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Anticancer, antimicrobial, antioxidant and DNA protective potential of mushroom *Leucopaxillus gentianeus* (Quél.) Kotl.

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Mushrooms are important natural materials used in complementary medicine. In this context, the present study investigates the biological activities and phenolic content of mushroom *Leucopaxillus gentianeus* (Quél.) Kotl. Methanol (MeOH) and dichloromethane (DCM) extracts of the mushroom was used in the study. Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) were measured with Rel assay kits. DNA protective activity was determined with pBR 322 supercoil DNA. The effects of the extracts on lung cancer A549 cell line were tested to determine the cytotoxic effects. Antimicrobial activities were tested using 9 microorganisms with the modified agar dilution method. The mushroom MeOH and DCM extracts exhibited the highest activity against *Candida glabrata*, *C. albicans* and *C. krusei*. It was also determined that *L. gentianeus* had a high antioxidant potential. Furthermore, it was identified that DNA protective and cytotoxic activities of the mushroom were also present. Analysis for phenolic contents using HPLC revealed the presence of gallic acid, catechin, epicatechin, cinnamic acid, chlorogenic acid and coumaric acid. Overall results suggest that the mushroom *L. gentianeus* has considerable pharmacological potential, particularly anticancer, antimicrobial, antioxidant and DNA protective action.

Keywords: Bitter false funnelcap, Cytotoxic, Oxidative stress index

Use of natural products in addition to synthetic drugs for the treatment of various diseases is not uncommon. However, side effects is an issue of concern with the synthetic drugs[1]. In this context, mushrooms are one of the important natural materials of the ecosystem[1,2]. Several mushroom species are used as natural nutrient and medicinal sources by people[3-5]. Mushrooms, like plants, contain compounds that possess different biological effects. Previous studies reported that these bioactive compounds have antioxidant, DNA protective, analgesic, antibacterial, antifungal, antiviral, antitumor, cytostatic, immunosuppressive, antiallergic, antiatherogenic, hypoglycemic, anti-inflammatory and hepato-protective activities[5-8]. Thus, determination of the biological activities of mushrooms is important to identify new natural resources.

Leucopaxillus gentianeus (Quél.) Kotl., commonly called Bitter false funnelcap or bitter brown leucopaxillus, grows under conifers and can be identified by its brown pileus, white lamellae and white stipe[9]. This study explored the phenolic compounds contents of *L. gentianeus* extract for its potential use as antioxidants, antimicrobials, DNA protective and anticancer resource.

Materials and Methods

The sample was collected during routine field surveys conducted in Gaziantep province in Turkey on 2017-2018. Morphological (shape, colour, size) and ecological properties of the samples were recorded in the field and the microscopic characteristics were recorded in the laboratory under suitable conditions with light microscopy using 3% KOH solution (Leica DM750). The samples were morphologically identified using the references reported by Dähncke[10] and Roux[11] The mushroom samples were dried at 40°C after the identification procedure. Dried mushroom samples were powdered with a mechanical grinder. Powdered mushroom samples were extracted with methanol (MeOH) and dichloromethane (DCM) in a Soxhlet device.

TAS, TOS and OSI tests

Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) of mushroom extract were determined with Rel Assay brand commercial kits (Assay Kit Rel Diagnostics, Turkey).

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Trolox was used as TAS calibrator and the results were expressed as mmol Trolox equiv./L[12]. Hydrogen peroxide was used as TOS calibrator and the results were expressed as μmol H_2O_2 equiv./L[13]. The OSI (AU: Arbitrary Unit) expressed as the ratio of TOS levels to TAS levels, while the mmol value in the TAS unit was converted to μmol units similar to the TOS unit[13]. Analyses were conducted in 5 replicates.

Antimicrobial activity test

Antimicrobial activity tests of methanol (MeOH) and dichloromethane (DCM) extracts of mushroom were conducted with the agar dilution method recommended by the Clinical and Laboratory Standards Institute (CLSI) and the European Committee on Antimicrobial Susceptibility Testing (EUCAST)[14-16]. Minimal inhibitor concentrations (MIC) for each extract were determined against standard bacterial and fungal strains. *Staphylococcus aureus* ATCC 29213, *S. aureus* MRSA ATCC 43300, and *Enterococcus faecalis* ATCC 29212 were used as Gram-positive bacteria. *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853 and *Acinetobacter baumannii* ATCC 19606 were used as Gram-negative bacteria. *Candida albicans* ATCC 10231, *C. krusei* ATCC 34135 ATCC 13803 and *C. glabrata* ATCC 90030 were used as fungi. Bacterial strains were pre-cultured in Muller Hinton Broth medium, while fungal strains were pre-cultured in RPMI 1640 Broth medium. To obtain standard inoculum, the turbidity of bacteria and fungi was adjusted based on McFarland 0.5 scale. All extracts were tested at 800-12.5 μg/mL concentrations and distilled water was used to dilute all extracts. Solvents used for the extracts were also tested for antimicrobial activity. Fluconazole and amphotericin B were used as reference drugs for fungi, whereas amikacin, ampicillin and ciprofloxacin were used as reference drugs for the bacteria. The minimum dilution inhibitor concentration that inhibits the growth of bacteria and fungi was determined as the minimum inhibitory concentration (MIC)[17-19].

DNA protective activity test

MeOH and DCM extract of mushroom DNA protective activities were determined using pBR 322 supercoil DNA. Standard solutions (25, 50, 100 and 200 μg/mL) were prepared using mushroom extracts. About 0.5 μg plasmid pBR 322 was added to supercoil DNA Eppendorfs and 10 μL of standard mushroom extract solutions were added. Further, 10 μL Fenton's agent (30 mM H_2O_2, 50 μM ascorbic acid and 80 μM $FeCl_3$) was added to the prepared solution and the product was incubated at room temperature (20°C) for 10 min. The final volume of the mixture was adjusted to 20 mL and hkept for 30 min at 37°C. The DNA was then analyzed with electrophoresis on a 1% agarose gel that contained ethidium bromide[20].

Antiproliferative effect test

The MTT assay (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide) was conducted on mushroom MeOH and DCM extract A549 cells to determine cell viability. Cells were separated in 3.0 mL Trypsin-EDTA solution (Sigma-Aldrich, MO, USA) after 70-80% confluence. Then, they were seeded on plates. The products were incubated for 24 h after seeding. Following the incubation, the extracts were diluted in different concentrations (5, 25, 50, 100 and 200 μg/mL) and the cells were incubated for 24 h. Controls were conducted with growth medium without FCS supplement. After 48 h of incubation, the supernatants were dissolved in growth medium and exchanged with 1.0 mg/mL MTT (Sigma) and the purple precipitate was incubated at 37°C until formation of purple sediment. The supernatants were then removed, dissolved with dimethylsulphoxide (DMSO) (Sigma-Aldrich, MO, USA) added to the MTT absorbed by the cells. Plates were then read at 570 nm with Epoch spectrophotometer (BioTek Instruments, Winooska, VT)[21].

Phenolics screening by HPLC

The mushroom extract phenolic content was determined using an HPLC instrument. DAD detector was used as a detector. Injection volume was set as 20 μL. As mobile phase, A: 3% acetic acid and B: methanol were used. The flow rate was adjusted to 0.8 mL per minute. Chromatographic separation was performed at 30°C with an Agilent Eclipse XDB-C18 clone (250×4.6 mm id 5 μm)[22].

Statistical analysis

All experiments were performed five times, and results expressed as mean ± standard deviation unless otherwise stated. Results were considered significant when $P < 0.05$.

Results and Discussion

Antimicrobial activity tests

Table 1 shows the antimicrobial activity of the methanol (MeOH) and dichloromethane (DCM) extracts of the Bitter false funnelcap *Leucopaxillus gentianeus* along with some common antibiotics

(amikacin, ampicillin and ciprofloxacin) and antifungal medications (amphotericin B and fluconazole) for comparison.

The MeOH extract exhibited higher activity on tested microorganisms. Antimicrobial activity of *L. gentianeus* has not been studied previously. However, different concentrations of *L. giganteus,* another *Leucopaxillus* species, has been reported to be effective against *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Morganella morganni*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Pasteurella multocida*[23]. In a different study, it was reported that different concentrations of *L. albissimus* (Peck) Singer was effective against *P. aeruginosa*, *Burkholderia cepacia*, *B. cenocepacia*, *B. multivorans*, *Stenotrophomonas maltophilia*, *Achromobacter xylosoxidans* and *Acinetobacter baumannii*[24]. In the present study,the antimicrobial activity of the MeOH and DCM extracts *L. gentianeus* demonstrated that both extracts exhibited highest activities against *C. glabrata*, *C. albicans* and *C. krusei* at 50 µg/mL extract concentration. Extracts were also found to be effective against *S. aureus*, *S. aureus MRSA*, *E. faecalis*, *E. coli*, *P. aeruginosa* and *A. baumannii* at 100-400 µg/mL concentration. In conclusion, the antimicrobial activities of *L. gentianeus* were studied for the first time in the present study and it was found that 50 to 400 µg/mL concentrations were active against the test microorganisms.

TAS, TOS and OSI

Total antioxidant status (TAS), total oxidant status (TOS) and oxidative stress index (OSI) values of mushroom *L. gentianeus* were determined with Rel Assay kits. The TAS was 3.683±0.086 mmol/L, TOS, 6.303±0.181 µmol/L, and the OSI was 0.171±0.006. Possibly, it is the first report on the TAS, TOS and OSI of *L. gentianeus*. In our previous oxidative stress studies on mushrooms, we have reported the TAS, TOS and OSI values of 1.748, 19.294 and 1.106 µmol/L, for *Lentinus tigrinus* (Bull.) Fr.[25]; 2.332, 26.945, and 1.156 µmol/L for *Terfezia boudieri* Chatin[26]; and 1.44L, 14.21 and 0.99 µmol/L for *Fomitopsis pinicola* (Sw.) P. Karst.[27], respectively In other studies, the TAS value of *Pleurotus eryngii* (DC.) Quél and *Cerioporus varius* (Pers.) Zmitr. & Kovalenko have been shown to be 1.93[28] and 2.31[29], respectively It is observed that *L. gentianeus,* used in the present study, had a higher TAS value compared to the above-reports. The TOS value of *L. gentianeus* is lower compared to that of *L. tigrinus, T. boudieri* and *F. pinicola*. This difference is due to the fact that *L. gentianeus* produces less reactive oxygen species in its native form. Furthermore, the low OSI value of *L. gentianeus* suggests that the mushroom tolerates the environmental and metabolic factors and oxidant compounds produced in the environment well through its endogenous antioxidant compounds. Overall, the mushroom *L. gentianeus* has been shown to have high antioxidant potential and it can be consumed as a natural antioxidant source.

Table 1 — Antimicrobial Activity of *Leucopaxillus gentianeus* extracts

	A	B	C	D	E	F	G	H	I
MeOH	100	100	200	200	200	100	50	50	50
DCM	100	100	400	200	400	200	50	50	50
Ampicillin	1.56	3.12	1.56	3.12	3.12	-	-	-	-
Amikacin	-	-	-	1.56	3.12	3.12	-	-	-
Ciprofloxacin	1.56	3.12	1.56	1.56	3.12	3.12	-	-	-
Fluconazole	-	-	-	-	-	-	3.12	3.12	-
Amphotericin B	-	-	-	-	-	-	3.12	3.12	3.12

[MIC values are presented in units of µg/mL. A, *Staphylococcus aureus*; B, *S. aureus* MRSA; C, *Enterococcus faecalis*; D, *Escherichia coli*; E, *Pseudomonas aeruginosa;* F, *Acinetobacter baumannii*; G, *Candida albicans*; H, *C. glabrata*; and I, *C. Krusei*. MeOH, Methanol; DCM, Dichloromethane]

DNA protective activity

The DNA protective activities of MeOH and DCM extracts of mushroom were determined using pBR 322 supercoil DNA. The findings are presented in Fig. 1, Oxidative damage on the DNA occurs due to production of ROS[30]. Free radicals can initiate or aggravate several diseases. Hydroxyl free radicals are known to be damage cellular DNA in humans, and they cause cellular cancer through partial damage to the DNA[31]. Fig. 1 demonstrates that 25, 50, 100 and 200 µg/mL *L. gentianeus* MeOH and

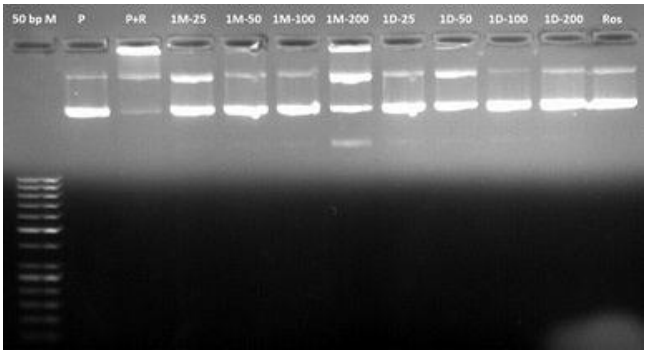

Fig. 1 — DNA Protective activity of *Leucopaxillus gentianeus* extracts [M, Marker; P, DNA; P+R, Negative control: pBR322+OH radical; and 1, *L. gentianeus*; M, Methanol; and D, Dichloromethane]

DCM extract concentrations exhibit significant protective activity against the hydroxyl radical. In the present study, rosmarinic acid was used as a positive control and exhibited strong DNA protection activity. MeOH extract @200 µg/mL resulted iin partial broke down Dof NA. Though the DNA protective activity of mushrooms in general are known, there are no reports available specific on *L. gentianeus*. Lee *et al*.[32] reported natural antioxidant and potential DNA protective activities of mushrooms viz. *Gloeophyllum trabeum* (Pers.) Murrill, *Daedalea dickinsii* Yasuda, *Pseudomerulius curtisii* (Berk.) Redhead & Ginns, *Stereum sanguinolentum* (Alb. & Schwein.) Fr., *Cryptoporus volvatus* (Peck) Shear and *Gloeophyllum abietinum* (Bull.) P. Karst.[32]. *Trametes gibbosa* (Pers.) Fr., *Fomes fomentarius* (L.) Fr., *Fuscoporia torulosa* (Pers.) T. Wagner & M. Fisch., *Daedalea quercina* (L.) Pers., *Inonotus hispidus* (Bull.) P. Karst. and *Trichaptum biforme* (Fr.) Ryvarden have also been reported to be potential DNA protectors[21], apart from *Laetiporus sulphureus* (Bull.) Murrill[33]. In our present study, we observed that *L. gentianeus* also exhibits potential DNA protective effect and could be used as a DNA protective agent from natural source.

Antiproliferative effect

MeOH and DCM extract of *L. gentianeus* standard solutions @25, 50, 100 and 200 µg/mL were prepared, and cell viability was tested with A549 lung cancer cell line. The findings are presented in Fig. 2. More than 60% of the deaths due to cancer and about half of the new cancer cases are witnessed in developing countries. Lung cancer is the most common type of cancer globally, followed by stomach, liver, colon, rectum and breast cancers[34]. It is the leading cause of cancer related deaths due to its high incidence, rapid progression and poor prognosis. Today, chemotherapy is widely used in the treatment of lung cancers. However, certain antitumor chemical drugs have serious adverse effects[35]. In this context, few mushrooms have been shown to possess anticancer potential. Previous studies have demonstrated that cucurbitacin B, cucurbitacin B esters, leucopaxillone A and leucopaxillone B isolated from *L. gentianeus* inhibit human lung carcinoma (A549), kidney carcinoma (CAKI 1), hepatoblastoma (HepG2), and breast adenocarcinoma (MCF-7) cell lines in different concentrations[36]. In the present study, *L. gentianeus* MeOH and DCM extracts demonostrated cytotoxic action on A549 cells. Fig. 2 shows strong antiproliferative effects of both MeOH and DCM extracts of *L. gentianeus* suggesting its anticancer potential.

Phenolic content

Phenolic acids are known for their antioxidant activity[37]. Gallic acid is reported to have antioxidant, antimicrobial, antitumor and anti-inflammatory properties[38,39]. Catechin and epicatechin are known to exhibit antioxidant, antimutagenic, antimicrobial and anticancer action[40,41]. Cinnamic acid possesses anti-inflammatory, antioxidative, antitumor, antimicrobial, antihypertensive and antihyperlipidemic activities[42]. Similary, chlorogenic acid exhibits antioxidant, antibacterial, anticancer, anti-inflammatory and DNA protective effects[43,44]. Coumaric acid has been shown to possess antioxidant, antimicrobial, immuno-modulatory, anti-inflammatory, antiangiogenic, anti-cancer, goitrogenic, antidiabetic and antihyper-lipidemic activities[45,46]. In the present study, we have shown that all the above mentioned phenolic compounds are present in *L. gentianeus* (Table 2) indicating its huge potential. These six compounds gallic acid, catechin, epicatechin, cinnamic acid, chlorogenic acid and coumaric acid present in *L. gentianeus*couold be responsible for its antioxidant, antimicrobial, DNA protective and cytotoxic effects as demonstrated by the above study.

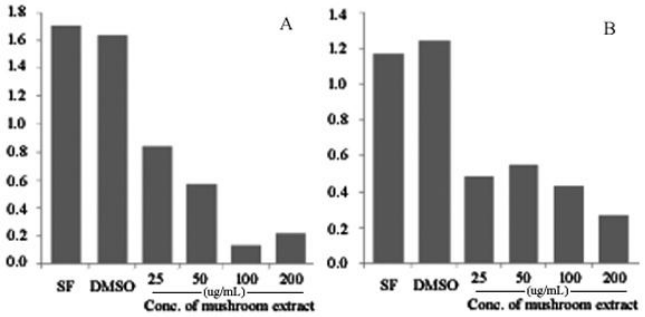

Fig. 2 — Antiproliferative effects of *Leucopaxillus gentianeus* extracts (conc. 25, 50, 100 and 200 µg/mL). (A) MeOH; and (B) DCM

Table 2 — Phenolic content of *Leucopaxillus gentianeus* (ppm)

Gallic acid	27.55
Catechin	130.77
Epicatechin	21.31
Chlorogenic acid	90.03
Coumaric acid	1.28
Cinnamic acid	34.37

Conclusion

Results of this study, demonstrated high antioxidant potential of methanol (MeOH) and dichloromethane (DCM) extracts of mushroom *Leucopaxillus gentianeus*. In addition, the extracts have been shown to possess DNA protective action and cytotoxic effect on A549 cells. The mushroom extracts exhibited normal antimicrobial activity on tested micro-organisms (*Staphylococcus aureus*, *S. aureus* MRSA, *Enterococcus faecalis*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and fungal strains). Their highest impact was on fungal strains (*C. albicans*, *C. glabrata* and *C. krusei*). Phenolic compounds such as gallic acid, catechin, epicatechin, cinnamic acid, chlorogenic acid and coumaric acid have been found to present in the mushroom. The above observations indicate the pharma-cological potential of the mushroom *L. gentianeus* as a natural source.

Conflict of interest

Authors declare no conflict of interests.

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