Cytotoxicity of some edible mushrooms extracts over liver hepatocellular carcinoma cells in conjunction with their antioxidant and antibacterial properties

Gökhan Sadi, Buğrahan Emsen, Abdullah Kaya, Aytaç Kocabaş, Seval Çınar, Deniz İrtem Kartal[1]

Department of Biology, Kamil Özdağ Faculty of Science, Karamanoğlu Mehmetbey University, Karaman, [1]Department of Biology, Biochemistry Graduate Programme, Middle East Technical University, Ankara, Turkey

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ABSTRACT

Background: Mushrooms have been valued for their nutritive content and as traditional medicines; several important medicinal properties of mushrooms have been recognized worldwide. **Objective:** The purpose of this study was to elucidate the cell growth inhibitory potential of four edible mushrooms; *Coprinus comatus* (O.F. Mull.) Pers. (Agaricaceae), *Tricholoma fracticum* (Britzelm.) Kreisel (Tricholomataceae), *Rhizopogon luteolus* Fr. and Nordholm (Rhizopogonaceae), *Lentinus tigrinus* (Bull.) Fr. (Polyporaceae) on hepatocellular carcinoma (HepG2) cells in conjunction with their antioxidant and antibacterial capacities. **Materials and Methods:** Five different extracts of edible mushrooms were obtained using water, methanol, acetone, *n*-hexane and chloroform as solvent systems for cytotoxic, antioxidant and antibacterial properties. **Results:** *C. comatus* showed substantial *in vitro* cytotoxic activity against HepG2 cell lines with all extracts especially with chloroform 50% inhibition (IC_{50} value of 0.086 mg/ml) and acetone (IC_{50} value of 0.420 mg/ml). Chloroform extract of *C. comatus* had maximum amount of β-carotene (25.94 µg/mg), total phenolic content (76.32 µg/mg) and lycopene (12.00 µg/mg), and *n*-hexane extract of *L. tigrinus* had maximum amount of flavonoid (3.67 µg/mg). While chloroform extract of *C. comatus* showed the highest 2,2-diphenyl-1-picrylhydrazyl (DPPH) capturing activity (1.579 mg/ml), the best result for metal chelating activity was obtained from methanolic extract (0.842 mg/ml). Moreover, all tested mushrooms demonstrated antibacterial activity and *n*-hexane extract of *L. tigrinus* and acetone extracts of *T. fracticum* were the most active against tested microorganism. **Conclusion:** These results indicate that different extracts of investigated mushroom have considerable cytotoxic, antioxidant and antibacterial properties and may be utilized as a promising source of therapeutics.

Key words: Cancer cells, cell toxicity, edible macrofungi, radical scavenging

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INTRODUCTION

Wild edible mushrooms have been consumed in fresh or dried forms by human for a long period due to its organoleptic properties such as flavor and texture.[1-3] Although thousands of mushrooms are present in nature, few of them are accepted as edible and commercial.[4,5] Studies on nutritional values of mushrooms showed richness in protein, dietary fibers, vitamins and minerals along with low fat and energy[2,3,6,7] enables to use as edible. Local consumption of wild mushrooms are also increasing because of their medicinal properties[4,6] due to the presence of secondary metabolites having pharmaceutical importance.[1,6]

Functional foods are new emerging area of food science and defined as food, which exerts health improving effects other than its nutritional value. Whereas, nutraceuticals are defined as concentrated phytochemicals derived from food and applied as a supplement.[8] Mushrooms are rich in nutraceuticals having antioxidant, antitumor and antimicrobial features.[1,4,9,10] There has been also a continuous search for new antimicrobial substances for decades and mushrooms are of interest for several researchers. Albeit nearly 60 antimicrobial compounds have been isolated from mushrooms; only the compounds from microscopic fungi have been present in the market as antibiotics until now.[11]

Address for correspondence:
Dr. Gökhan Sadi, Department of Biology, Kamil Özdağ Faculty of Science, Karamanoğlu Mehmetbey University, 70100, Karaman, Turkey.
E-mail: sadi@kmu.edu.tr

Antioxidants protect the living systems from free radicals, which results in oxidative damage of protein and nucleic acids. Even though humans are well protected against free radicals, many diseases such as cancer may result from the uncontrolled production of reactive oxygen species.[12] Therefore, antioxidant supplementation either as foods or drugs helps people to reduce the risk resulted from oxidative stress. Drugs of herbal origin have been used in traditional systems of medicine since ancient times, and many researchers have benefited from medicinal plants to prevent oxidative stress.[13-17]

Although, antioxidant capacities of mushrooms are well-established, anticancerogenic properties of wild edible mushrooms in clinical studies[8] is of immense important due to very few reports. Therefore, this study focused on analysis of bioactive feature of some wild edible mushrooms. In this concern, we elucidated the cell growth inhibitory potential of four edible mushrooms; *Coprinus comatus* (O.F. Mull.) Pers. (Agaricaceae), *Tricholoma fracticum* (Britzelm.) Kreisel (Tricholomataceae), *Rhizopogon luteolus* Fr. and Nordholm (Rhizopogonaceae), *Lentinus tigrinus* (Bull.) Fr. (Polyporaceae) on liver hepatocellular carcinoma (HepG2) cells in conjunction with their antioxidant and antibacterial capacities.

MATERIALS AND METHODS

Collection and identification of mushroom samples

Samples were collected in 2012 from different localities within Karaman province of Central Anatolian Region of Turkey. Necessary morphological and ecological characteristics of the samples were recorded, and they were photographed in their natural habitats. Then they were brought to the fungarium and microstructural properties were investigated. Comparing the obtained macroscopic and microscopic data with literature,[18] they were identified as *T. fracticum* (Kaya 7482), *L. tigrinus* (Kaya 7454), *C. comatus* (Kaya 7483) and *R. luteolus* (Kaya 7455). The samples are kept at Karamanoğlu Mehmetbey University, Science Faculty, Department of Biology.

Extraction of bioactive ingredients

After drying under mild heat evaporator, entire mushroom samples (cap and stripe) were powdered with liquid nitrogen, mortar and paste. Then bioactive ingredients were extracted by 250 ml of different solvent systems such as, water, methanol, chloroform, acetone and *n*-hexane using Soxhlet extraction apparatus throughout 2 days. After extraction, solvents were evaporated with rotary evaporator (IKA, Staufen Germany) under vacuum to dryness and lyophilized to get ultra dry powders that were solubilized with minimum amount of sterile distilled water. They were diluted to different concentrations according to experiments before the experimental setup.

Determination of cell growth inhibitory potential of mushrooms on hepatocellular carcinoma cells

Cell viability was quantified using 2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide (XTT) *in vitro* cellular toxicity assay.[19] Accordingly, HepG2 cells were cultured in RPMI 1640 medium with L-glutamine and 25 mM HEPES (Lonza, USA) supplemented with 10% fetal bovine serum and 0.2% gentamycin sulfate. They were grown in a humidified incubator at 37°C with 5% CO_2. After growing to expected confluency (90%), they were harvested by 2 ml 0.05% trypsin/ethylenediaminetetraacetic acid (EDTA), complete detachment was obtained by incubation at 37°C for 5 min. Later, trypsin activity was brought to end by the addition of 5 ml growth medium. Cell suspension was counted with trypan blue dye and cell concentration was adjusted to 100,000 cells/ml. In sterile microtiter culture disc, 50 µl HepG2 cell in fresh complete medium and 50 µl extracts in different concentration (0.5–10 mg/ml) were mixed and incubated for 48 h or 72 h at 37°C in 5% CO_2.

Cell growth inhibitory potential of the mushrooms on HepG2 cells was studied using commercial XTT cell proliferation kit (Biological Industries, Israel). 100 µl of activation solution (PMS-N methyl dibenzopyrazine methyl sulfate) was added to 5 ml XTT reagent (supplied with kit) and mixed. Then, 50 µl of this solution was added to each well containing cells and extracts and incubated for 10 h with activated XTT under 5% CO_2 at 37°C in a humidified incubator. Finally, the intensity of the formazan was measured at 415 nm with Multiscan Go microplate reader (Thermo Scientific, USA), and 50% inhibition (IC_{50}) values were calculated.

Determination of antioxidant potentials

Possible antioxidant potential of different extracts of tested mushrooms were evaluated by measuring their total flavanoids, β-carotene and lycopene amount and phenolic contents as well as reducing power, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and metal chelating activity. The methods used to evaluate these indicators of antioxidative capacity are given in the following parts.

Determination of total phenolic contents

Concentration of total phenolic compounds in different extracts of *T. fracticum, L. tigrinus, C. comatus* and *R. luteolus* was determined spectrophotometrically according to the method described previously by[20] with slight modifications. In this method, gallic acid with various concentrations (0.01–1.0 mM) was used as a standard

phenolic compound. This method was adapted to microtiter plate measurements in triplicate. A volume of 20 µl of standards or different extracts (10 mg/ml) were mixed with the same amount of Folin and Ciocalteu's phenol reagent (2N) and kept in dark for 3 min. Afterward, 20 µl of 35% sodium carbonate (w/v) and 140 µl dH_2O were added to start incubation period for 10 min. After that, the absorbance was measured at 725 nm and the results were calculated as mean ± standard error of mean (SEM) from gallic acid calibration curve and expressed as mg of gallic acid equivalents/mg of extracts.

Determination of total flavonoid contents

Total flavonoids of water, methanol, chloroform, acetone and *n*-hexane extracts obtained from edible mushrooms were determined using protocols reported by[21] with slight modifications. A volume of 50 µl of extracts (10 mg/ml) were mixed with 215 µl of ethyl alcohol (80% v/v), 5 µl of aluminum nitrate (10% w/v) and 5 µl potassium acetate (1 M) in microtiter plates and incubated for 40 min at room temperature. After reading at 415 nm, total flavonoid contents were calculated according to following equation:

$$\text{Total flavonoid contents (µg/mg extract)} = (A_{415} + 0.01089)/0.002108$$

Determination of β-carotene and lycopene contents

Different extracts with water, methanol, *n*-hexane, acetone and chloroform that were obtained from four edible mushroom was re-extracted with 10 ml of acetone: hexane (4:6) mixture and filtered through Whatman No. 4 filter paper in order to determine β-carotene and lycopene contents. After filtration, absorbance of the filtrates was measured at 453, 505 and 663 nm. β-carotene and lycopene contents were determined according to following equations.[21]

$$\text{β-carotene content (mg/100 mg)} = 0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$$

$$\text{Lycopene content (mg/100 mg)} = -0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$$

DPPH radical scavenging activity

Free radical scavenging activities (RSA) of extracts obtained from *T. fracticum, L. tigrinus, C. comatus* and *R. luteolus* were determined by monitoring DPPH reduction. Gallic acid (0.01–0.5 mM) was used as standard antioxidant molecule and to determine RSA, 20 µl of various concentrations of standard or extracts were mixed with 180 µl of DPPH solution (0.06 mM in methanol) and incubated for 1 h at dark in microtiter plates. Blank measurements without standard or extracts were also performed. After reading absorbance at 517 nm, reduction of the DPPH radical was determined as percent discoloration of DPPH which was calculated according to following formula;

$$\text{Radical Scavenging Activity (RSA) (\%)} = 100 \times \frac{\text{DPPH absorbance - DPPH and extract absorbance}}{\text{DPPH absorbance}}$$

The extract concentrations providing the IC_{50} was calculated from the graph of RSA versus extract amount that was used for the comparison of different extracts of tested mushrooms.[22]

Determination of metal chelating activity

Chelating abilities of various extracts from edible mushrooms were determined using EDTA as a standard chelating agent.[23] Different concentrations (50 µl) of extracts (2, 4, 6, 8, 10 mg/ml) and standard (0.1–5 mM) were added to microplate wells and mixed with 10 µl ferrozine (5 mM), 5 µl iron (II) chloride (2 mM) and 185 µl absolute methanol. After incubation for 10 min at room temperature, absorbances were read at 562 nm, and IC_{50} values were calculated.

Determination of reducing power

Reducing powers of different extracts were determined according to the method[24] with an adaptation to microplate measurement. Gallic acid (0.01–0.1 mM) was used as a standard antioxidant. In this method, various concentrations of 50 µl mushroom extracts (2, 4, 6, 8, 10 mg/ml) were mixed with 75 µl phosphate buffer (0.2 M pH: 6.6) and 75 µl potassium ferricyanide (1% w/v) in a total volume of 200 µl and incubated at 50°C for 20 min. After adding 75 µl trichloroacetic acid (10% w/v), samples were centrifuged for 10 min at 1000 × *g*. Supernatant (75 µl) were transferred to another microtiter plate and mixed with 75 µl distilled water and 15 µl iron (III) chloride (0.1% w/v). After reading the absorbance at 700 nm, effective concentrations (EC_{50}) at which the absorbance was 0.5 for reducing power was calculated.

Determination of antibacterial activities

The antibacterial activities of different extracts of four edible mushrooms (40 µg/µl) were determined against *Bacillus subtilis* (Ehrenberg) Cohn, *Bacillus licheniformis, Staphylococcus aureus* Rosenbach, *Escherichia coli* T. Escherich and *Agrobacterium tumefaciens* Smith and Townsend by the disc diffusion susceptibility method.[25] Penicillin, gentamicin and tetracycline were used as the standard antibiotic discs. Microorganisms were grown in Müller Hilton broth at 35°C and 28°C for 18 h and then cell density were adjusted according to 0.5 McFarland standards. After that, 100 µl of adjusted microorganism suspension was inoculated onto Müller Hilton Agar by spread plate

technique. A volume of 40 µl of extract (40 µg/µL) loaded discs and standard antibiotic discs were placed on inoculated Petri dishes. Negative controls were prepared with water alone. Inhibition zones (IZ) around the discs after overnight incubation were measured to determine antibacterial activities of the extracts. The study was performed in triplicates of each sample.

Statistical analyses

All the assays were carried out at least in triplicate measurements. The results are expressed as mean values and SEM. Antioxidant, antibacterial and cytotoxicity activities of mushrooms were compared with Analysis of Variance (ANOVA) test followed by the appropriate *post-hoc* test (Duncan Test) and values with $P < 0.05$ were considered as significantly different. IC_{50} and EC_{50} values were calculated with Probit Regression Analysis and associated 95% confidence limits for each treatment. Relations among the variables were tested by Bivariate Correlation Analysis. These calculations were carried out using Statistical Package for Social Sciences (SPSS®, version 21.0, IBM Corporation, Armonk, NY, USA).

RESULTS AND DISCUSSION

Naturally occurring substances present in edible mushrooms have beneficial effects on health. Several species of mushroom show free RSA and high growth inhibitory potential against cancer cells and microorganisms[23,26-33] that's why, mushrooms have recently become attractive as nutritionally beneficial foods and as a source material for the development of some drugs. This study presents the comparison of four different edible mushrooms; *T. fracticum, L. tigrinus, C. comatus* and *R. luteolus* which are consumed locally in different regions of Turkey in terms of their important medicinal properties such as cytotoxic, antioxidant and antibacterial effects.

Cell growth inhibitory potential of mushrooms on liver hepatocellular carcinoma cells

Liver tissue is involved in the metabolism of various nutrients including edible mushrooms and their digestive products in the form of glucose, amino acids and fatty acids. Furthermore, it contains thousands of enzymes essential to perform vital metabolic functions to metabolize both beneficial and harmful substances and store some of the compounds having antioxidant properties.[26,34,35]

Cancer diseases are one of the main causes of death worldwide and hepatocellular carcinoma (also called malignant hepatoma) is the most common type of liver cancer.[26] Liver HepG2 cells are the model cell lines to study liver functions *in vitro* since they maintain several liver properties. Our study reports on the investigation into the cytotoxic activity of different extracts of four edible mushrooms on HepG2 cells that have not been reported previously.

To study the growth inhibitory activity of the four edible mushrooms *in vitro*, HepG2 cell lines were incubated with various concentrations of four edible mushrooms obtained from different solvent extraction. The potentially cytotoxic effect of the mushroom extracts was assessed using *in vitro* XTT assay. Findings from our study clearly demonstrated that elevated concentrations of mushroom extracts are cytotoxic to human liver carcinoma (HepG2) cells. In particular, *C. comatus* displayed the strongest growth inhibitory potential on HepG2 cells. However, all extracts of *C. comatus* are more effective anticancer agent than other mushrooms tested with very low IC_{50} values (0.086 mg/ml for chloroform and 0.42 mg/ml for acetone extracts). In addition to *C. comatus, L. tigrinus* also possess high degree of growth inhibitory potential over HepG2 cells for all extracts (IC_{50} value of 0.376 mg/ml for acetone, 0.434 mg/ml for *n*-hexane and 2.854 mg/ml for chloroform). In contrast, *T. fracticum* was the least effective cytotoxic material among the all tested mushroom since nearly tenfold higher amount of extract was needed to obtain IC_{50} values as compared with the most effective *C. comatus*. IC_{50} value of acetone extract was 3.851 mg/ml, 5.164 mg/ml for chloroform and 51.982 mg/ml for water extract of *T. fracticum* that was significantly higher than those of other mushrooms. Cytotoxic activities of all tested extracts were summarized in Table 1.

Antioxidant components

Substances that can inhibit or retard oxidation of substances are called antioxidants and recently there has been increasing interest in discovering natural antioxidants, especially those of macrofungal origin. Various macrofungi species, plants, lichens and algae have antioxidant constituents which are important materials due to the fact that these components have anti-inflammatory, antiallergic, antiviral, anti-aging, anticarcinogenic properties and behave like biological response modifiers.[36-39] Therefore, they are used in medicinal and pharmaceutical fields by many researchers. The present study was undertaken with the aim to carry out a systematic comparative antioxidant study on different extracts gained from four edible mushrooms using different *in vitro* methods, and to find any correlation between the antioxidant activity and bioactive ingredients such as total phenolic, total flavonoid, lycopene and β-carotene contents of the extracts. To evaluate the total antioxidant properties of four edible mushrooms, three different assays were carried out namely; scavenging activity on DPPH radicals, reducing power and metal chelating activity.

Table 1: 48 and 72 h IC_{50} values (mg/ml) of different extracts obtained from four mushrooms for cytotoxicity test

Treatment	Species	48 h IC_{50} (limits)	72 h IC_{50} (limits)
Acetone[a]	*T. fracticum*	3.851 (3.529-4.194) K[b]	3.079 (2.827-3.331) M[b]
	L. tigrinus	0.376 (0.093-0.650) B	0.362 (0.100-0.610) D
	C. comatus	0.420 (0.143-0.692) C	0.307 (0.082-0.544) B
	R. luteolus	1.539 (1.289-1.763) F	1.410 (1.177-1.618) G
Chloroform[a]	*T. fracticum*	5.164 (4.617-5.827) O	3.675 (3.324-4.052) O
	L. tigrinus	2.854 (1.398-60.414) I	2.576 (1.329-34.778) L
	C. comatus	0.086 (0.015-0.149) A	0.078 (0.009-0.141) A
	R. luteolus	2.167 (1.843-2.491) H	1.824 (1.518-2.106) H
n-Hexane[a]	*T. fracticum*	16.548 (4.347-4656.340) S	11.962 (3.729-570.714) S
	L. tigrinus	0.434 (0.400-0.471) D	0.322 (0.301-0.341) C
	C. comatus	0.864 (0.721-1.188) E	0.523 (0.481-0.576) E
	R. luteolus	1.604 (1.437-1.762) G	1.327 (1.166-1.474) F
Methanol[a]	*T. fracticum*	11.096 (9.743-13.133) Q	8.786 (7.516-10.629) Q
	L. tigrinus	7.446 (6.607-8.607) P	5.046 (4.613-5.558) P
	C. comatus	3.899 (3.590-4.238) L	3.217 (2.921-3.541) N
	R. luteolus	4.319 (3.992-4.684) N	2.445 (2.222-2.677) K
Water[a]	*T. fracticum*	51.982 (22.611-548.396) T	44.063 (20.480-330.053) T
	L. tigrinus	3.902 (3.356-4.584) M	2.201 (1.947-2.469) I
	C. comatus	3.404 (2.960-3.969) J	2.339 (2.041-2.669) J
	R. luteolus	12.641 (10.116-17.344) R	11.519 (9.058-16.161) R

[a]Extract; [b]Values followed by different capital letters in the same column differ significantly at $P<0.05$. *T. fracticum: Tricholoma fracticum, L. tigrinus: Lentinus tigrinus, C. comatus: Coprinus comatus, R. luteolus: Rhizopogon luteolus*, IC_{50}: 50% inhibition

Scavenging ability on DPPH radicals

The model of scavenging DPPH radical is a widely used method to evaluate the free RSA of antioxidants. DPPH is a stable free radical, and when antioxidants react with DPPH, unpaired electron is paired off and the DPPH solution is decolorized. Therefore, decolorization degree demonstrates the radical scavenging activity of an antioxidant containing materials.

Previous studies showed that extracts or chemical constituents obtained from various mushrooms possess DPPH scavenging activities due to their antioxidant compounds. Studies carried out with *Laetiporus sulphureus*;[40] *Pleurotus ostreatus*;[31,41] *Lentinus edodes*, *Sparassis crispa* and *Mycoleptodonoides aitchisonii*;[42,43] *Stereum hirsutum*;[44] *Russula virescens*;[45] *Pleurotus eryngii*;[46] *Macrolepiota procera*;[27,47] *Pleurotus flabellatus*;[38] *Russula laurocerasi*;[48] *Suillus bovinus*;[49] *Phellinus fastuosus*, *Phellinus grenadensis*, *Phellinus merrillii* and *Phellinus badius*;[50] *Lenzites betulina*;[51] *Lactarius piperatus*;[52] *Cordyceps militaris*;[53] *Tricholoma portentosum* and *Lactarius deliciosus*;[54] *Pholiota nameko*[55] revealed the high DPPH scavenging activities of these fungi correlated with their antioxidant compounds.

In the present study, all the mushroom species showed DPPH free RSA that were found to rise with increasing concentration. Maximum concentrations (10 mg/ml) of methanol extracts from *R. luteolus* and *T. fracticum* and chloroform extract from *C. comatus* showed DPPH scavenging activities over 70% (71.38, 71.19 and 70.28%, respectively). Among all the mushroom extracts, highest activity was obtained from the lowest concentration (2 mg/ml) of chloroform extract from *C. comatus* (53.56%). Comparing activity rates of the extracts of four mushrooms, chloroform extracts gave best results and all activity rates were over 60%. The scavenging effects of four mushrooms (in chloroform extract) on DPPH radical were in the descending order of *C. comatus* >*T. fracticum* >*L. tigrinus* >*R. luteolus*. The lowest activity values belonged to acetone extract. In acetone extract, DPPH scavenging activity rates of four mushrooms ranged from 13.67% to 40.08% for maximum concentration (10 mg/ml) and was in the order of *L. tigrinus* <*T. fracticum* <*C. comatus* <*R. luteolus* [Figure 1]. The chloroform extract from *C. comatus* showed strong DPPH radical scavenger that was reflected by its low IC_{50} value (1.579 mg/ml). Other extracts that had low IC_{50} values (under 3 mg/ml) are methanol and chloroform extract from *R. luteolus* (2.398 and 2.518 mg/ml) and methanol extract from *C. comatus* (2.566 mg/ml). IC_{50} value of standard (gallic acid) was found as 0.037 mg/ml and there was statistically ($P < 0.05$) significant difference between this value and the values belonging to all mushroom extracts [Table 2].

Chelating abilities of metal ions

The presence of metal ions; especially Fe^{2+} lead the production of reactive species such as hydroxyl radicals and metal-oxygen complex in biological systems and cellular

Table 2: IC_{50} values (mg/ml) of different extracts from four mushrooms and standard for scavenging activity on DPPH radicals

Treatment	Species	IC_{50} (limits)	Slope±SEM
Acetone[a]	*T. fracticum*	44.122 (27.256-106.624) Q[c]	1.214±0.187
	L. tigrinus	68.591 (36.524-249.140) R	1.359±0.242
	C. comatus	26.226 (18.622-46.629) P	1.195±0.163
	R. luteolus	17.089 (13.072-26.471) N	1.087±0.147
Chloroform[a]	*T. fracticum*	4.731 (4.094-5.406) I	1.114±0.134
	L. tigrinus	4.472 (3.693-5.267) H	0.902±0.133
	C. comatus	1.579 (0.568-2.415) B	0.581±0.133
	R. luteolus	2.518 (1.203-3.490) D	0.549±0.132
n-Hexane[a]	*T. fracticum*	128.817 (37.953-16039.858) U	0.471±0.145
	L. tigrinus	85.547 (31.499-2261.451) T	0.524±0.144
	C. comatus	76.708 (29.311-1778.957) S	0.521±0.143
	R. luteolus	15.857 (12.214-24.349) M	1.045±0.145
Methanol[a]	*T. fracticum*	3.257 (2.642-3.800) G	1.095±0.134
	L. tigrinus	7.451 (5.669-12.070) K	0.542±0.132
	C. comatus	2.566 (1.734-3.242) E	0.815±0.133
	R. luteolus	2.398 (1.571-3.070) C	0.810±0.133
Water[a]	*T. fracticum*	10.161 (8.152-14.595) L	0.846±0.136
	L. tigrinus	22.638 (13.523-87.929) O	0.566±0.137
	C. comatus	6.072 (5.121-7.409) J	0.866±0.134
	R. luteolus	3.193 (0.817-4.783) F	0.366±0.131
Gallic acid[b]	-	0.037 (0.026-0.048) A	0.601±0.053

[a]Extract, [b]Standard solution, [c]Values followed by different capital letters in the same column differ significantly at $P<0.05$. *T. fracticum: Tricholoma fracticum, L. tigrinus: Lentinus tigrinus, C. comatus: Coprinus comatus, R. luteolus: Rhizopogon luteolus*, SEM: Standard error of mean, DPPH: 2,2-diphenyl-1-picrylhydrazyl, IC_{50}: 50% inhibition

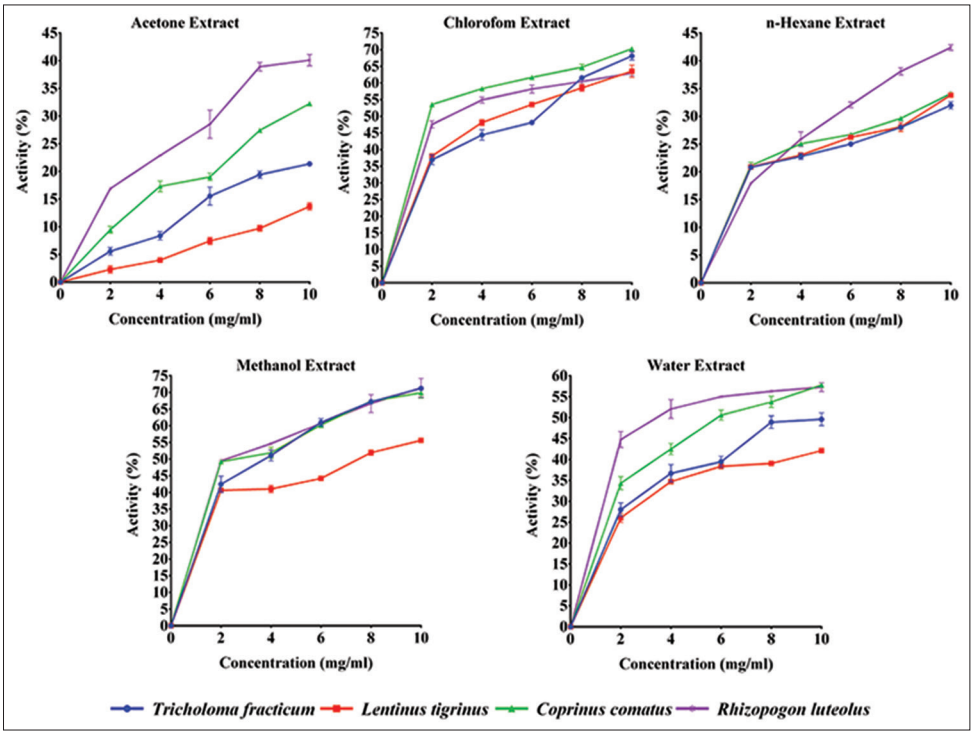

Figure 1: Scavenging abilities of extracts in different concentrations from four mushrooms on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals

damage eventually occurs with metal catalyzed reactions.[56] To prevent oxidative stress caused by metal ions, edible mushrooms that have metal chelating abilities are very important in foods. In the determination of metal chelating activities, extracts of the mushroom species was used to suppress the formation of ferrous and ferrozine complex,

indicating their chelating activity and capture ferrous ion before the production of ferrozine in the system.

Many researchers have previously examined chelating activities of different extracts of some mushroom species and chemical constituents of different fungi in their studies and achieved positive results.[38,40,45,46,57-62]

The present study has also been focused on metal chelating activities of different extracts from *T. fracticum*, *L. tigrinus*, *C. comatus* and *R. luteolus*. Correlations between chelating capacities of various extracts of four mushrooms and their concentrations were observed. Methanol extracts at concentrations of 8 and 10 mg/ml and chloroform extract at concentration of 10 mg/ml of of *C. comatus* displayed over 70% metal chelating activity (70.17, 78.01 and 70.52%, respectively). Methanol extract gave higher results compared to other extracts. For the chelating effect of ferrous ion, four mushrooms in methanol extract at concentration of 10 mg/ml were effective in the following order: *C. comatus* >*R. luteolus* >*T. fracticum* >*L. tigrinus* [Figure 2].

As shown in Table 3, for chelating on ferrous ions, highly potent inhibitions were found by methanol extract from *C. comatus* (IC_{50} = 0.842 mg/ml), methanol extract from *R. luteolus*, (IC_{50} = 2.397 mg/ml) and chloroform extract from *C. comatus* (IC_{50} = 5.650 mg/ml) when compared with standard (EDTA) inhibitor (IC_{50} = 0.158 mg/ml) [Table 3].

Determination of reducing power

The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity.[63] In the used protocol, an increase in the color of the test solution depends on the reducing power of each compound. The presence of reducers (antioxidants) causes the conversion of the Fe^{3+}/ferricyanide complex used in this method to the ferrous form. Higher absorbance indicated higher reducing power of the selected extracts.

Previously, researchers reported that many mushroom species have reducing power.[52,54,60] They also reported that methanolic extracts of some mushroom species had high reducing power activity.

In the present study, reducing power of the various extracts of edible mushrooms increased with concentration. Based on the reducing power, among the mushroom extracts predominant one was acetone extract from *R. luteolus* (EC_{50} = 1.754 mg/ml). While the highest reducing powers in chloroform and methanol extracts were belonged to *C. comatus* (EC_{50} = 4.045 and 2.541 mg/ml, respectively), in *n*-hexane and water extracts *R. luteolus* showed the highest reducing capacities (EC_{50} = 4.869 and 2.468 mg/ml, respectively) [Table 4].

However, as shown in Figure 3, *L. tigrinus* demonstrably had the lowest reducing activity in acetone and chloroform

Table 3: IC_{50} values (mg/ml) of different extracts from four mushrooms and standard for chelating on ferrous ions

Extract	Species	IC_{50} (Limits)	Slope±SEM
Acetone[a]	*T. fracticum*	10.488 (9.109-12.697) J[c]	1.476±0.148
	L. tigrinus	10.996 (9.848-12.669) K	2.086±0.171
	C. comatus	11.632 (9.944-14.480) M	1.448±0.150
	R. luteolus	24.468 (17.846-41.007) T	1.261±0.165
Chloroform[a]	*T. fracticum*	8.946 (7.616-11.224) F	1.096±0.138
	L. tigrinus	9.564 (8.525-11.079) H	1.706±0.151
	C. comatus	5.650 (5.157-6.208) D	1.654±0.140
	R. luteolus	66.157 (30.383-457.340) U	0.687±0.152
n-Hexane[a]	*T. fracticum*	13.339 (10.739-18.677) P	1.117±0.144
	L. tigrinus	15.088 (11.953-21.729) S	1.154±0.147
	C. comatus	12.740 (11.099-15.336) O	1.891±0.169
	R. luteolus	14.664 (12.115-19.359) R	1.458±0.157
Methanol[a]	*T. fracticum*	7.467 (6.597-8.712) E	1.287±0.139
	L. tigrinus	14.119 (11.809-18.229) Q	1.523±0.158
	C. comatus	0.842 (0.207-1.504) B	0.618±0.136
	R. luteolus	2.397 (1.460-3.141) C	0.725±0.133
Water[a]	*T. fracticum*	12.337 (10.619-15.147) N	1.629±0.156
	L. tigrinus	9.513 (8.136-11.841) G	1.193±0.140
	C. comatus	11.534 (10.052-13.875) L	1.681±0.156
	R. luteolus	9.596 (8.712-10.818) I	2.082±0.163
EDTA[b]	–	0.158 (0.135-0.183) A	1.207±0.064

[a]Extract, [b]Standard solution, [c]Values followed by different capital letters in the same column differ significantly at $P<0.05$. EDTA: Ethylenediaminetetraacetic acid, *T. fracticum: Tricholoma fracticum, L. tigrinus: Lentinus tigrinus, C. comatus: Coprinus comatus, R. luteolus: Rhizopogon luteolus*, SEM: Standard error of mean, IC_{50}: 50% inhibition

Table 4: EC_{50} values (mg/ml) of different extracts from four mushrooms and standard for reducing power

Treatment	Species	EC_{50}
Acetone[a]	*T. fracticum*	5.757 K[c]
	L. tigrinus	15.185 T
	C. comatus	4.429 G
	R. luteolus	1.754 B
Chloroform[a]	*T. fracticum*	6.899 N
	L. tigrinus	11.368 R
	C. comatus	4.045 E
	R. luteolus	6.667 M
n-Hexane[a]	*T. fracticum*	8.493 O
	L. tigrinus	5.664 J
	C. comatus	8.722 P
	R. luteolus	4.869 H
Methanol[a]	*T. fracticum*	6.624 L
	L. tigrinus	5.498 I
	C. comatus	2.541 D
	R. luteolus	4.104 F
Water[a]	*T. fracticum*	13.011 S
	L. tigrinus	9.850 Q
	C. comatus	17.139 U
	R. luteolus	2.468 C
Gallic acid[b]	-	0.365 A

[a]Extract, [b]Standard solution, [c]Values followed by different capital letters in the same column differ significantly at $P<0.05$. *T. fracticum: Tricholoma fracticum, L. tigrinus: Lentinus tigrinus, C. comatus: Coprinus comatus, R. luteolus: Rhizopogon luteolus*, EC_{50}: Effective concentrations

extracts. Whereas in *n*-hexane and water extracts, *T. fracticum* had minimum reducing power. Similarly, in methanol extract, lower reducing power were determined in *L. tigrinus* and *T. fracticum*.

Amount of bioactive components working as antioxidants

Significant differences between the chemical composition of wild and commercial edible mushrooms have been reported.[6] Natural antioxidants derived from macrofungi are of considerable interest since these compounds have important constituents with free radical scavenging ability and hence may contribute directly to the antioxidative action. Among them, phenolic compounds, flavonoids, β-carotene and lycopene contents correlates well with the antioxidant potential of the edible mushrooms. Numerous studies carried out by scientists with some mushrooms demonstrated that these materials had antioxidant components such as β-carotene, flavonoid, lycopene and phenol.[31,38,41,52,54,60-62,64,65] Previous studies also revealed that many other edible mushrooms such as *Agaricus bisporus*,[60,62] *Amanita vaginata*,[66] *Grifola frondosa*,[61] *P. eryngii*,[46] *P. ostreatus*,[31,41] *Russula delica*[60] and *S. bovinus*[49] had high rate of phenol, flavonoids, ascorbic acid, β-carotene and lycopene. For that reason, various extracts from *T. fracticum*, *L. tigrinus*, *C. comatus* and *R. luteolus* were analyzed

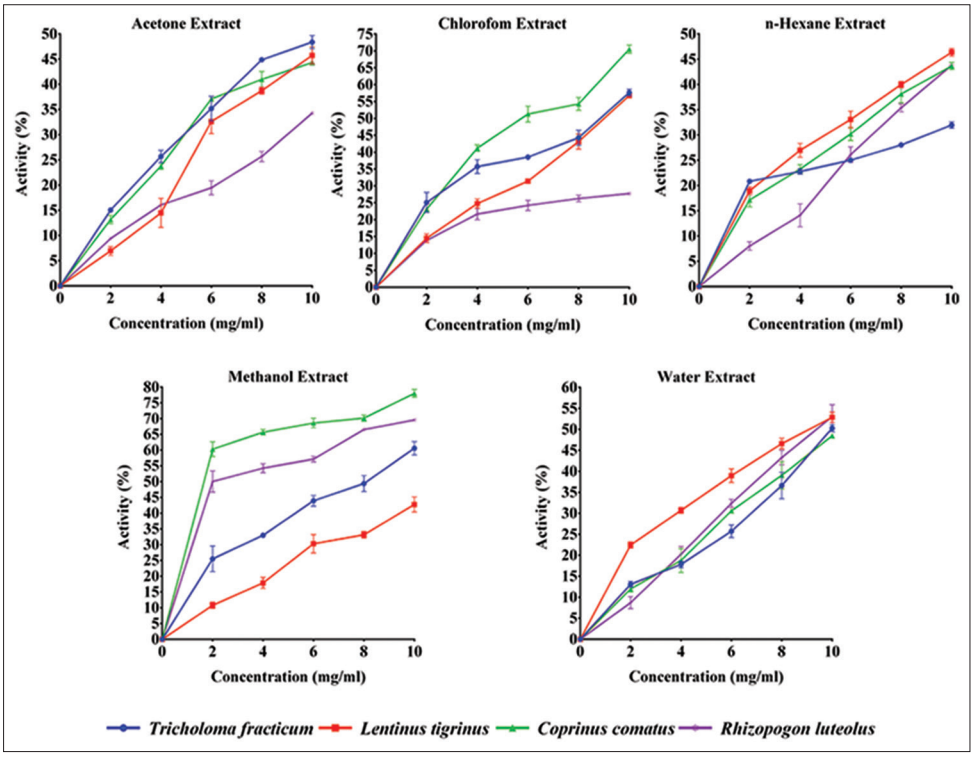

Figure 2: Chelating abilities of extracts in different concentrations from four mushrooms on ferrous ions

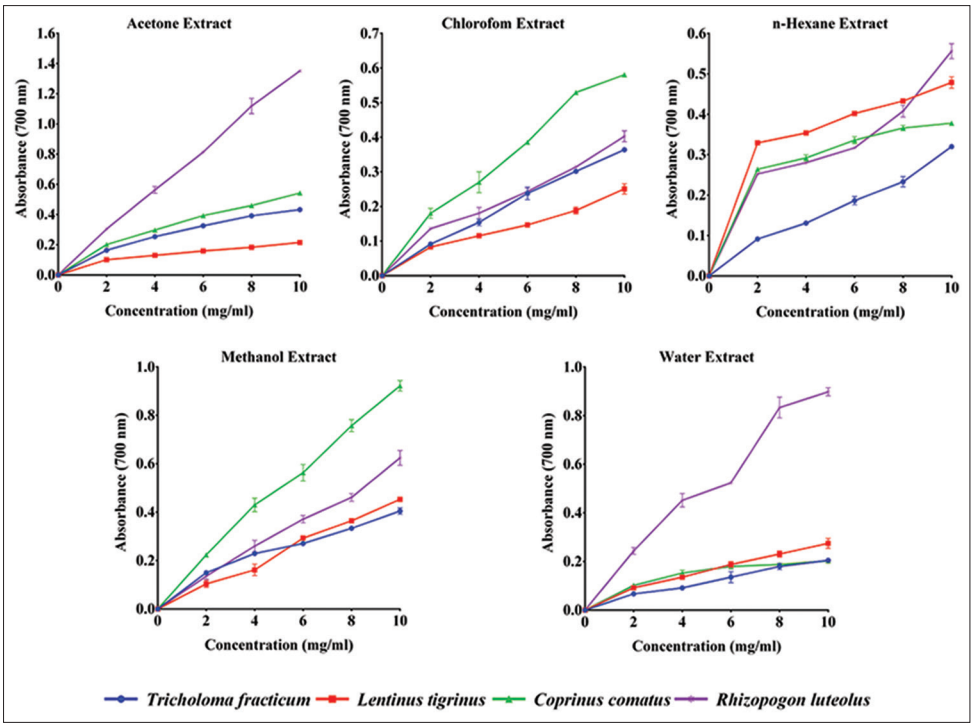

Figure 3: Reducing powers of various extracts from four mushrooms

for their antioxidant components including β-carotene, flavonoids, lycopene and total phenols.

As for the total phenolic contents, highest quantities were found in chloroform and acetone extract of *C. comatus* (76.32 and 61.09 μg/mg, respectively). At the same time, considerable phenolic content was determined in water extract of *L. tigrinus* (46.76 μg/mg). The total phenolic content in other mushroom extracts was lower than 37.00 μg/mg.

Considering flavonoid content, *n*-hexane and water extract of *L. tigrinus* showed the maximum amounts (3.67 and 3.22 μg/mg respectively). The flavonoid content in other mushroom extracts was lower than 3.00 μg/mg.

Among the mushroom extracts, chloroform extract of *C. comatus* had the highest β-carotene content (25.94 μg/mg). Furthermore acetone and methanolic extracts of this mushroom had higher β-carotene level than the other edible mushrooms (23.32 and 6.55 μg/mg, respectively), *n*-hexane and water extracts of *L. tigrinus* (17.28 and 13.13 μg/mg, respectively) had also considerable amount.

Chloroform and acetone extract of *C. comatus* revealed higher lycopene content (12.00 and 9.16 μg/mg, respectively) as compared with other mushroom extracts. However, lycopene contents in other mushroom extracts were found in lower amounts (0.96-6.25 μg/mg) [Table 5].

In this study, it was also determined that there is a positive correlation between antioxidant activity and bioactive constituents of tested edible mushrooms [Table 6]. Our results suggested that phenolic acids and flavonoids may be the second major contributors for the antioxidant activity as the IC_{50} values of radical scavenging activity of various soluble fractions of edible mushrooms and also the contents of phenolics or flavonoids exhibited significant correlation. Present study also revealed that DPPH radical scavenging activity was in significant correlation with the amount of phenolics, lycopene, flavonodis and β-carotene. Furthermore, reducing capacity of edible mushrooms was in positive correlation with the amount of flavonoids. Therefore, many foods that have a high rate of phenolics or flavonoids can be used for the purpose of medical therapies.

Antibacterial activities of different extracts

Mushrooms are rich sources of natural antibiotics, and many of the secondary metabolites may combat with many bacteria and viruses.[67] With an increasing number of bacteria developing resistance to commercial antibiotics, extracts and derivatives from mushrooms hold great promise for novel medicines. Therefore, antibacterial activity of tested edible mushrooms were analyzed by disc diffusion method on two Gram-negative (*E. coli* and *A. tumefaciens*) and three Gram-positive (*B. subtilis, B. licheniformis, S. aureus*) bacteria. All four tested mushrooms showed antibacterial activity at least on one

Table 5: Antioxidant contents of different extracts from four edible mushrooms

Content (μg/mg)	Species	Extract[a]				
		Acetone	Chloroform	*n*-Hexane	Methanol	Water
β-Carotene	*T. fracticum*	18.70±0.78	18.40±1.18	14.70±0.25	7.03±0.19	12.11±0.18
	L. tigrinus	2.58±1.36	9.41±2.58	17.28±1.10	2.71±0.08	13.13±0.15
	C. comatus	23.32±0.28	25.94±0.63	4.80±0.85	6.55±0.30	12.87±0.94
	R. luteolus	8.02±0.19	6.11±0.10	3.42±0.81	4.85±0.20	5.41±0.19
Flavonoid	*T. fracticum*	1.51±0.05	1.36±0.17	2.17±0.10	1.60±0.24	1.93±0.01
	L. tigrinus	0.41±0.07	0.94±0.02	3.67±0.05	1.16±0.13	3.22±0.01
	C. comatus	0.88±0.04	2.88±0.09	0.27±0.05	2.33±0.14	0.72±0.01
	R. luteolus	2.28±0.20	0.63±0.08	1.23±0.05	2.99±0.07	1.62±0.26
Total phenols	*T. fracticum*	32.72±0.12	30.36±0.34	5.93±0.04	2.52±0.38	36.81±0.09
	L. tigrinus	18.66±0.21	24.74±0.07	19.36±0.29	3.81±0.13	46.76±1.19
	C. comatus	61.09±0.32	76.32±1.11	5.02±0.01	8.40±0.28	32.37±16.19
	R. luteolus	12.16±0.72	7.70±0.60	8.03±0.19	4.13±0.23	6.69±0.18
Lycopene	*T. fracticum*	6.12±0.37	6.25±0.50	5.94±0.02	5.69±0.27	3.37±0.18
	L. tigrinus	1.06±0.21	3.52±1.68	3.43±2.14	1.51±0.05	4.21±0.08
	C. comatus	9.16±0.32	12.00±0.48	1.99±0.11	3.87±0.01	5.41±0.38
	R. luteolus	4.44±0.11	1.08±0.03	1.77±1.33	3.11±0.06	0.96±0.03

[a]Each value is expressed as mean±SEM (*n*=3). *T. fracticum: Tricholoma fracticum; L. tigrinus: Lentinus tigrinus; C. comatus: Coprinus comatus; R. luteolus: Rhizopogon luteolus*; SEM: Standard error of mean

Table 6: Correlations between amounts of bioactive components and antioxidant activities

Correlation	Pearson correlation coefficient				
	Acetone[b]	Chloroform[b]	*n*-Hexane[b]	Methanol[b]	Water[b]
DPPH activity *β*-Carotene	0.287	0.984[a]	−0.687	0.955[a]	−0.522
DPPH activity Flavonoid	0.751	0.979[a]	−0.306	0.574	−0.934
DPPH activity Total phenols	0.087	0.954[a]	−0.083	0.372	−0.767
DPPH activity Lycopene	0.514	0.983[a]	−0.680	0.827	−0.218
Metal chelating activity *β*-Carotene	0.335	0.956[a]	0.760	0.731	−0.533
Metal chelating activity Flavonoid	−0.693	0.952[a]	0.604	0.806	0.714
Metal chelating activity Total phenols	0.446	0.970[a]	0.113	0.649	−0.247
Metal chelating activity Lycopene	0.087	0.982[a]	0.965[a]	0.484	−0.692
Reducing power activity *β*-Carotene	0.062	0.858	−0.330	0.335	−0.980[a]
Reducing power activity Flavonoid	0.961[a]	0.956[a]	0.126	0.586	−0.075
Reducing power activity Total phenols	−0.285	0.924	0.439	0.968[a]	−0.898
Reducing power activity Lycopene	0.246	0.902	−0.677	−0.021	−0.897

[a]Correlation is significant at the 0.05 level; [b]Extract. DPPH: 2,2-diphenyl-1-picrylhydrazyl

microorganism. On the other hand, 10 out of 20 extracts (five different extracts of each four mushrooms, total 20 extracts) had stronger antimicrobial potential. Table 7 summarizes the antibacterial effects of edible mushroom extracts. Accordingly, the largest IZ was measured as 13 mm with chloroform extract of *R. luteolus* on *B. subtilis*. Besides, wide range of activity was observed with *n*-hexane extract of *L. tigrinus*. It inhibited the growth of entire microorganism used in this survey, including *E. coli*, because, none of the other extracts did inhibit the growth of *E. coli*. Strong antibacterial activity of *L. tigrinus* was followed by *T. fracticum* in such that its acetone extract showed antimicrobial activity against all microorganisms other than *E. coli*. The results revealed that for all tested

Table 7: Antibacterial activities of different extracts obtained from four mushrooms

Species	Extract	*Escherichia coli*	*Bacillus subtilis*	*Bacillus licheniformis*	*Staphylococcus aureus*	*Agrobacterium tumefaciens*
Tricholoma fracticum (IZ; mm)	Water	-	-	6	-	-
	n-Hexane	-	6	8	-	8
	Chloroform	-	6	6	-	-
	Acetone	-	6	6	9	6
	Methanol	-	-	-	-	-
Lentinus tigrinus (IZ; mm)	Water	-	8	6	-	-
	n-Hexane	6	6	8	6	8
	Chloroform	-	-	-	-	-
	Acetone	-	-	-	-	-
	Methanol	-	-	-	-	-
Coprinus comatus (IZ; mm)	Water	-	-	6	-	-
	n-Hexane	-	-	-	-	-
	Chloroform	-	-	-	-	-
	Acetone	-	-	-	-	-
	Methanol	-	-	-	-	-
Rhizopogon luteolus (IZ; mm)	Water	-	-	-	-	-
	n-Hexane	-	-	6	-	6
	Chloroform	-	13	6	-	6
	Acetone	-	-	8	6	7
	Methanol	-	-	-	-	-

IZ: Inhibition zones

extracts *Bacillus* sp. were more susceptible, since at least one extract from this mushroom showed antibacterial activity. Moreover, water extract of *C. comatus* has antibacterial activity against *B. licheniformis*. Indeed it is well known that Gram-negative microorganism more resistant to antibacterial than Gram-positives.

This study provided consistent result with earlier reports that mushrooms produce wide variety of antimicrobial compounds.[68,69] It was previously reported that *C. comatus* had antimicrobial acitivity against *S. aureus* and *B. subtilis*[70] whereas in this study it showed its activity against *B. licheniformis*. This discrepancy could result from cultivation from the different environment because climate, environment and the soil type can effect chemical composition of the mushrooms. On the other hand, similar result was obtained with *L. tigrinus* that it showed a wide range of antimicrobial activity.

CONCLUSION

Edible macrofungi have gained increasing reputation in recent decades, not only due to their nutritive value, but also for their possible health benefits and therapeutic uses. Therefore, wild beneficial sources bring new natural products into the pharmaceutical industry. Our results on four edible mushrooms elucidates that, *C. comatus* showed substantial *in vitro* cytotoxic activity against HepG2 cell lines that was in parallel with its high β-carotene, total phenolic and lycopene contents. High antioxidant potential of *C. comatus* obtained from highest DPPH capturing, and metal chelating activity was in significant positive correlation with the abovementioned bioactive ingredients. As for antibacterial activity, *L. tigrinus* and *T. fracticum* were the most active against tested microorganism. These results indicate that different extracts of investigated edible mushrooms have considerable cytotoxic, antioxidant and antibacterial properties and may be utilized as a promising source of therapeutics. Therefore, edible mushrooms might provide an appropriate source of antioxidant and cytotoxic natural compounds and could also be searched as a potent antibacterial drug against infectious diseases. *C. comatus* deserves further studies due to its incredible cytotoxic and antioxidant potential, and it can be a promising mushroom that would be benefitted in the field of pharmacology with safer and better effectiveness.

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