Chemical Diversity and Cytotoxicity of *Ganoderma lucidum* from Two Different Localities of Pakistan

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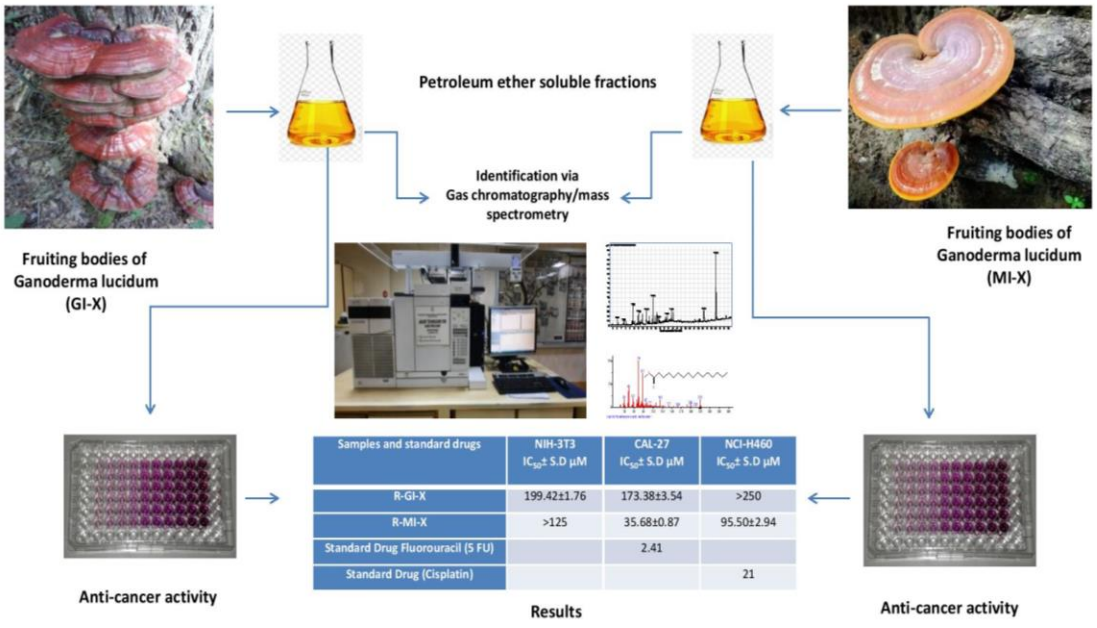
(Received on 15th July 2021, accepted in revised form 20th September 2021)

Summary: *Ganoderma lucidum* is a medicinal mushroom of family Ganodermataceae. It is found in Japan, China, Pakistan and some other parts of Asia. It is reported to exhibit numerous pharmacological properties. In Pakistan this mushroom inhabits areas with different soil and climate conditions which may affect its chemical composition and biological activity. In this context, this study was aimed to investigate the chemical constituents and cytotoxicity of wild growing *Ganoderma lucidum* from two different localities of Pakistan**,** Bhawalpur, Punjab (GI-X) and Nawabshah, Sindh (MI-X). These yielded an oily residue (R-GI-X; 2.3 g) and (R-MI-X; 20 g) on extraction with petroleum ether at room temperature. Their cytotoxicity was determined for oral adenosquamous carcinoma (CAL-27) and non-small-lung cancer (NCI-460) cell lines. Only R-MI-X showed toxicity for these cell lines and CAL-27 cells were found more sensitive (IC_{50} 35.68 µM) than NCI-H460 cells which were moderately sensitive (IC_{50} 95.50 µM). R-GI- X was not active for these cell lines. Fractions R-GI-X and R-MI-X contained 40 and 22 compounds respectively. Common compounds were mainly saturated and unsaturated fatty acids and their esters. A major difference was the presence of two ergosterols, 5, 6-dihydroergosterol and (20E)-3-(acetyloxy) ergosta-9(11),20(22)-dien-6-yl acetate in MI-X which may be responsible for its antiproliferative activity as many ergosterols are reported to possess anti-cancer activity against various cell lines.

Keywords: *Ganoderma lucidum; GC-MS; ergosterols; antiproliferative activity; CAL-27 and NCI-460 cell lines*



Samples and standard drugs	NIH-3T3 IC_{50}± S.D µM	CAL-27 IC_{50}± S.D µM	NCI-H460 IC_{50}± S.D µM
R-GI-X	199.42±1.76	173.38±3.54	>250
R-MI-X	>125	35.68±0.87	95.50±2.94
Standard Drug Fluorouracil (5 FU)		2.41	
Standard Drug (Cisplatin)			21

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Introduction

Ganoderma lucidum (family: Polyporaceae OR Ganodermataceae) grows commonly in East Asian countries. It is known as "lingzi" in Chinese and "Reishi" in Japanese and has been used in Chinese Traditional Medicine over the past two millennia [1]. It is a group of wood degrading mushrooms with hard fruiting bodies, existing in many colors like red, brown, blue and white. Its extracts and constituents have been reported to exhibit numerous pharmacological activities such as immunomodulatory, antihepatotoxic [1], anti-cancer [2], aldose-reductase inhibitory [3], anti-tumor, anti-inflammatory [4] and anti-microbial [5]. Triterpenoids and polysaccharides are major constituents reported while steroids, proteins, nucleotides, fatty acids, vitamins, and minerals comprise the minor mass of this medicinal mushroom [6]. A recent review compiled the pharmacologically active compounds of *G. lucidum* which possess anti cancer activity demonstrating that this mushroom has a potential to combat cancer as a natural alternative [7]. In Pakistan *G. lucidum* is known as "khumbi" and is naturally grown in different regions of Punjab and interior Sindh. The aim of this study was to find out the chemical diversity of the chemical constituents of wild growing *G.lucidum*from two different localities of Pakistan and evaluation of their cytotoxicity against two cancer cell lines CAL-27 and NCI-460.

Experimental

Materials

These studies were conducted at HEJ Research Institute of Chemistry, I.C.C.B.S, University of Karachi. Analytical grade solvents were purchased from Sigma Aldrich (Munich, Germany), Scharlau (Barcelona, Spain). Distilled solvents were used for solvent separation.The cancer cell lines were purchased from American Type Tissue Culture Collection (ATCC; USA).

Mushroom collection and extraction

Wild samples of *G. lucidum* were collected by Dr. Muhammad Ismail Bhatti from two different regions of Pakistan namely LalSuhanra National park, district Bahawalpur Punjab (labeled as GI-X) and PAE Forest near Sakrand city of Benazir Abad (Nawabshah) Sindh (labeled as MI-X) and were authenticated by the Mycology section of Botanical Sciences Division, Pakistan Museum of Natural History, Garden Avenue Shakarparian Islamabad Pakistan with Myco Accession No. 15525and 15711 respectively. Fruiting bodies were dried at room temperature and crushed to a raw powder to obtain GI-X (1kg) and MI-X (0.5 kg). Each of these was soaked in petroleum ether for three days at room temperature. The clear supernatant was decanted and the process was repeated three times. The combined extract of each was vacuum dried in a rot vapor. The residue from GI-X yielded an oily residue (R-GI-X; 2.3 g) while from MI-X a powdery residue (R-MI-X; 20 g) was obtained.

Gas chromatography-mass spectrometry

Analysis was performed on an Agilent 7890A instrument equipped with GC-MS mass-selective detector (Agilent 5975C with tri axial detector). The carrier gas was He at flow rate of 1 mL/min, OPTIMA 23102-72OPTIMA-5 (30m x 250µm x 0.25µm) capillary chromatography column, injection temperature 325℃ , column thermostat at 50 ℃ for 3 min then at 10 ℃/min to 200℃ for 15 min; 320℃ for 10 min with the run time of 70 min. The detector interface temperature was 250℃. The quadrupole mass selective detector scanned in the range 40-800 m/z. Constituents were identified by comparing mass spectra with NIST-electronic library [8] and retention indices of compounds were calculated by using Kovats equation [9], using standard mixture of *n*-alkane in the range of C_{12}–C_{60} (Sigma-Aldrich).A portion (2 mg each) of R-GI-X and R-MI-X was subjected to GC analysis on an Agilent 7890A instrument equipped with GC-MS mass-selective detector (Agilent 5975C with tri axial detector). The carrier gas was He at flow rate of 1 mL/min, OPTIMA 23102-72 OPTIMA-5 (30m x 250µm x 0.25µm) capillary chromatography column, injection temperature 325℃ , column thermostat at 50 ℃ for 3 min then at 10 ℃/min to 200℃ for 15 min; 320℃ for 10 min with the run time of 70 min. The detector interface temperature was 250℃. The quadrupole mass selective detector scanned in the range *m/z* 40-800. R-GI-X and R-MI-X contained 40 and 22 compounds respectively (Table-1). Common compounds were mainly saturated and unsaturated fatty acids and their esters. A major difference noted was the presence of two ergosterols, 5, 6-dihydroergosterol and (20E)-3-(acetyloxy) ergosta-9(11), 20 (22)-dien-6-yl acetate in R-MI-X in 15.39 and 21.68 % respectively.

Cytotoxicity

Both R-GI-X and R-MI-X were tested for cytotoxicity against human oral adenosquamous

carcinoma cell line CAL-27 and human non-small lung cancer (NCI-H460). The cytotoxicity was determined by using MTT assay. The cells were cultured in complete DMEM media containing 10% FBS and 1% of sodium pyruvate, amphotericin B, penicillin and streptomycin. Cisplatin (for NCI-H460 cells) and fluorouracil 5-FU (for CAL-27 cells) were used as standard drugs. The test fractions were dissolved in DMSO. Cells treated with DMSO only were also included in this study as a vehicle control.

3-(4, 5-Dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) Assay:

MTT assay was used to determine cytotoxicity of R-GI-X and R-MI-X and control drugs on CAL-27 and NCI-H460 cells, as well as on normal primary mouse embryonic fibroblast (NIH-3T3) cells. Briefly, for monolayer formation, almost 10^4cells/200 µL (of NCI-H460 or CAL-87 cells) were dispensed to each well of 96-wells plate and incubated for 24 h at 37ºC. Following incubation, media was aspirated. R-GI-X and R-MI-X were added in the wells having NCI-H460 and CAL-27 cells. For NCI-H460 cells, cisplatin was used as a drug control whereas, for CAL-27 cells fluorouracil5-FU was used as a standard drug. In all the wells, the DMSO concentration was maintained at ≤ 1%. The cells were then incubated for 24 h at 37ºC in 5% CO_2 incubator. After incubation, supernatant was aspirated and 200 µL of 5 mg/mL MTT dye was added to each well. Following 3 h incubation supernatant was removed and 100 µL of DMSO was added into each well. The plates were then kept for shaking on an orbital shaker for 10-20 min until formazan crystals were solubilize. The absorbance was then measured at 560 nm by using spectrophotometer and the IC_{50} values were calculated.

Results and discussion

The petroleum soluble fractions R-GI-X and R-MI-X obtained from two samples of *G. lucidum* collected from Punjab and Sindh regions of Pakistan as described in the Experimental were analyzed by GC-MS. These were found to contain 40 and 22 compounds respectively. The results showed that CAL-27 cells were more sensitive (IC_{50} 35.68 µM) to R-MI-X cells than NCI-H460 cells (IC_{50} 95.50 µM), and showed a higher IC_{50} (>125 µM) for the normal NIH-3T3 cells. On the other hand, the IC_{50} values of R-GI-X for CAL-27 and NCI-H460 cells were close to or greater than the IC_{50} dose that produced cytotoxicity on the normal NIH-3T3 cells. It was noted that saturated and unsaturated fatty acids and their esters were common in the two samples. However, a major difference noted in R-MI-X was the presence of two ergosterols, 5, 6-dihydroergosterol and (20E)-3-(acetyloxy) ergosta-9(11), 20(22)-dien-6-yl acetate in 15.39 and 21.68 % respectively. Their presence may be responsible for the better antiproliferative activity of R-MI-X as many ergosterols are reported in literature to possess anti-cancer activity against various cell lines [10].

Conclusion

The study manifests that geographical and environmental conditions play an important role in chemical composition and biological activity of natural products. Hence, R-MI-X demonstrated a potent inhibitory activity (IC_{50} 35.68 µM) for CAL-27 cells and moderate (IC_{50} 95.50 µM) activity against NCI-H460 cells (Table-2) and a higher IC_{50} (>125 µM) for the normal NIH-3T3 cells. On the other hand the IC_{50} values of R-GI-X for CAL-27 and NCI-H460 cells were close to or greater than the IC_{50} dose that produced cytotoxicity on the normal NIH-3T3 cells.

Table-1: Constituents of petroleum ether soluble fraction R-GI-X and R-MI-X.

S. No.	RT	Compounds	RI	R-GI-X (%)	R-MI-X (%)
1	20.91	Methyl tetradecanoate (Methyl myristate).($C_{15}H_{30}O_2$).	1709	1.11	-
2	21.89	Ethyl (2E)-3-(4-methoxyphenyl)-2-propenoate,($C_{12}H_{14}O_3$).	1761	0.35	-
3	22.38	Ethyl tetradecanoate (Ethyl myristate)($C_{16}H_{32}O_2$).	1791	0.65	-
4	23.21	Methyl pentadecanoate, ($C_{16}H_{32}O_2$).	1819	4.54	-
5	25.65	Methyl (9Z)-9-hexadecenoate (Methyl palmitoleate)($C_{17}H_{32}O_2$).	1890	2.68	-
6	26.85	Methyl hexadecanoate (Methyl palmitate)($C_{17}H_{34}O_2$).	1925	19.9	1.19
7	29.07	Ethyl hexadecanoate (Ethyl palmitate)($C_{18}H_{36}O_2$).	1989	14.08	6.09
8	29.49	Methyl 14-methylhexadecanoate($C_{18}H_{36}O_2$).	2001	2.18	-
9	30.57	Methyl heptadecanoate (Methyl margarate)($C_{18}H_{36}O_2$).	2028	3.03	-
10	34.53	Methyl 9,12-Octadecadienoate.	2128	1.59	0.69
11	34.84	Methyl 11-Octadecenoate, ($C_{19}H_{34}O_2$).	2136	14.12	-
12	35.43	Stearic Acid,($C_{18}H_{36}O_2$).	2151	0.31	0.49
13	36.45	Ethyl linoleate,($C_{20}H_{36}O_2$).	2177	2.37	-
14	36.65	Ethyl 9(Z)-Octadecenoate (Ethyl oleate.)($C_{20}H_{38}O_2$).	2182	11.06	6.69
15	37.22	Ethyl Octadecanoate (Ethyl Stearate)($C_{20}H_{40}O_2$).	2190	5.84	3.33

16	38.28	Methyl 6,9,12-Octadecatrienoate,($C_{19}H_{32}O_2$).	2256	0.06	-
17	38.50	Methyl 2-hydroxyoctadecanoate, ($C_{19}H_{38}O_3$).	2270	0.37	-
18	38.62	n-Propyl 9,12-octadecadienoate (n-Propyl linoleate)($C_{21}H_{38}O_2$).	2278	0.07	1.33
19	38.71	*n*-Propyl 9-octadecenoate,($C_{21}H_{40}O_2$).	2283	0.36	-
20	38.88	3-Octyl-oxiraneoctanoic acid, *cis*-($C_{18}H_{34}O_3$).	2293	0.15	-
21	38.99	Methyl (7E,10E,13E)-7,10,13-icosatrienoate,($C_{21}H_{36}O_2$).	2300	0.22	-
22	39.09	Isobutyl stearate,($C_{22}H_{44}O_2$).	2307	0.19	-
23	39.22	Methyl (10E,13E)-10,13-icosadienoate,($C_{21}H_{38}O_2$).	2315	0.14	-
24	39.30	*cis*-13-Eicosenoic acid,($C_{20}H_{38}O_2$).	2320	0.27	-
25	39.68	Methyl (10E)-14-oxo-10-nonadecenoate,($C_{20}H_{36}O_3$).	2343	0.42	-
26	40.54	7-Methyl-Z-tetradecen-1-ol acetate,($C_{17}H_{32}O_2$).	2393	-	0.24
27	40.55	Ethyl eicosanoate,($C_{22}H_{44}O_2$).	2393	0.82	-
28	40.90	*i*-Propyl 9,12-octadecenadienoate, ($C_{21}H_{38}O_2$).	2428	0.44	-
29	41.32	Cyclohexylhexadecanoate, ($C_{22}H_{42}O_2$). Cyclohexylpalmitate.	2467	-	2.83
30	41.57	Oxiraneoctanoic acid, 3-octyl-, *cis*- (Epoxyoleic acid)($C_{18}H_{34}O_3$).	2489	0.11	-
31	41.92	Glycerol 1-monopalmitate.	2521	0.09	0.34
32	42.06	Methyl docosanoate (Methyl behenate)($C_{23}H_{46}O_2$).	2534	0.11	-
33	42.70	Ethyl docosanoate (Ethyl behenate)($C_{24}H_{48}O_2$).	2593	0.21	-
34	42.73	*Z*-5-Methyl-6-heneicosen-11-one,($C_{22}H_{42}O$).	2595	-	0.22
35	42.97	3,7,11-Trihydroxypregnan-20-one.	2622	0.14	2.71
36	43.04	Methyl 21-methyldocosanoate, (Methyl tricosanoate)($C_{24}H_{48}O_2$).	2630	0.08	-
37	43.08	2-[(2E)-5-(2,2-Dimethyl-6-methylenecyclohexyl)-3-methyl-2-pentenyl]benzo-1,4-quinone,($C_{21}H_{28}O_2$).	2635	-	2.55
38	43.22	2,3-Dihydroxypropyl elaidate (Monoelaiden)($C_{21}H_{40}O_4$).	2651	-	1.31
39	43.42	Decyloctadecanoate (Decyl stearate)($C_{28}H_{56}O_2$).	2676	Unidentified	0.8
40	43.64	Heptacosane,($C_{27}H_{56}$).	2699	-	0.58
41	43.88	Glycerol 2-monooleate.	2727	2.86	-
42	44.46	Methyl tetracosanoate,($C_{25}H_{50}O_2$).	2793	-	2.55
43	45.26	Nonacosane,($C_{29}H_{60}$).	2893	-	3.16
44	46.45	9(11)-Dehydroergosteryl benzoate,($C_{35}H_{46}O_2$).	3031	0.41	-
45	46.80	3*α*,5*α*-Cyclo-ergosta-7,9(11),22t-triene-6*β*-ol, ($C_{28}H_{42}O$).	3096	-	1.43
46	47.77	Stigmasterol,($C_{29}H_{48}O$).	3167	-	1.28
47	49.18	(20E)-3-(Acetyloxy)ergosta-9(11),20(22)-dien-6-yl acetate.	3235	1.50	21.68
48	49.43	Ergosta-5,24(28)-dien-3-ol, (3*β*)-($C_{28}H_{46}O$).	3243	1.38	-
49	49.58	5,6-Dihydroergosterol.	3296	-	15.39
50	49.82	4,22-Cholestadien-3-one,($C_{27}H_{42}O$).	3312	0.71	-
51	50.19	*α*-Ergostenol,($C_{28}H_{48}O$).	3336	0.32	-
52	51.78	Ergosta-4,6,8(14),22-tetraen-3-one,($C_{28}H_{40}O$).	3439	0.55	

RT: Retention time; RI: Retention Index

Table-2: IC_{50} of R-GI-X and R-MI-X on CAL-27 and NCI-460 cell lines.

Samples and standard drugs	NIH-3T3 IC_{50}± S.D µM	CAL-27 IC_{50}± S.D µM	NCI-H460 IC_{50}± S.D µM
R-GI-X	199.42±1.76	173.38±3.54	>250
R-MI-X	>125	35.68±0.87	95.50±2.94
Standard Drug Fluorouracil (5 FU)	-	2.41	-
Standard Drug (Cisplatin)	-	-	21

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