathways and enzyme catalysts during different stages of maturity [31].

In general, it is apparent that many beneficial metabolites with multifunctional roles are found in various types of mushrooms, including *L. rhinocerus.* Essential fatty acids and sterols are recognized as anti-cardiovascular disease metabolites and immune enhancer metabolites [19,32], alcohols as anti-microbial agents [19], triterpenoids and sterols as hepato protective agents [33] and metabolites that enhances food flavoring and aromas, such as alcohols, aldehydes, amides, amines, carboxylic acid, esters, ketones, terpenoids, thiols and mercapto[19]. Nonetheless, compounds detected in the GCMS analysis of *L. rhinocerus*

were comparable with other mushrooms species and plants [2,3,4,7,8], thus distinguishing either similarities or differences among plants of various species and genus.

However, our study has limitations, such as the lack of identification of eminent immunomodulating compounds (β-glucan), which are water soluble polysaccharides that are reported to be more suitable for investigation using other methods, such as liquid chromatography/mass spectrometry [34], high performance liquid chromatography [35], X-ray crystallography [36] or atomic force microscopy [37]. Furthermore, the major volatiles present in fresh *L. rhinocerus* samples may be different from those reported in this study where samples have been cultivated. In addition, the non-volatile components of *L. rhinocerus* remain to be investigated. It is noteworthy to note that the spectral library matching performed to identify volatile compounds in this study gives rather a more preliminary presumptive data; therefore, future studies which include authentic standards should be conducted to further confirm the presence of the volatile compounds. An additional methylation steps to confirm the presence of long chain fatty acids as recommended by Mendez et al (2008) will be an added advantage [38].

CONCLUSION

We identified 44 volatile constituents of *L. rhinocerus*. Extraction by methanol resulted in the highest number of constituents (22 compounds) and is perhaps the best organic solvent for extracting active constituents from *L. rhinocerus*. Although linoleic acid is the substrate for the eight-carbon component of most mushrooms, in *L. rhinocerus* linoleic acid is the main constituent. Interestingly, *L. rhinocerus* contains an ester called lignoceric acid methyl ester.

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