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Evaluation of antioxidant activity of C*alocybe indica* P&C and analysis of its chemical constituents by GC-MS

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Ethanolic extract was prepared from fruit body of the summer mushroom *Calocybe indica* grown on paddy straw and the *in vitro* antioxidant activity as well as the chemical constituents of ethanolic extract was evaluated. Antioxidant activity was evaluated by using free radical scavenging activity and reducing power activity. The chemical compound profiling was done through GC-MS analysis and the existence of unsaturated fatty acid, polysaccharides derivatives, terpenoid, alkaloids and phenols in ethanolic extract were revealed. The maximum polysaccharide detected was dianhydromannitol (3.42 %) with retention time 15.44 min. Pentadecanoic acid, a saturated fatty acid rare in nature, which has important role in human blood serum metabolism was identified in this mushroom. The result of the present study brings out some new compounds which can be further worked upon for better understanding of the different properties of the mushroom.

Key words: *Calocybe indica, GC-MS, fatty acids, terpenoids, polysaccharides*

INTRODUCTION

Free radicals play a causative role in a variety of diseases including heart disease, cancer, Parkinson's and Alzheimer's disease, impairment of immune function, cataracts and muscular degeneration in elderly people. All organisms are protected against free radical damage by defense systems involving superoxide dismutase and catalase or antioxidants such as ascorbic acid, tocopherol and glutathione. Improved antioxidant status may have an immuno-stimulatory effect. The antioxidant status of humans reflects the dynamic balance between the antioxidant defense and pro-oxidant conditions. When the mechanism of antioxidant protection becomes unbalanced by factors such as ageing, impaired physiological functioning may occur, resulting in diseases and accelerated ageing. Antioxidant supplements or foods containing high concentrations of antioxidants may help to reduce oxidative damage. Several mushrooms, both wild and cultivated, have been reported to possess antioxidative and other bioactive properties (Ferreira *et al.* 2009; Jananie *et al.* 2012; Orhan and Ustan (2011) have determined the total phenol content as well as the antioxidant activity of some selected wild mushrooms and stated that mushrooms might be used as nutraceuticals or directly eaten in the diet to maintain good health. The mushroom-derived polysaccharides lentinan, schizophyllan, and krestin have been accepted as immune-ceuticals in Japan, Korea and China (Zhang *et al.* 2016). Some of the mushrooms produce substances having potential medicinal effects and a large number of bioactive molecules namely polysaccharides, terpenoids, sterols, protein complexes have been isolated and identified from mushrooms. Polysaccharides are the best known and most potent mushroom derived substances with antitumor and immuno-modulating, antioxidant properties. Therapeutic metabolites of different edible mushrooms and their bioactivity has been reviewed by Barman *et al.*(2018). Antioxidant and antidiabetic activity of *Pleurotus djamor* have also been evaluated by Roy *et al.*(2018). Plants possess pharmaceutical properties due to the presence of various

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secondary metabolites like flavonoids, alkaloids and steroids that are utilized to combat different pathogens (Hussain *et al.* 2011). Mushrooms are also considered to be an important source of biologically active compounds of medicinal value. Mushrooms are nutritional source of phytosterols and the most abundant sterol in mushroom is ergosterol. Ergosterol has been linked with antioxidative activity and is the natural precursor of Vitamin D2, which is formed under UV light irradiation (Shao *et al.* 2010; Philips and Rasor, 2016). Villares *et al.* (2012, 2014) have stated that the ergosterol in mushroom is found in forms both free and esterified. In the present investigation attempts have been made to detect antioxidants activity and to analyse the chemical constituents of *Calocybe indica* by GC-MS.

MATERIALS AND METHODS

Sample collection and preparation

Fresh 6-day old fruit body of *Calocybe indica* cultivated in paddy straw was collected from the mushroom production unit, North Bengal University. The sample was cleaned, washed under tap water several times to remove the dirt and was freeze dried and finally powdered using a mixing grinder. The mushroom powdered sample was stored in clean bottles at normal temperature (27±2 °C) until use.

Preparation of crude extract

The crude extracts from the mushroom sample was obtained by means of cold extraction method. About 30 g of the powdered mushroom sample was added separately to 300 ml of ethanol in conical flask, covered with aluminium foil and kept on a rotary shaker for 24 hrs at room temperature. The solution was filtered with the help of Whatman No.1 filter paper and the filtrate was evaporated by rotary evaporator at 40°C. The dried extracts were then dissolved in ethanol at the rate of 25 mg/ml and utilized for analysis of antioxidant activity and GC-MS analysis.

Reducing Power Ability (RPA)

The reducing power of ethanolic extract (ME) of *C. indica* was determined according to standard procedure. One ml of mushroom extract sample was mixed with an equal volume of distilled water and 2.5 ml of 0.2 M phosphate buffer, pH 6.6, and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min. 2.5 ml of 10% trichloro acetic acid was added to the mixture and centrifuged at 10000 *g* for 15 min. The upper layer of the solution was mixed with 2.5 ml of distilled water and 0.3 ml of 0.1% $FeCl_3$ and the absorbance was measured at 700 nm. Gallic acid (5-40 mg/ml) was used as control.

Free Radical Scavenging (FRS) Activity

The effect of ME of *C. indica*on DPPH radical was estimated where 1000 µl of mushroom extracts were mixed with 50 µl of DPPH solution that was made by addition of 2 ml ethanol in 5 mg of DPPH powder. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{Inhibition of DPPH free radical (\%)} = \frac{\text{A control} - \text{A sample}}{\text{Absorbance of control}} \times 100$$

An antioxidant value of 100% indicates the strongest antioxidant activity and 95% ethanol as blank and the DPPH and Methanol mixture solution as control was used for absorbance.

Gas chromatography-mass spectrometry

The GC-MS analysis extract of the extract was done in AIRF, JNU. For analysis GC-MS-QP2010 ultra-gas chromatograph was equipped with direct injector with linear velocity. A split injection was used for sample introduction and the split ratio was set to 10:0. The oven temperature was programmed to start at 50ÚC, hold for 2 minutes then ramp at 20Úc per minute to 280ÚC and hold for 20 minutes. The helium carrier gas was set to 1.21ml/minute flow rate. Total GC running time was 60 min. ACQ top double focusing magnetic sector mass spectrometer operating in electron ionization (EI) mode with TSS-3333 software was used for compound analyses. High resolution mass spectra were acquired at a resolving power of 5000 (20% height definition) and scanning the magnet from m/z 40 to m/z 650 at 1 second per scan.

RESULTS AND DISCUSSION

General observation during sample preparation

A total of 7 days was required to dry the fresh mushroom fruit bodies. From 1 kg fresh fruit body,

140 g of dry mushroom powder was obtained. The harvested fresh and dried fruit body and mushroom powder is presented in Fig. 1.

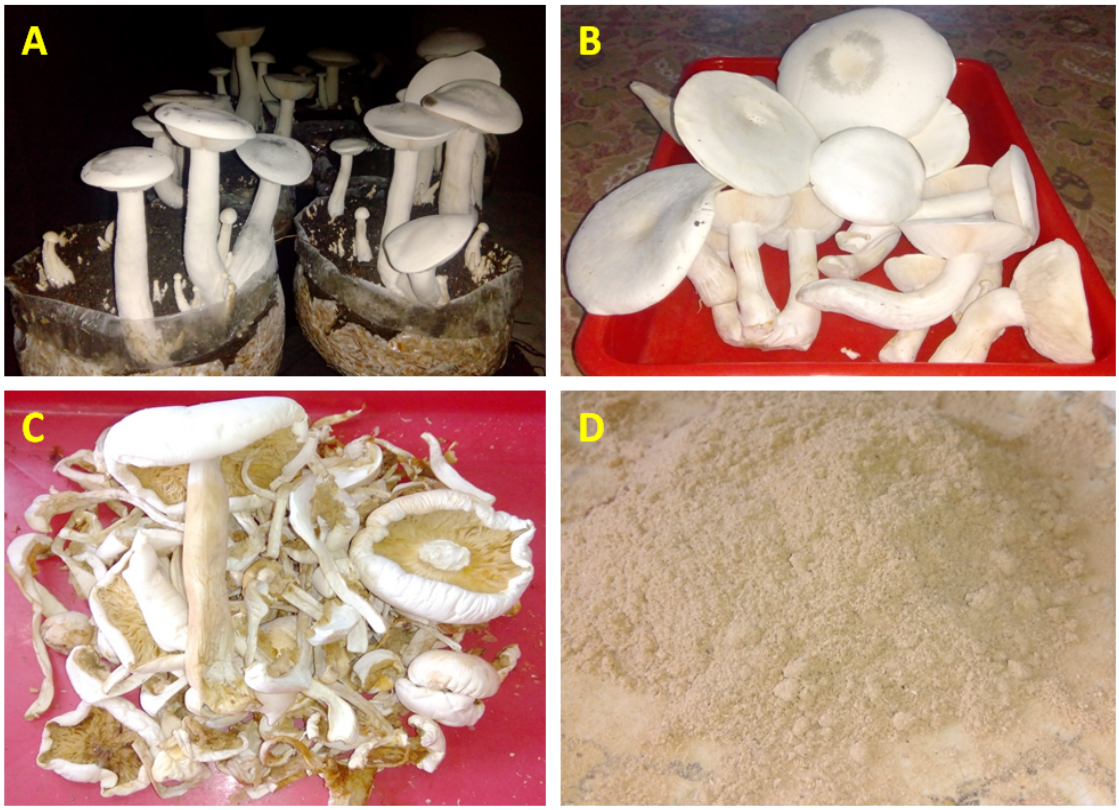

Fig.1: *Calocybe indica* (A)growing fruit body, (B) harvested fruit body, (C), freeze dried fruit body and (D) mushroom powder

Antioxidant Activity

Ferric (Fe^{+3}) reducing activity power was estimated using the ethanolic extract of *C. indica* and it was found that the mushroom species showed appreciable reducing power activity in different concentrations (5-35 µg//ml) with the highest activity being recorded (0.48±0.0173) at the highest extract concentration of 35 µg/ml concentration (Fig.2A). Ethanolic extract of *C. indica* fruit body showed positive antioxidant activity by fading the violet colour of DPPH solution to yellow to pale violet colour. Results revealed that the scavenging activity of DPPH were directly proportional with the concentration of the samples used. As the concentration of the sample increased, the scavenging activity of free radicals was also enhanced. It was found that *Calocybe indica* showed DPPH scavenging activity as 52% at 500µg/ml concentration (Fig 2B). Earlier studies have also reported that mushroom extracts possess antioxidant activity which increases with concentration **(**Mujic *et al.* 2010; Vidovic *et al.* 2010). Mushroom extracts have a strong antioxidant and antimicrobial activity *in-vitro* (Lo *et al.* 2005; Kosanic *et al.* 2012). The antioxidant

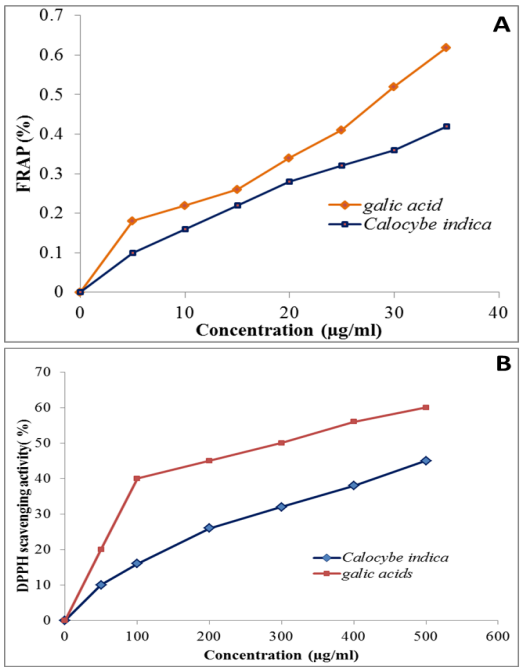

Fig.2: Antioxidant activity of ethanolic extract of *C. indica,* A. Ferric Reducing Activity Power (FRAP %) and B. DPPH Scavenging Activity (%)

Table 1 : Compounds detected in Ethanolic extract of mature fruit body of *Calocybe indiaca* by GC-MS analysis

Peak	R Time	Area%	Compounds	Mol. formula	Mol. Wt.
1	4.554	0.07	1-propanamine, 2-methyl-n-(2-methylpropylidene	$C_8H_{17}N$	127
2	5.833	0.03	2-heptanone	$C_7H_{14}O$	114
3	6.977	0.06	N-(2-Methylbutylidene)isobutylami	$C_9H_{19}N$	141
4	7.041	0.02	N-ethylpyrrolidine	$C_6H_{13}N$	99
5	7.082	0.01	Piperidine, 1-methyl-	$C_6H_{13}N$	99
6	8.566	0.03	Furan, 2-pentyl-	$C_9H_{14}O$	138
7	9.856	0.11	1-Butanamine, 2-methyl-N-(2-methylbutylidene)-	$C_{10}H_{21}N$	155
8	10.15	0.17	3-methylbutyl-(3-methylbutylidene)amine	$C_{10}H_{21}N$	155
9	12.39	3.00	1,2,3-propanetriol	$C_3H_8O_3$	92
10	15.44	3.42	Dianhydromannitol	$C_6H_{10}O_4$	146
11	18.53	0.06	N,4-dimethylpentanamide	$C_7H_{15}NO$	192
12	18.71	0.11	Octanoic acid, 7-oxo-	$C_8H_{14}O_3$	158
13	19.25	0.03	4-pentylbutan-4-olide	$C_9H_{16}O_2$	156
14	20.57	0.04	Bicyclo[3.1.1]heptan-3-one, 6,6-dimethyl-2-(2-oxopropyl)-, isomer 2	$C_{12}H_{18}O_2$	194
15	22.08	0.03	2-methyl-n-phenylacrylamide	$C_{10}H_{11}NO$	161
16	22.22	0.08	1-dodecanol	$C_{12}H_{26}O$	186
17	23.13	0.10	Phenol, 2,4-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O$	206
18	25.00	0.03	1-hexadecene	$C_{16}H_{32}$	224
19	29.46	0.04	Heptadecanoic acid, ethyl ester	$C_{19}H_{38}O_2$	298
20	30.54	0.09	1H-Cyclopropa[a]naphthalene,1a,2,3,3a,4,5,6,7b-octahydro-1,1,3a,7-tetramethyl	$C_{15}H_{24}$	204
21	31.51	0.04	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284
22	32.75	0.08	Dibutyl phthalate	$C_{16}H_{22}O_4$	278
23	32.90	0.08	1h-purin-6-amine	C5H5N5	135
24	33.51	5.24	Hexadecanoic acid, ethyl ester	$C_{18}H_{36}O_2$	284
25	33.82	7.11	Hexadecanoic acid	$C_{16}H_{32}O_2$	256
26	36.62	3.24	N-Propyl 9,12-octadecadienoate	$C_{21}H_{38}O_2$	322
27	36.81	5.69	Ethyl 9-octadecenoate	$C_{20}H_{38}O_2$	310
28	37.69	66.29	Octadec-9-enoic acid	$C_{18}H_{34}O_2$	282
29	40.21	0.28	9-Octadecenal, (Z)-	$C_{18}H_{34}O$	266
30	41.64	0.14	Hexadecanal, 2-methyl-	$C_{17}H_{34}O$	254
31	42.04	0.22	Oleic anhydride	$C_{36}H_{66}O3$	546
32	42.77	0.08	tert-Hexadecanethiol	$C_{16}H_{34}S$	258
33	43.02	0.19	1-Hexadecyne	$C_{16}H_{30}$	222
34	43.56	0.10	2-(7-Hydroxymethyl-3,11-dimethyl-dodeca-2,6,10-trienyl)-[1,4]benzoquinone	$C_{21}H_{28}O3$	328
35	44.28	0.07	S-[2-[N,N-Dimethylamino]ethyl] N,N-dimethy Lcarbamoylthiocarbo-hydroximate	$C_8H_{17}N_3O_2S$	219
36	44.50	0.18	2-[12-(2-oxiranyl)dodecyl]oxirane	$C_{16}H_{30}O_2$	254
37	45.30	0.08	9-Octadecenoic acid (Z)-, octadecyl ester	$C_{36}H_{70}O2$	534
38	45.80	0.05	Ethyl tetracosanoate	$C_{26}H_{52}O_2$	396
39	45.99	0.10	Squalene	$C_{30}H_{50}$	410
40	46.52	0.05	4-nonanol, 2,6,8-trimethyl-	$C_{12}H_{26}O$	186
41	46.63	0.05	Oxalic acid, hexadecyl 2-phenylethyl ester	$C_{26}H_{42}O_4$	418
42	47.97	0.30	Anthraergostatetraenol benzoate	$C_{35}H_{46}O2$	498
43	48.19	0.05	9(11)-Dehydroergosteryl benzoate	$C_{35}H_{46}O2$	498
44	49.19	0.12	Oleic anhydride	$C_{36}H_{66}O3$	546
45	52.39	2.10	Ergosta-7,22-dien-3-ol,(3.beta.,5.alpha.,22E)	$C_{28}H_{46}O$	398
46	52.71	0.24			

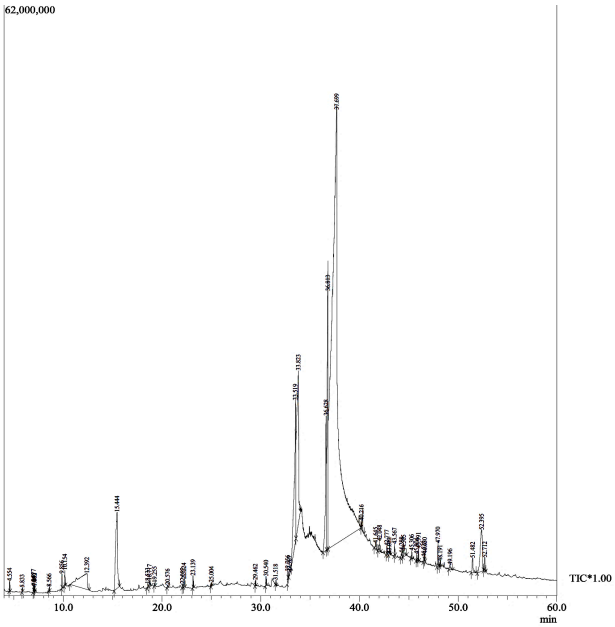

Fig. 3: Total ion Chromatogram (TIC) of Ethanolic extract of mature fruit body of *Calocybe indica*

study of Liu *et al.* (2013) have clearly indicated that ethanolic extract of *A. bisporus* had significant antioxidant activity against various antioxidant systems *in vitro*.

GC-MS analysis

Interpretation of mass spectrum GC-MS was conducted using the database of National Institute Standard and Technology (NIST) having more than 62,000 patterns. The spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight of the components detected in the test materials are presented in Table-1. From the GC-MS -TIC spectrum (Fig. 3), the ethanol extract from *C. indica* resulted in the identification of 46 different peaks of compounds in different retention time. In our study, maximum (66.29%) unsaturated fatty acid

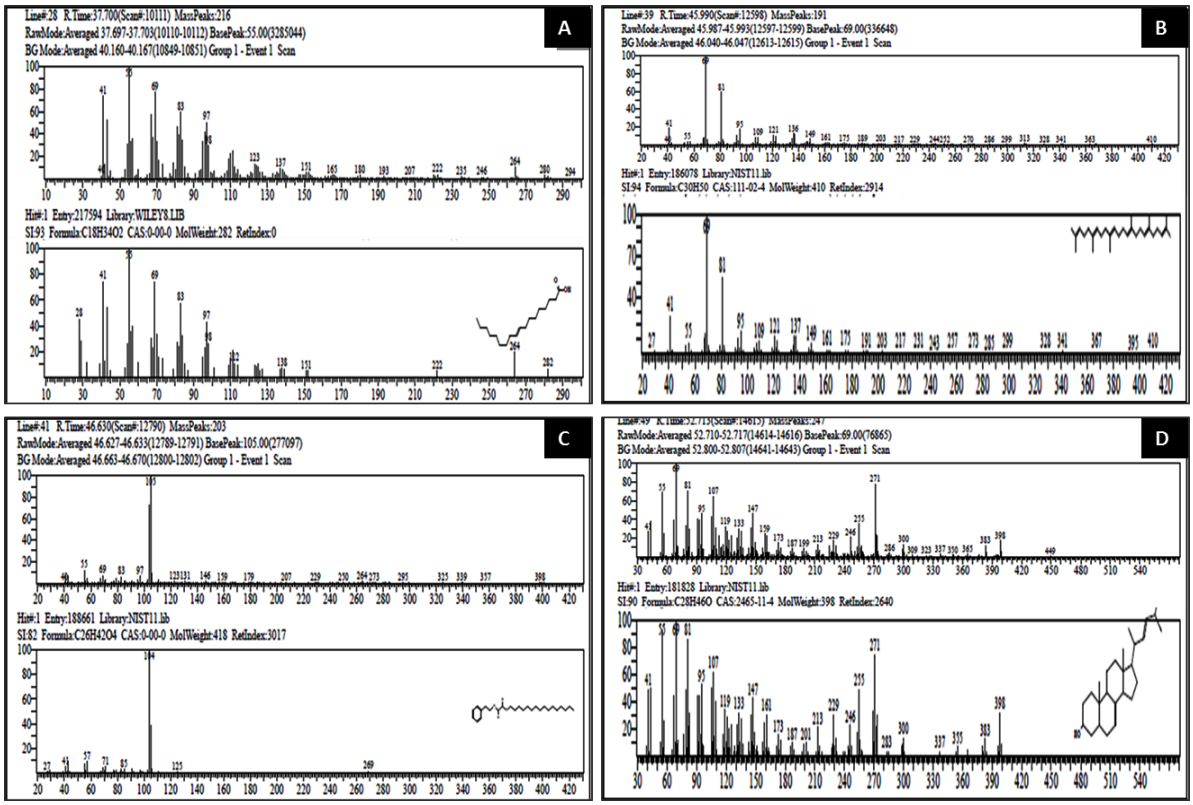

Fig 4: Major Compounds identified by MS analyses in Ethanolic extract of mature fruit body (*C. indica),* A. Octadec-9-enoic acid, B. Squalene, C. Oxalic acid, hexadecyl 2-phenylethyl ester, D. Ergosta-7,22--dien-3-ol, (3.beta.,5.alpha.,22E).

activity of different mushroom species was evaluated by Keles *et al.* (2011) and Reis *et al.* (2012) and they stated that the ethanolic extract of wild mushroom have significant *in vitro*. The as octadec-9-enoic acid along with hexa-decanoic acids (15%) and moderate amount of steroids as ergosta was detected. Major Compounds identified in ethanolic extract of mature fruit body

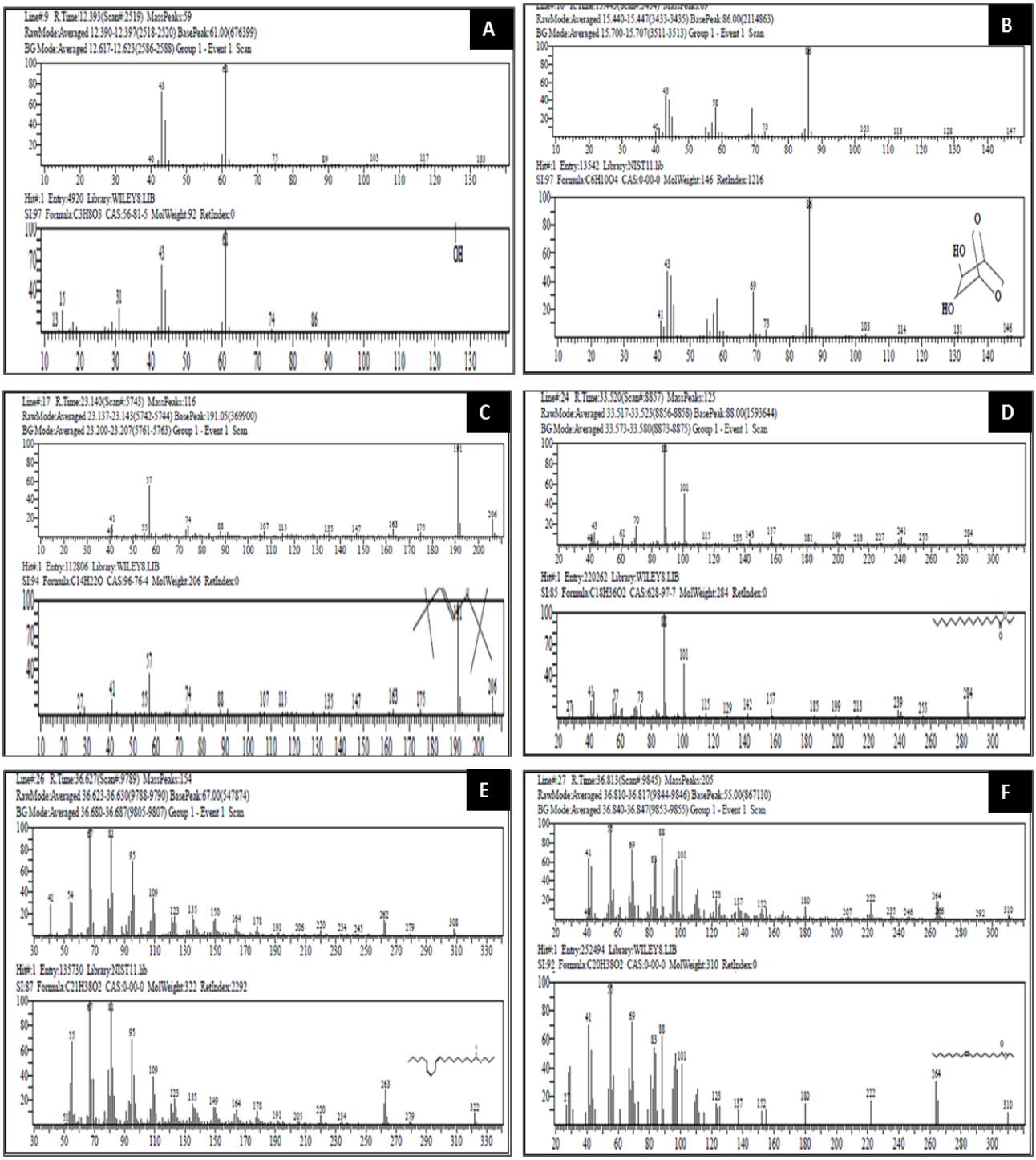

Fig.5: Major Compounds identified by MS analyses in Ethanolic extract of mature fruit body (*C. indica*) A.1,2,3-propanetriol, B. Dianhydromannitol, C. Phenol, 2,4-bis(1,1-dimethylethyl),D. Hexadecanoic acid, ethyl ester, E.N-Propyl 9,12-octadecadienoate, F. Ethyl 9-octadecenoate

of *C. indica* using MS analyses were Octadec -9-enoic acid, squalene, oxalic acid, hexadecyl 2-phenylethyl ester, ergosta-7,22-dien-3-ol, (3. beta.,5.alpha.,22E) have been presented in Fig. 4(A-D) as well as 1,2,3-propanetriol, dianhydromannitol, phenol, 2,4-bis(1,1-dimethylethyl), hexadecanoic acid, ethyl ester, N-Propyl 9,12-octadecadienoate and ethyl 9-octadecenoate (Figure 5 A-F). Sugar derivative dianhydramannitol (3.43%) was detected in the sample which is a carbohydrate derivative used as sweetener in diabetic food and as a medication; it

is used to decrease high pressure in the eye (glaucoma) and to lower increased intracranial pressure (head trauma). Various steroid derivatives were also detected. Squalene a terpenoid known as super-antioxidant was detected in ethanolic extract. The results were in line with the GC-MS studies on petroleum ether soxhlet extract of dried fruiting bodies of *Pleurotus ostreatus* by Suseem *et al.* (2011). Oxalic acid was detected as organic acid in the sample which also contributes to the anti-oxidative activity of the mushroom sample. The GC-MS analysis of the purified mushroom sample was reported to have compounds like alkanes and fatty acids which possess therapeutic properties (Lakshmi and Raja Lakshmi, 2011). But Shao *et al.* (2010) investigated different parts of button mushrooms at various development stages and found different fatty acid and detected non esterified ergosterol. Ribeiro *et al.* (2011) identified free amino acids composition and thirty fatty acids from twelve wild edible mushroom species by ion trapping method in GC-MS. Mohammed and Farghaly (2014) used GC-MS analysis and reported alcohol, alkane, amides, esters, fatty acids, terpenoid, heterocyclic and phenols in ethanolic extract of *Pleurotus ostreatus.* Phenol was also detected in our study. Ragasa *et al.* (2016) reported the presence of sterols and lipids in dichloromethane extract of *A. bisporus* detected in GC-MS analysis. The presence of sterol and lipids was detected by GC-MS in *Pleurotus florida* (Ragasa *et al.* 2015) and *Pleurotus djamor* (Ragasa *et al.* 2016a). GC-MS analysis of *Pleurotus djamor* also indicated the presence of different bioactive compounds having therapeutic value essential for human health (Roy *et al.* 2018).

The summer mushroom *C. indica* may be used as an antioxidative food supplement. The presence of compounds in *C. indica* responsible for antioxidative activity was confirmed by the GC-MS analysis. The GC-MS analysis revealed a wide range of chemical constituents which includes fatty acid, steroids, and polysaccharides in the summer mushroom *C. indica*. The fatty acids that were identified in the present study contributes two significance to human; the first is their natural origin which is safer to people and environment, the second is that they have been considered at low risk for resistance development by pathogenic microorganisms. From the above results it can be concluded that *C. indica* can be used as bio-resource of important bioactive compound like ester, fatty acids, terpenoid, polysaccharides. This mushroom can be good and safe natural sources of antioxidants and could be of significance in making the food and in human therapy, animal and plant diseases.

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