Comparative cytotoxic effect of metabolites from wild and mutant strains of *Schizophylum commune* grown in submerged liquid medium

Aina, D.A.[1*],Oloke, J.K.[2], Awoyinka, O.A[3]., Adebayo, E.A[2]., Akoni, O.I[4], Agbolade, J.O.[5] and Odeniyi, K.M[4]

[1] Department of Biosciences and Biotechnology, School of Basic and Applied Sciences, Babcock University, Ilisan-Remo, Ogun State. P.M.B. 21244 Ikeja, Lagos, Nigeria
[2] Department of Pure and Applied Biology, Ladoke Akintola University of Technology,Ogbomoso,Oyo State.
[3] Department of Medical Biochemistry, Ekiti State University, Nigeria,
[4] Department of Biochemistry, Ben Carson School of Medicine, Babcock University, Nigeria
[5] Department of Biological sciences, Oduduwa University, Ipetumodu, Ile-Ife, P.M.B. 5533 Osun State
Corresponding author: ainatayo2002@yahoo.com

ABSTRACT

The increasing incidence of cancer coupled with clinical failure of orthodox medicines and rising costs of irradiation and surgical interventions that impact negatively on access to care particularly in low and middle income countries including Nigeria have become a serious global health problem. Hence an edible mushroom, *Schizophyllum commune* was explored in this study, for the discovery, development and delivery of novel anticancer agents. *S. commune* wild type and mutant strains generated by exposing the wild type organism to Ultraviolet rays at various time intervals of 30,60 and 90 minutes coded as SCW, SCM1, SCM2 and SCM3 respectively were grown in submerged liquid medium containing *Hibiscus sabdariffa* calyx extract enriched with yeast extract and peptone water under continuous aeration for six days. Thereafter Gas Chromatography-Mass Spectrophotometric and Cytotoxicity assay using Hep2 cells were carried out on the broth. The GC-MS inferred over hundred diverse organic compounds present in all the

broth but with each respective *S. commune* producing distinct and peculiar compounds as their own secondary metabolites. Cytotoxicity assay revealed greatest cell proliferation inhibitory activity against Hep2 cell by broth from SCM3 with IC_{50} of 425 ug/mL followed by SCM2, 520 ug/mL, SM1, 560 ug/mL and SCW, 600 ug/mL ($P < 0.05$). Hence the *S. commune* irradiated with ultraviolet rays for 90 minutes possesses high anticancer properties than the wild type and other mutant strains.

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INTRODUCTION

In recent times there has been a relevant passion in research of natural products due to repeated failure of alternative drug discovery methods to deliver many vital compounds in biological activities such as immune-suppression, antibiotics, anticancer, antioxidants and other metabolic ailments (Mau *et al.,* 2002). It is now increasingly recognized that correct diet controls modulates many functions of the human body and, consequently, participates in the maintenance of good health, or homeostasis, necessary to reduce the risk of many diseases (Smith and Sullivan, 2002, Lindequist *et al.,* 2005).

Previously, mushrooms have been reported to have great potential as a nutritionally functional food and a source of physiologically beneficial and non-toxic medicines (Wasser, 1999; Zaidman *et al*., 2005). Mushroom similar to plants, have a great potential for the production of useful

bioactive metabolites and they are prolific resource for drugs with anticancer activities (Thomas and Earl, 1994). Mushrooms characteristically contain a tremendous, variety of secondary metabolites and the bioactivity of these compounds depend on how the mushroom is prepared and consumed. The vast structural diversity of natural compounds found in mushrooms provide potential opportunities for discovering new drugs that rationally target the abnormal molecular and biochemical signals leading to cancer (Russo *et al*., 2006).

Many other researchers supported the idea that mushrooms have become attractive as a functional food and as a source for development of drugs and nutraceuticals. Mushrooms are consumed much more for their texture and flavor than for their functional and medicinal properties. Most of the edible mushrooms, however, do not have medicinal value (e.g. *Agaricus bisporus*) compared to some non-edible mushrooms (e.g. *Ganoderma, Coriolus*) which have gained important medicinal usage (Wasser and Weis, 1999a). The edible mushrooms which demonstrate medicinal or functional properties include species of *Lentinula, Auricularia, Hericium, Grifola, Flammulina*, and *Pleurotus* while others known for their medicinal properties include *Ganoderma* and *Trametes* (*Coriolus*) are definitely non-edible due to their coarse and hard texture or bitter taste. The historical evolution of these essentially scarce, forest derived mushrooms would most certainly not have been as fresh, whole mushroom but as hot water extracts, concentrate, liquors or powders and used in health tonics, in many forms. Thus their original human use was medicinal rather than nutritional (Smith and Sullivan, 2002).

When used for therapeutic intention, the medicinal mushrooms are normally consumed as powdered concentrates or extracts in hot water, and the extract concentrated and used as drink or freezes dried or spray-dried to form granular powders which allow easier handling, transportation and consumption (Mizumo *et al*., 1995). As such, these liquid concentrates or

dried powdered mushrooms contained in capsules can be considered as dietary supplements (DS) with potential health benefits (Chang and Buswell, 1996). Regular intake of these concentrates is believed to enhance the immune responses of the human body, thereby increasing resistance to diseases and, in some cases, causing regression of the disease state (Jong *et al*., 1991). It is appropriate to note that these mushroom compounds show little or no side effects to humans unlike many highly purified and widely used modern pharmaceuticals (Smith & Sullivan, 2002). The immune system of an organism plays an important role in the defense against infections and tumour formation. Several purified mushroom compounds e.g. *Lentinan* and *Grifolan* D have been shown to enhance or potentiate host resistance in the treatment of various cancers, immunodeficiency diseases or immune suppression after drug treatments as adjuvants for vaccines and for combination therapy with antibodies (Smith and Sullivan, 2002).

Against this backdrop *S. commune* that has long been acknowledged for its medicinal properties (Oso, 1991; Han *et al.*, 2005) is chosen for this present study. It is extremely important because it produces the polysaccharide schizophyllan which shows considerably medicinal properties. Wasser (2002) reported that the polysaccharide prevents oncogenesis shows anti-tumour activities against various allogeneic and syngeneic tumours, and prevent tumour metastasis. Prior to this report, the secondary metabolites of *S. commune* were known to show anti-candida, anti-tumour, and anti-viral properties (Ooi and Lui, 1999). However, the present study was carried out to compare the anticancer activity of the wild type and mutant strains of *S. commune* grown in submerged liquid medium.

MATERIALS AND METHODS

Collection of microbial sample

S. commune was collected from dead wood of *Mangifera indica* at Ogbomoso, Oyo State, Nigeria. It was characteristics in the department of Pure and Applied Biology, Ladoke Akintola, University of Technology,Ogbomoso,Nigeria, following the method of Zoberi (1972) and Alexopolous *et al*.,(1996).

Sample preparation and establishment of mycelial cultures

Tissue culture was carried out on fresh carpophores of *S.commune* using the method of Jonathan *et al* (2009).The mycelial thus generated were cultured on plates of potatoes dextrose agar (PDA).

Production of *Schizophyllum commune* mutants

Various mutants of *S. commune* were prepared as follows; Fresh plates of *S. commune* were allowed to sporulate. The spores were removed with sterile distilled water and re-inoculated on three different freshly prepared plates. The plates were exposed to UV light at 260nm at various time intervals to induce mutation (Peak *et al*., 1984) with modifications .The first plate was exposed to UV for 30 minutes and labelled as *S. commune* mutant I (SCM1). The second plate was exposed to UV for 60 minutes and labelled as *S. commune* mutant II (SCM2).The third plate was exposed to UV for 90 minutes and labelled as *S. commune* mutant III (SCM3). Fresh plate of the organism before exposing to UV radiation was also prepared and labelled as *S. commune* wild type (SCW). Four different strains that eventually resulted were SCW, SCM1, SCM2 and SCM3.

Culture preparation for metabolites production

The basal medium used consist of 100mls of *Hibiscus sabdariffa* solution added with 6g Glucose , 1.6g Malt extracts, 2g Peptone, 1.2g Yeast extracts, 0.8g KH_2PO4, 0.4g $MgSO4.7H_20$, 0.4g Urea and pH adjusted to 5.8 (Yap and Ng 2001) with modification. *S. commune* wild type and mutants (SCW,SCM1,SCM2 and SCM3) were initially sub-cultured on PDA plates and then 6mm plug of the vigorous growing agar plate culture (5- day old) was removed using sterile cork borer. The sterilized basal medium was inoculated with this mycelia disc of *S. commune* wild type and mutants. The fermentation experiments were set-up under aerobic condition for 6 days.

Extraction of metabolites from submerged culture medium

The organisms were cultivated in the chemically defined medium as described above. One hundred millilitres of the chemically defined medium were dispensed into 250mls bottles and pH adjusted by using 0.1N HCl and 0.1N NaOH to 5.8, and sterilized, then inoculated with 6mm agar disc plug of the fungus and incubated at 28^oC, for 6days. On the 6^{th} day of fermentation, the experiment was terminated. The liquid fermented products were centrifuged at 4,000rpm for 20mins using electrical centrifuge. After centrifugation, the residues were discarded and the filtrates that contained the metabolites were transferred into separating funnels. Equal volume of acetone was used to extract the metabolites in the separating funnel. This was achieved by thoroughly shaking the acetone and the filtrate and thereafter allowed to settle. Two different layers were formed; the upper layer which contained the acetone and the lower layer containing the metabolites. The mutant that produced the highest metabolites was noted alongside the wild type.

Collection of Cancer Cell Lines

The cryovial of Hep 2 cells were obtained from National Agency for Food and Drug Administration Control, Yaba, Lagos, Nigeria at -192^{0}c.

Extraction of Lectin from mycelia mat of *S. commune*

The mycelia mat that was collected after submerged fermentation into 20 ml of 0.01M phosphate buffer saline through surface scraping with a sterilized blade. The resulting solution was kept in the refrigerator for 30 minutes.

Purification of Spent Culture Supernatant (SCS)

This step involves intense work carried out on the fermentation broth (SCS) which has been kept in the fridge over time. The process wisely involves three main techniques which involve extraction, fractionation and isolation/purification of fraction as described by Kumala *et al*., 2007.

Extraction

Separation of cell mass from the SCS containing the secondary metabolites of interest was carried out in cold centrifuge at 4^{0}C at 2000rpm for 20 minutes. The pH of the supernatant was adjusted to 3-4 using 1M acetic acid and then extracted by acetone three times, Sodium sulphate was added to remove the last trace of water from the acetone extract.

Fractionation Analysis

The extracted secondary metabolite produced by the fungus was further purified by silica gel short column chromatography using non polar solvent mixtures of Hexane: Ethyl acetate in turn 100:0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100 and followed by semi polar solvent mixture of Ethyl acetate: Methanol 99:1, 97:3, 95:5, 93:7, 90:10, 85:15, 80:20, 75:25, 70:30, 65:35, 60:40, 55:45, 50:50, 45:55, 40:60, 35:65, 30:70, 25:75, 15:85, 5:95, 0:100. This fractionation was analyzed using TLC (Thin Layer Chromatography) and further by GC-MS. In the TLC the different fractions were run against a standard Sterigmatocystin.

Identification of Fractions by GC-MS

This step involves the use of GC-MS model agilent technologies7890A. Anhydrous sodium sulphate was added to the fractionated broth from SCW, SCM1, SCM2, & SCM3 and was poured through sterile cotton wool which served as separating medium. The anhydrous sodium sulphate absorbed the remaining water in the sample 1µl of the filtrate was then injected into the machine. The carrier gas pushes the filtrate into the column where the separation takes place.

Bioassay Using Hep2C Cell Line

Prior to Cytotoxicity test, the acetone fraction was lyophilized and reconstituted by serial dilution in 1% methanol containing PBS (pH 7.4) at 50 – 1000 ug.mL concentrations. The assay was carried out using monolayer culture of Hep2 cells in DMEM medium supplemented with amphotericin (50 ug/mL), gentamicin (100 ug.mL) and glutamine 1 ug.mL under 5% CO_2 for 48 h. for the determination of 50% end point ($CCID_{50}$) and IC_{50}. Cyclophosphamide at 1 – 10

ug/mL and 1% methanol were used as positive and negative controls respectively. The IC_{50} was calculated using Reed and Muench calculation method:

$Log_{10}CCID_{50}$ = -L-D(S-D).

Where L= Total number of viable cell without treatment

S= Total number of viable cell with treatment, and

D= Difference between each cell

RESULTS AND DISCUSSION

Results derived from GC-MS reference over hundred organic compounds to be present in the broth. As shown in the Table, Broth from the Wild type showed forty nine organic compounds. Out of these, five were found to also be present in all the broth from other three mutant strains- SCM1, SCM2 and SCM3. These compounds are pentadecanoic acid, 14 methyl esters, Hexadecanoic acid, Methyl esters and Octadecanoic acid. The broth from SCM1 was also found to distinctly consist of about forty six compounds out of which only four namely Cis- 13 Octadecanoic acid and Cis- Vaccenic acid, Oximic and Furanone compounds were also found in SCM2 broth. Well a total forty seven compounds were also strictly found in SCM2 broth with fifteen out of interestingly found in the Wild type. However, a total of thirty three organic compounds were found to be associated with the broth from SCM3. Over 50% of these compounds were found to be strictly present in the SCM3 broth alone but not found in any other broth.

Medicinal mushrooms are known to accumulate a wide variety of bioactive compounds including terpernoids, steroids, phenols, nucleotides and their derivatives glycoproteins and polysaccharides that display a broad range of biological activities (Borchers *et al.,* 1999; Teissedre and Landrault, 2000). These different bioactive compounds have been extracted from the fruiting body, mycelia and culture medium such as *L. edodes, G. lucidum, S. commune, Trametes versicolor, Inonotus obliquus and Flammulina velutipes* (Wasser and Weis, 1999). In this study the inference of seventeen distinct compounds found strictly to be associated with broth from SCM3 could be a clear indication for its high cytotoxic potency compare to the broth from other strain of the *S. commune.* These distinct compounds are:- 1-Methyl-4-(1-Methylethenyl, 2,5-Dihydroxypropiophenone, Trans-2-ethylbicyclo decane, 4-amino-5-formamidomethyl, 2-methyl pyrimidine, Benzo(b) tetrahydrofuran-3-one,5,6 dihydroxy ethyl vanillin, Butyl octyl ester, Isobutyl octyl ester, Buty-1,2-Methylpropyl ester, Cyclohexylmethyl butyl ester, Pentanoic acid, 10-Undecenyl ester, Myristic acid vinyl ester, Tritetracontane, 2,3-Dihydroxypropyl ester, 2-hydroxy-1-(hydroxymethyl) ethyl ester.

However, Figure 1 below shows the percentage growth inhibition of the cancer cell line (Hep2 Cells) against the concentration of the broth samples (SCW, SCM1, SCM2 & SCM3). It can be observed that the inhibitory effect of the cancer cells were found to increase as the concentration of the broth increased. At very low concentration of 25µg/ml, all the samples were found not to elicit inhibitory activity but at a high concentration of 900µg/ml to 1000µg/ml, there was no record of the cells lines as all had been inhibited by the high concentration of all the broths.

From the plot of growth inhibitory profile of Hep2 cells below (Figure 1), the 50% inhibition (IC_{50}) of all the broths were found to be at varying concentration with SCM3 having the lowest

concentration of 425µg/mL while IC_{50}, 520 µg/mL, 560 µg/mL, and 600µg/mL for SCM2, SCM1 and SCW respectively.

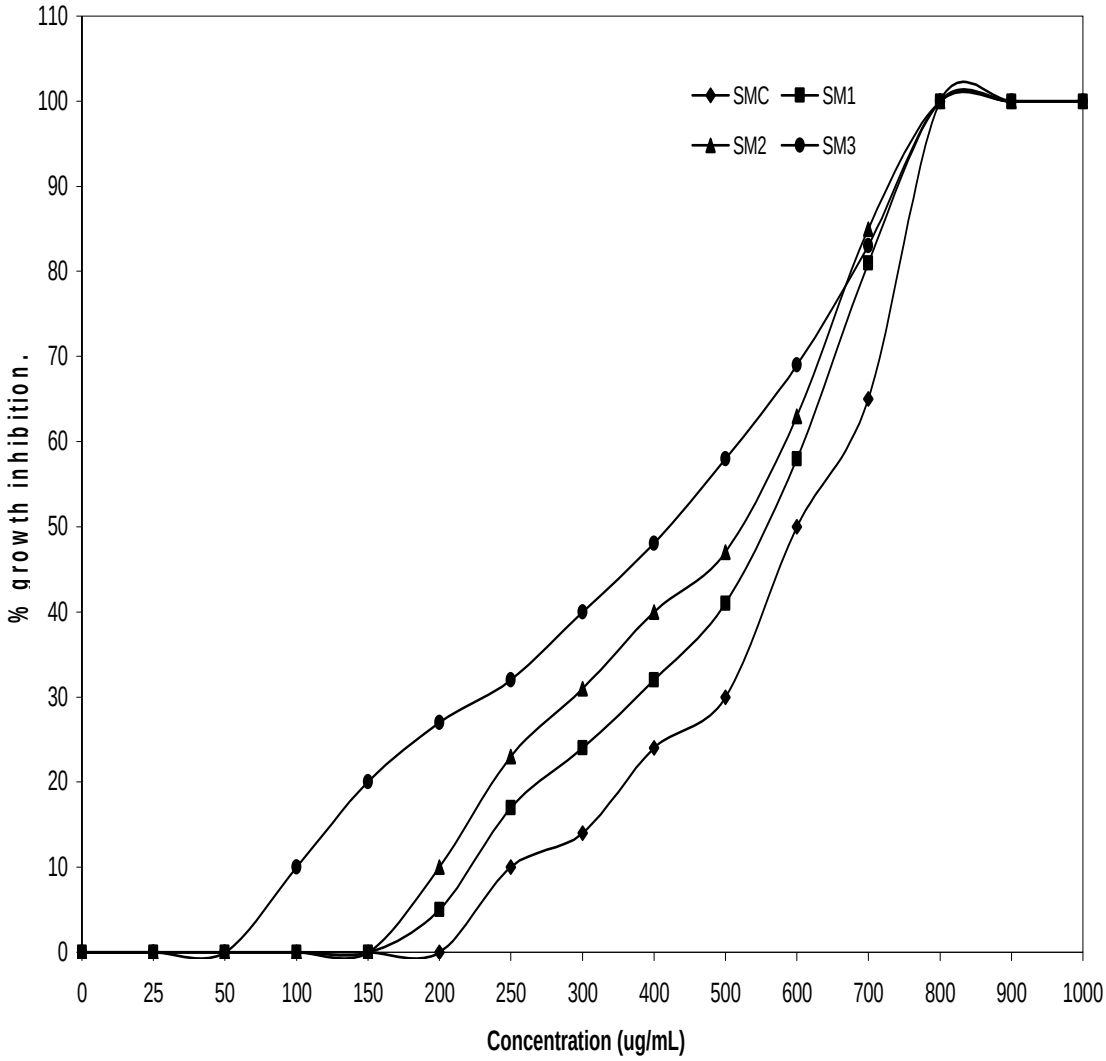

Figure 1: Growth inhibitory profile of Hep2 cells exposed to various concentrations of wild type and mutant *S. commune* strains.

Table1: GC/MS result of fractionation of metabolites of wild and mutant strains of *S.commune*

S/N	Metabolites	SCW	SCM1	SCM2	SCM3
1	11,13-Tetradecadien-1-ol	+	-	-	-
2	Pentafluoropropionic acid	+	-	-	-
3	n-Tridecan-1-ol	+	-	+	-
4	Dodecyl ester	+	-	-	-
5	2-Propenoic acid	+	-	+	-
6	Oxybis (methyl 1,2, 1-ethanediyl) ester	+	-	+	-
7	Cyclotetradecan	+	-	-	-
8	n-Tridecahexyl estern-1-ol	+	-	-	-
9	Phthalic acid	+	-	+	+
10	2-ehtylhexyl isobutyl ester	+	-	-	-
11	Isobutyl undecyl ester	+	-	+	+
12	1,2-Benzenedicarboxylic acid	+	-	+	+
13	Butyl cyclohexyl ester	+	-	-	-
14	Butyl hexyl ester	+	-	+	-
15	Butyl decyl ester	+	-	-	-
16	Dibutyl phthalate	+	-	-	-
17	Pentadecanoic acid	+	+	+	+
18	14- methyl ester	+	+	+	+
19	Hexadecanoic acid	+	+	+	+

20	**Methyl ester**	+	+	+	+
21	**Butyl 2-methylpropyl ester**	+	-	+	-
22	**Isobutyl nonyl ester**	+	-	-	-
23	**Ethyl pentadecyl ester**	+	-	-	-
24	**9- Octadecenoic acid**	+	+	-	+
25	**10- Octadecenoic acid**	+	-	-	-
26	**Octadecanoic acid**	+	+	+	+
27	**Olean-12-ene**	+	-	-	-
28	**4,4,6a,6b,8a,11,12,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-Octadecahydro-2H-Picen-3-one.**	+	-	-	-
29	**1-Hydroxypyrene**	+	-	-	-
30	**2-Ethylhexyl isohexyl ester**	+	-	+	+
31	**Neopentyl undecyl ester**	+	-	-	-
32	**2-Ethylhexyl hexyl ester**	+	-	+	-
33	**Beta sitosterol**	+	-	-	-
34	**Gamma sitosterol**	+	-	-	-
35	**17-(1,5-dimethylhexyl)-10,13-dimethyl-4-vinylhexadecahydrocyclopenta (a) phenanthren-3-ol.**	+	-	-	-
36	**Urs-12-ene**	+	-	-	-
37	**Benzo (b) naphtha (2,3-d) furan**	+	-	-	-
38	**Cholest-4-en-3-one**	+	-	-	-

39	14-methyl- Benzene methanol	+	-	-	-
40	4-hydroxy-4.alpha.	+	-	-	-
41	5-cyclo-A-homo-5.alpha	+	-	-	-
42	Cholestan-6-one	+	-	-	-
43	Ergost-5,8 (14)-dien-3-ol	+	-	-	-
44	4.alpha	+	-	-	-
45	5-cyclo-A-homo-5-alpha	+	-	-	-
46	2,3- Diaminophenol	+	-	-	-
47	Bicyclo(2.2.2) octane	+	-	-	-
48	2-methyl ergost-25-ene-3,5,6,12-tetrol	+	-	-	-
49	Beta 5.alpha,6.beta.,12.beta ergosta-5,7-dien-3-ol	+	-	-	-
50	Oxime	-	+	+	-
51	Methoxy-phenyl-2-amino-5-methylbenzoic acid	-	+	-	-
52	4-ethylbenzoic acid	-	+	-	-
53	2-methylbutyl ester	-	+	-	-
54	2(5H)- Furanone	-	+	+	-
55	2(3H)- Furanone	-	+	+	-
56	3- Hexane	-	+	-	-
57	4H- Pyran-4-one	-	+	-	-
58	2,3-dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	-	+	-	-
59	9-Eicosene	-	+	-	-

60	**Trichloroacetic acid**	-	+	-	-
61	**Dodecyl ester**	-	+	-	-
62	**Tridecyl ester**	-	+	-	-
63	**1-Octedecene**	-	+	-	-
64	**1-Docosene**	-	+	-	-
65	**9-Eicosene**	-	+	-	-
66	**Oxirane**	-	+	-	-
67	**Heptadecyl**	-	+	-	-
68	**Hexadecyl**	-	+	-	-
69	**Bicyclo (3.1.1) Heptane**	-	+	-	-
70	**2,6,6-Trimethyl-(1.alpha.,2.beta.,5.alpha)**	-	+	-	-
71	**Tetradecanoic acid**	-	+	-	-
72	**Cycloeicosane**	-	+	-	-
73	**17- Pentatriacotene**	-	+	-	-
74	**9,12- Octadecadienoic acid**	-	+	-	-
75	**10- Heneicosene**	-	+	-	-
76	**Cyclohexane**	-	+	-	+
77	**4-(4-ethylcyclohexyl)-1-Pentyl**	-	+	-	-
78	**Octadecane**	-	+	-	+
79	**1-(ethenyloxy)-11-Octadecenoic acid**	-	+	-	-
80	**2-Piperidinone**	-	+	-	-
81	**N-(4-bromo-n-butyl)- Octatriacontyl pentafluoropropionate 1-decanol**	-	+	-	-

82	**2-hexyl**	-	+	-	-
83	**Heptadecanoic acid**	-	+	-	+
84	**16-methyl ester**	-	+	-	+
85	**Triacontyl pentafluoropropionate Dotriacontyl pentafluoropropionate**	-	+	-	-
86	**Cis-13-Octadecanoic acid**	-	+	+	-
87	**9,17-Octadecadienal**	-	+	-	-
88	**(Z) Cis-Vaccenic acid**	-	+	+	-
89	**2-Piperidinone**	-	+	-	-
90	**N-(4-bromo-n-butyl)**	-	+	-	-
91	**1-Octylnonyl-1-bromo-3-(2-bromoethyl) Octane**	-	+	-	-
92	**6,11- Undecadiene**	-	+	-	-
93	**1-Acetoxy-3,7-dimethyl**	-	+	-	-
94	**2,6,10,14,18,22-Tetracosahexane**	-	+	-	-
95	**2,6,10,15,19,23-hexamethyl**	-	+	-	-
96	**Squalene**	-	+	-	-
97	**9-Methyl-Z-10-Tetradecen-1-ol**	-	+	-	-
98	**Acetate**	-	+	-	-
99	**Methanesulfonic acid**	-	+	-	-
100	**2,7-Dioxatricyclo (4.3.1.0(3,8)) Dec-5-yl ester 4-pyridinol**	-	+	-	-
101	**Cyclohexane ethanol**	-	+	-	-
102	**4-Methyl-beta-methylene**	-	+	-	-

103	**Trans-1H-Indene**	-	+	-	-
104	**1-(1,5-dimethyl 1-2-hexenyl)-Octahydro-7a-methyl**	-	+	-	-
105	**1,5,9- Decatriene**	-	+	-	-
106	**2,3,5,8- Tetrametthyl**	-	+	-	-
107	**2-amino-6-methylbenzoic acid**	-	-	+	-
108	**Methoxy- phenyl- 4- ethylbenzoic acid**	-	-	+	-
109	**2-butyl ester**	-	-	+	-
110	**2H-pyran**	-	-	+	-
111	**3,4-dihydro**	-	-	+	-
112	**4H- Pyran-4-one**	-	-	+	-
113	**2,3-dihydro-3,5-dihydroxy-6-methyl**	-	-	+	-
114	**Cyclododecane**	-	-	+	-
115	**1-Tetradecanol**	-	-	+	-
116	**4-Heptafluorobutyryl oxyhexadecane**	-	-	+	-
117	**Dodecyl acrylate**	-	-	+	-
118	**Bis(2-methylpropyl) ester**	-	-	+	+
119	**Isobutyl 2-Pentyl ester**	-	-	+	-
120	**Isohexyl propyl ester**	-	-	+	-
121	**8-Octadecenoic acid**	-	-	+	-
122	**Methyl-16-methyl-heptadecanoate**	-	-	+	-
123	**Oleic acid**	-	-	+	-
124	**Gamma Tocopherol**	-	-	+	-

125	Beta-Tocopherol	-	-	+	-
126	26-Nor-5-Cholesten-30beta.-ol-25-one	-	-	+	-
127	Cholesstane	-	-	+	-
128	4,5-epoxy	-	-	+	-
129	Cholest-5-en-3-ol	-	-	+	-
130	Cyclohexyl 2-pentyl ester	-	-	+	+
131	Vitamin E	-	-	+	-
132	Alpha Tocopherol	-	-	+	-
133	4,5,6,7- Tetrahydro-benzo© thiopene-1-carboxylic acid allylamide	-	-	+	-
134	Campesterol	-	-	+	-
135	Ergost-5-en-3-ol	-	-	+	-
136	D-Limonene	-	-	-	+
137	1-Methyl-4-(1-Methylethenyl0	-	-	-	+
138	2,5-Dihydroxypropiophenone trans	-	-	-	+
139	Trans-2-ethylbicyclo decane	-	-	-	+
140	4-amino-5-formamidomethyl-2-methyl pyrimidine	-	-	-	+
141	Benzo(b) tetrahydrofuran-3-one,5,6-dihydroxy ethyl vanillin	-	-	-	+
142	Butyl octyl ester	-	-	-	+
143	Isobutyl octyl ester	-	-	-	+
144	Buty-1,2-Methylpropyl ester	-	-	-	+

145	**Cyclohexylmethyl butyl ester**	-	-	-	+
146	**Pentanoic acid**	-	-	-	+
147	**10-Undecenyl ester**	-	-	-	+
148	**Myristic acid vinyl ester**	-	-	-	+
149	**Tritetracontane**	-	-	-	+
150	**2,3-Dihydroxypropyl ester**	-	-	-	+
151	**2-hydroxy-1-(hydroxymethyl) ethyl ester.**	-	-	-	+

CONCLUSIONS

From this study, lectin derived from all the strains of *S. commune* can be a good source for medicinal lectin investigation and the broth culture derived after six day fermentation can readily be used to combat cancerous cases such as that associated with Larynx.

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