IJPSR (2018), Volume 9, Issue 10 (Research Article)

INTERNATIONAL JOURNAL OF PHARMACEUTICAL SCIENCES AND RESEARCH

Received on 04 February, 2018; received in revised form, 04 September, 2018; accepted, 11 September, 2018; published 01 October, 2018

MYCOCHEMICAL PROFILING OF *LENTINUS SQUARROSULUS* MONT., A WILD EDIBLE MACROFUNGI USING GC-MS

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Keywords:

Western Ghats, ITS sequence analysis, Mycochemicals, GC-MS

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ABSTRACT: Studies to exploit wild mushrooms as a source of biologically active compounds are gaining importance in the recent years. In that view, the present study was carried out to evaluate the bioactives of wild edible macrofungi, *Lentinus squarrosulus* Mont. The specimen was subjected to various biochemical tests with the view to establish standards for their identity and chemical composition. To identify the chemical constituents the dried sporocarp was subjected to successive solvent extraction like petroleum ether, chloroform and ethanol using a Soxhlet extractor. The qualitative mycochemical analysis have shown the presence of some important chemical constituents *viz*., alkaloids, terpenoids, flavonoids, cardiac glycosides, steroids, phenols, fats and oils in all the extractives. Further, GC-MS analysis of ethanolic extracts was carried to identify the important volatile constituents. This data may provide baseline information about the bioactive constituents of these species in providing valuable compounds of substantial medicinal importance. The study also envisaged the proper identity of the target specimen by molecular identification. The present study illustrates the value of molecular tools, especially when coupled with traditional taxonomic tools in disclosing the hidden macrofungal diversity.

INTRODUCTION: The Western Ghats of India have a huge wealth of mushroom flora. Bhadra Wildlife Sanctuary, tropical forest lying midst of the Western Ghats is a protected area and Tiger Reserve. Bhadra Wildlife Sanctuary is a biodiversity hotspot and has a wide range of flora and fauna. The diversity of geographical and climatic conditions prevalent in this sanctuary makes the region a natural habitat for a number of medicinal and edible mushrooms.

QUICK RESPONSE CODE

DOI: 10.13040/IJPSR.0975-8232.9(10).4349-54

Article can be accessed online on: www.ijpsr.com

DOI link: http://dx.doi.org/10.13040/IJPSR.0975-8232.9(10).4349-54

One such mushroom of importance is *Lentinus squarrosulus* Mont., an edible macrofungi commonly found in the study area belonging to the family Polyporaceae. The genus *Lentinus* belongs to class Agaricomycetes, family Polyporaceae and contains forty species [1]. There are a total of 20 valid species of genus *Lentinus* encountered from India [2].

Upon scrutiny of the latest authentic literature, Index Fungorum, a nomenclature database for fungi accepts 120 species of *Lentinus* [3]. *L. squarrosulus* is a paleotropical species showing wide distribution extending throughout equatorial Africa, Southeast Asia, the Pacific islands, and Australasia [4]. *L. squarrosulus* is a white rot saprophytic fungus. Morphologically, the basidiocarp is characterized by either whitish or greyish with notable conspicuous squamules on the surface [5].

The macrofungi is usually found on fallen tree trunks, old stumps, and buried or exposed roots of trees. It usually grows in caespitose clusters, consisting of three to six basidiocarps, but sometimes, a tuft of up to thirty basidiocarps may be found [6]. In its natural habitat, the mushroom grows rapidly with a short life-span, quickly decays, and is easily shredded by rain [7]. *L. squarrosulus* have been reported from the Western Ghats of Tamil Nadu [8, 9, 10, 11] and Maharashtra [4, 12]. But reports on this macrofungi are very scanty in Karnataka.

Traditional identification of macrofungi was done based on the morphologies of fruiting body and microscopic features. There are some species of macrofungi that have unique characters that makes difficult to distinguish them to species level using morphology alone. Hence, to avoid the confusion of wrong identification molecular technique is performed. Many of these problems can be overcome by molecular characterization by analyzing the ITS regions of rDNA from dried fruit body.

Scientific information on wild macrofungi is essential to understand its pharmaceutical and nutraceutical importance. Mushrooms as a source of mycopharmaceuticals and myconutraceuticals is likely to be lost if not documented and screened properly. These species were subjected to various biochemical tests with the view to establish standards for their identity and chemical composition. Although several surveys have been conducted on the diversity of mushrooms of the Western Ghats, very little is known on their nutritional status [13].

So its medicinal potential is relatively unknown to the communities in different parts of the world and remains underutilized so far. In the last few years, Gas Chromatography Mass Spectrometry (GC-MS) has become firmly established as a key technological platform for secondary metabolite profiling in both plant and non-plant species [14]. Hence, the present work was carried out to elucidate the mycochemical content and thereby making an attempt to discover and identify the bioactive constituents of the ethanolic extracts of *L. squarrosulus* using Gas Chromatography coupled with Mass Spectroscopy (GC-MS).

MATERIALS AND METHODS:

Survey Area and Collection: The geographical location of Bhadra Wildlife Sanctuary is 13º43′38.7 N latitude and 75º42′55.2′E longitude with an altitude of 659 m. It is located 38 km Northwest of Chikkamagaluru district of Karnataka state. Temperatures vary from 10-35 °C and mean annual rainfall varies from 1200 mm to 2600 mm. The vegetations are mostly dry deciduous, moist deciduous and semi evergreen forests. The fresh sporocarps of *L. squarrosulus* was collected from the decaying wood of *Mangifera indica* L. from Bhadra Wildlife Sanctuary during the month of August 2016.

Morphological Identification of Macrofungi: The material was brought to the laboratory and taxonomically studied and identified. Initial identification was done on the basis of micro and macroscopic features according to the published descriptions and manuals. Morphological characters such as colour, size, texture shape and margin of fruit body, other features such as odour, stipe and stipe length, pileus length, gill attachment and spacing were considered. The nomenclature was based on the Index Fungorum and Mycobank.

Molecular Characterization of *L. squarrosulus*: Genomic DNA was isolated from the dried fruiting body using manufacturer's kit (Chromous Biotech Private Limited, Bangalore). Two universal primers ITS1 and ITS4 were used for the amplification of ITS regions of mushrooms [15]. Using these primers the PCR amplified products from the referenced sample was sequenced in a forward and reverse direction.

The PCR tubes were placed in a thermal cycler for amplification and was carried out according to the following protocol: an initial denaturation at 94 ºC for 5 min, followed by 35 cycles at 94 ºC for 30 sec, 52 º C for 30 sec and 72 ºC for 45 sec and a final extension at 72 °C for 5 min. The amplified PCR products were separated by gel electrophoresis. The DNA fragments obtained from agarose gel were purified using chromous gel extraction kit and directly sequenced. To make DNA sequences two universal primers (ITS1 and ITS4) were used. The nucleotide sequences were determined by chain termination method using an ABI 3500 XL Genetic Analyzer.

The resulting sequences were compared with those available in the National Centre for Biotechnology Information (NCBI) database for *L. squarrosulus* using the Basic Local Alignment Search Tool (BLAST) software.

Preliminary Mycochemical Screening: In the present study, the fruiting body was shade dried, ground to a coarse powder using a domestic blender and subjected to Soxhlet method of extraction [16] using solvent systems like petroleum ether, chloroform and ethanol depending on their dielectric constants from non polar to polar system. All the chemicals were of analytical grade. The extracts are then concentrated to dryness in vacuum using a rotary flash evaporator. The extractives yield was determined and stored at 4 ºC for further biochemical analysis. The chemical screening of various fruiting body extracts of *L. squarrosulus* was carried out following the procedures described by [17, 18, 19].

GC-MS Analysis: Among all the extractives, the yield was found to be more in ethanol. Moreover, preliminary mycochemical screening revealed more number of compounds in ethanol extraction. Hence, further work to isolate and identify the potent volatile constituents from ethanolic extracts was carried out by Gas Chromatography coupled with Mass Spectroscopy (GC Model: Thermo Trace GC Ultra, MS Model: Thermo DSQ II, at Vittal Mallya Scientific Research Foundation, Bangalore). The spectrum of the unknown components was compared with the spectrum of the known components in their library to identify the compounds. The chemical name, molecular formula, molecular weight, chemical structure of the components of the test samples was also determined. The total GC-MS running time was 35 min.

RESULTS AND DISCUSSION:

Morphology of the Macrofungi: The wild basidiomata of *L. sqaurrosulus* **Fig. 1** and **Fig. 2** perfectly matched morphological features that are well described in several mycological treatises [4, 6, 20] and electronic sources. It exists as a valid species in Mycobank (MB#477648). Some of the identifying characters are described as follows: sporocarp entirely white, caespitose, usually consisting of three to six basidiomes but occasionally a tuft of up to thirty basidiomes pileus 2-10 cm wide, gills cream in colour, umbilicate to infundibuli form, furfuraceous, with concentric zones of small squamules, deeply decurrent, crowded, stem 1-6 cm × 2-7 mm, flesh thick in the centre of the pileus, putrescent, mature fruiting body is tough. Microscopically, this is identified by 6-8 × 1.5-2.5 µm sized, subcylindric, thin walled, smooth and hyaline basidiospores.

FIG. 1: COLONIES OF *L. SQUARROSULUS* GROWING IN TUFT ON DECAYING WOOD OF *MANGIFERA INDICA*

FIG. 2: FRUITING BODIES OF *L. SQUARROSULUS*

Molecular Characterization of *L. squarrosulus*: The species confirmation was done by PCR analysis as it is one of the most advanced techniques to confirm the biological species. The sequences obtained were compared to NCBI database. According to the taxonomic reports of BLAST, the studied macrofungi was *L. squarrosulus*. The ITS sequence isolated gave about 666bp showing 99% similarity, accession number (KT273380.1) and confirmed its identity. The nucleotide sequences were deposited in the NCBI GenBank and obtained the accession number (MH053154). The aligned sequence data of *L. squarrosulus* is shown in the **Fig. 3**.

AGGGGTACCTGCGGAAGGATCATTATCGAGTTTGAAACGGGTTGTAGCTGGC
CTTCCGAGGCATGTGCACGCCCTGCTCATCCACTCTACACCTGTGCACTTACT
GTGGGTTTCAGGAGCTTCGAAAGCGAGAAAAGGGGCCTTCACGGGCTTTTTT
CTTGCCTAGTTGTTACTGGGCCTACGTTTCACTACAAACACTTATAAAGTATC
AGAATGTGTATTGCGATGTAACGCATCTATATACAACTTTCAGCAACGGATCT
CTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAA
TTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACCTTGCGCTCCTTGGT
ATTCCGAGGAGCATGCCTGTTTGAGTGTCATGAAATTCTCAACCTAACGGGTT
CTTAACGGGACTTGCTTTAGGCTTGGACTTGGAGGTTCTTGTCGGCTTGCTTC
AATGTCAAGTCGGCTCCTCTTAAATGCATTAGCTTGGTTCCTGTGCGGATCGG
CTCACGGTGTGATAATTGTCTACGCCGCGACCGTTGAAGCGTTTTTATAGGCC
AGCTTCTAGTCGTCTCTTTACGAGACAATAATCATCGAACTCTGACCTCAAAT
CAGGTAGGACTACCCGCTGAACTTAAGCATATC

FIG. 3: ALIGNED ITS SEQUENCE DATA OF *L. SQUARROSULUS*

Preliminary Mycochemical Screening: The qualitative chemical screening of crude extracts of *L. squarrosulus* was performed for petroleum ether, chloroform and ethanol. The extraction yields ranged from 0.422% for petroleum ether followed by 0.686% for chloroform and 7.25% for ethanol. It can be seen that the extraction yield of ethanol is more than chloroform and petroleum ether. This shows that the extraction yield increases with the increase in polarity. Similar findings were reported [22] which matched the present study.

The yield of extraction depends on the solvent with varying polarity, pH, temperature, extraction time, and composition of the sample. Under the same extraction time and temperature, solvent and composition of sample are known as the most important parameters. The results of mycochemical screening are presented and tabulated in the **Table 1**.

The mycochemical analysis showed positive results for alkaloids, terpenoids, flavonoids, cardiac glycosides, steroids, phenols, fats and oils. Saponins was found to be absent in all the extracts. The presence of phytochemical constituents is known to exhibit medicinal as well as physiological activities [17]. These mycochemicals alone or in combination may have tremendous therapeutic potential in curing various ailments. Further study is aimed at establishing its pharmaceutical and nutraceutical importance. Catechin, a major group of phenolic compounds isolated from *L. squarrosulus* exhibited antioxidant activity [23].

TABLE 1: MYCOCHEMICALS OF *L. SQUARROSULUS* MONT.

Solvent Extracts	Mycochemicals							
	Alkaloids	Terpenoids	Flavonoids	Cardiac glycosides	Saponins	Phenols	Steroids	Fats & oils
Petroleum ether	+	+	+	+	-	-	+	+
Chloroform	+	+	+	+	-	+	-	+
Ethanol	+	+	+	+	-	+	+	+

'+' = Present, '-' = Absent

GC-MS Analysis: GC-MS is proved to be suitable for the interpretation of the volatile compositional characteristics of the mushrooms [21]. The distinct bouquets of macrofungi such as mushrooms and truffles, highly valued in the culinary arts, include mixtures of different volatile organic compounds, of which alcohols, aldehydes, terpenes, aromatics and thiols dominate [24, 25, 26]. In this study, the GC-MS chromatogram analysis of ethanolic extracts of *L. squarrosulus* lead to the identification of 38 volatiles in varying proportions **Fig. 4**. All the 38 mycochemicals with their retention time, molecular formula, molecular weight and concentration (peak area %) are presented in the **Table 2.**

On comparison of the mass spectra of the constituents with their library, the five major peaks representing five major compounds were characterized and identified. The mass spectrometer analyzes the compounds eluted at different times to identify the nature and structure of the compounds. The individual mass fragmentation of five major compounds is illustrated in the **Fig. 5.**

The most prevailing compounds were methyl 2-oxo-1-pyrrolidine actetate (10.40%), hexadecanoic acid methyl ester (10.74%), 9, 12- octadecadienoic acid (Z, Z) (24.21%), ergosterol (16.98%) and ergosta-7, 22-dien-3-ol, (3.beta, 5.alpha 22E) (4.62%).

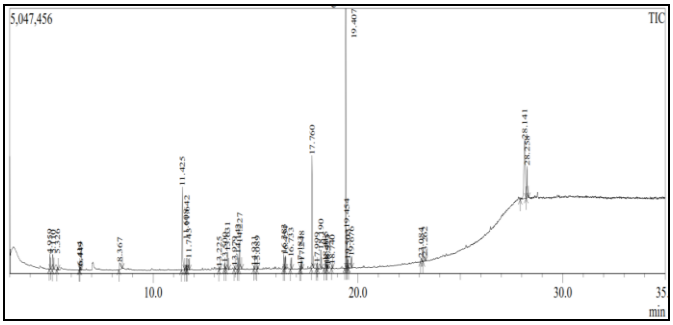
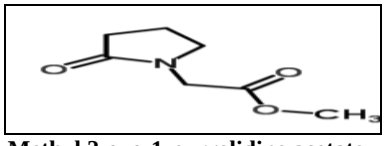


FIG. 4: GC-MS CHROMATOGRAM OF ETHANOLIC EXTRACT OF *L. SQUARROSULUS*

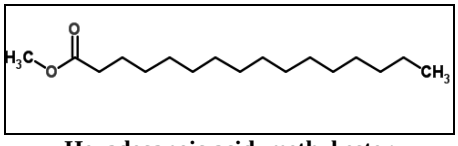
TABLE 2: LIST OF COMPOUNDS IDENTIFIED IN THE ETHANOLIC EXTRACT OF *L. SQUARROSULUS* BY GC-MS

Peaks	RT	Compound name	MF	MW	Peak area%
1	4.959	1-Ethylbutyl hydroperoxide	$C_6H_{14}O_2$	118	1.39
2	5.110	Pentane, 3-ethyl-2,4-dimethyl	C_9H_20	128	1.47
3	5.326	Methyl 1-butenyl ketone	$C_6H_{10}O$	98	0.26
4	6.414	D-Pantolactone	$C_6H_{10}O_3$	130	0.41
5	6.449	2H-Pyran-2-one, 5,6-dihydro-	$C_5H_6O_2$	98	0.30
6	8.367	2(3H)-Furanone, dihydro-4-hydroxy-	$C_4H_6O_3$	102	1.90
7	11.425	Methyl -2-oxo-1-pyrrolidine acetate	$C_7H_{11}NO_3$	157	10.40
8	11.608	2H-2,4a-Ethanonaphthalene, 1, 3, 4, 5, 6, 7 hexahydro-2,5,5-trimethyl	$C_{15}H_{24}$	204	0.65
9	11.642	1-Dodecanol(CAS)n-Dodecanol	$C_{12}H_{26}O$	186	0.49
10	11.743	Tetradecane (CAS) n-Tetradecane	$C_{14}H_{30}$	198	1.06
11	13.225	(E,E,E)-3,7,11,15-Tetramethylhexa deca-1,3,6,10,14-pentaene	$C_{20}H_{32}$	272	0.21
12	13.500	2-Butenedioic acid (Z)-, dibutyl ester	$C_{12}H_{20}O_4$	228	0.62
13	13.631	alpha- Caryophyllene	$C_{15}H_{24}$	204	2.02
14	13.979	Propylphosphonic acid, fluoroanhydride 4-methylcyclohexyl ester	$C_{10}H_{20}FO_2P$	222	0.29
15	14.142	1-Pentadecene	$C_{15}H_{30}$	210	1.34
16	14.227	Tetradecane(CAS)n-Tetradecane	$C_{14}H_{30}$	198	3.42
17	14.931	n-Nonylcyclohexane	$C_{15}H_{30}$	210	0.26
18	15.089	8-Pentadecanone	$C_{15}H_{30}O$	226	0.24
19	16.383	1-Heptadecene	$C_{17}H_{34}$	238	1.37
20	16.454	Octadecane	C18H38	254	1.28
21	16.733	Pentadecanoic acid, methyl ester	$C_{16}H_{32}O_2$	256	1.03
22	17.183	Cyclohexane, undecyl-(CAS) Undecy cyclohexane	$C_{17}H_{34}$	238	0.28
23	17.248	1,2-Benzenedicarboxylic acid, dinonyl Ester	$C_{26}H_{42}O_4$	418	0.90
24	17.760	Hexadecanoic acid, methyl ester	$C_{17}H_{34}O2$	270	10.74
25	17.999	Benzenepropanoic acid, 3,5-bis(1,1-dimethylethyl)-4-hydroxy-, methyl ester	$C_{18}H_{28}O_3$	292	0.52
26	18.190	Dibutyl phthalate	$C_{16}H_{22}O_4$	278	2.36
27	18.415	1-Heptadecene	$C_{17}H_{34}$	238	0.80
28	18.475	Tricosane (CAS)n-Tricosane	$C_{23}H_{48}$	324	0.42
29	18.523	7-Hexadecenoic acid, methyl ester, (Z)	$C_{17}H_{32}O_2$	268	0.29
30	18.740	Hexadecanoic acid, 15-methyl-, methyl ester	$C_{18}H_{36}O_2$	284	0.27
31	19.407	9,12-Octadecanoic acid(Z,Z-), methyl Ester	$C_{19}H_{34}O_2$	294	24.21
32	19.454	9-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	296	4.18
33	19.503	9-Octadecanoic acid(Z-),methyl ester	$C_{19}H_{36}O_2$	296	0.92
34	19.676	Octadecanoic acid, methyl ester	$C_{19}H_{38}O_2$	298	1.03
35	23.084	Palmitaldehyde	$C_{16}H_{32}O$	240	0.42
36	23.262	1,2-Benzenedicarboxylic acid,bis(2-et)	$C_{24}H_{38}O_4$	390	0.63
37	28.141	Ergosterol	$C_{28}H_{44}O$	396	16.98
38	28.258	Ergosta-7,22-dien-3-ol, (3.beta.,5.alpha 22E)	$C_{28}H_{46}O$	398	4.62

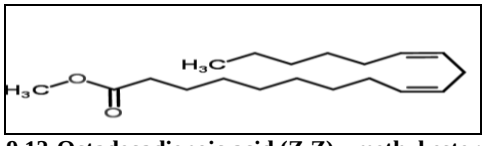
RT- Retention time, MF-Molecular formula, MW- Molecular weight



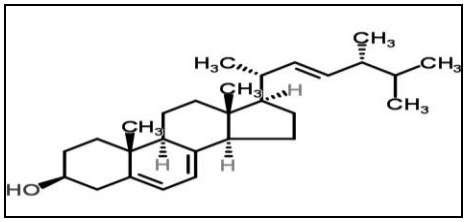
Methyl 2-oxo-1-pyrrolidine acetate



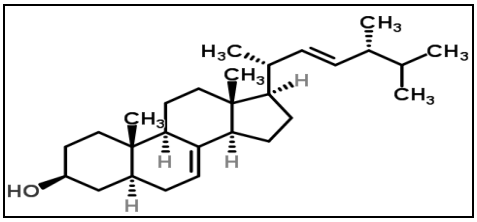
Hexadecanoic acid, methyl ester



9,12-Octadecadienoic acid (Z,Z)-, methyl ester



Ergosterol



Ergosta-7, 22-dien-3-ol, (3.beta, 5.alpha 22E)

FIG. 5: STRUCTURE OF CHARACTERIZED COMPOUNDS FROM THE ETHANOLIC EXTRACTS OF *L. SQUARROSULUS*

CONCLUSION: The characterization on the chemical profiles of *L. squarrosulus* revealed the presence of number of secondary metabolites that was qualitatively and quantitatively determined. As far as we know, this work is the first approach to report the volatile constituents of *L. squarrosulus* by using GC-MS. The volatiles represent a new frontier in bioprospecting, and the study of these gas-phase compounds promises the discovery of new products for human exploitation and will provide basis for future research work. Moreover, the studies on molecular analysis suggested that the Internal Transcribed Spacer (ITS) regions can be considered as a standard barcode for fungal identification and established a powerful tool. Further, studies on isolation and identification of bioactive compounds have to be performed to determine the bioactivity of each compound.

ACKNOWLEDGEMENT: The authors gratefully acknowledge the Chairman, Department of Post Graduate Studies and Research in Applied Botany, Kuvempu University, Shankaraghatta for providing the lab facilities. We also thank Mr. Vinu K., Research Scholar by extending his help in field visits for access to the mushroom specimen.

CONFLICT OF INTEREST: There is no conflict of interest regarding this publication.

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How to cite this article:
Roy DR and Krishnappa M: Mycochemical profiling of *Lentinus squarrosulus* Mont., a wild edible macrofungi using GC-MS. Int J Pharm Sci & Res 2018; 9(10): 4349-54. doi: 10.13040/IJPSR.0975-8232.9(10).4349-54.