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Antioxidant, antimicrobial and DNA protection activities of phenolic content of *Tricholoma virgatum* (Fr.) P.Kumm.

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ABSTRACT

Tricholoma is one of the famous genera of Basidiomycota division. Although some species of this genus have been used as culinary mushrooms, very negligible investigations have been conducted on *Tricholoma* genus phenolic contents and their biological activities. In the present study, the antioxidant, antimicrobial, and DNA protection properties of total phenolic contents of *Tricholoma virgatum* (Fr.) P. Kumm. (54% methanolic extract) were assessed. *T. virgatum* phenolic content was determined by an analytic high-performance liquid chromatography (HPLC) method based on compression with standard phenolic compounds including gallic acid, catechin, chlorogenic acid, epicatechin, and coumaric acid. Total antioxidant status (TAS), total oxidant status (TOS), and oxidative stress index (OSI) values were determined using Rel Assay kits. For DNA protective potential assay, pBR322 supercoiled DNA method was used. The antimicrobial activity assay was done based on the agar dilution method on six different microorganisms include *Staphylococcus aureus, Enterococcus faecalis, Escherichia coli*, *Pseudomonas aeruginosa*, *Candida albicans*, and *Candida tropicalis*. The total phenolic contents of this mushroom lied within range of 2.02-56.85 ppm based on coumaric acid and chlorogenic acid as standards. TAS, TOS, and OSI values were determined as 3.754±0.088 mmol/L, 8.362 ±0.085 µmol/L, and 0.223 ±0.007, respectively. *T. virgatum* methanol extract could exhibit a protective effect on DNA against the hydroxyl radical at 100 µg/mL concentration. Although *T. virgatum* is not recommended as an edible mushroom, according to our results, this mushroom could be considered as valuable source for phenolic compounds with significant antioxidant/antimicrobial effects.

Keywords: *Tricholoma virgatum; mushroom; antioxidant activity; antimicrobial activity; DNA protection; phenolic content.*

1. INTRODUCTION

Mushrooms are known as a major human edible source for a long time in many nations. Based on the Food and Agriculture Organization of the United Nations (FAO) statistics, the global mushroom production between 1997 and 2009 was 2.18 to 3.5 million tons in the world [1]. Chemically, mushrooms are rich sources of valuable constituents such as carotenoids, polyphenol, alkaloids, and dietary fiber [2]. Mushrooms and their metabolites have been investigated extensively for different biological activities such as antioxidant activities, anticancer activities, DNA protection and antimicrobial potential [3]. Many studies have shown that mushroom's chemical constitutions could be health-promoting, and thus these compositions can be considered as a complementary and functional food in many supplementary products [4]. Due to commercial and industrial requirements, identification of the biological potencies as well as chemical content of mushrooms is an important field in natural products research [5]. Various medicinal uses of mushroom as well as their edibility have engendered a great deal of attention to their pharmacological potencies including antioxidant, antibiotic, and antineoplastic activity [6-9]. One of the most important research fields in this regard is oxidative stress related diseases. Oxidative stress is a state of imbalance between oxidants and antioxidants in favor of the oxidants, leading to chronic and sub-chronic detrimental effects. The reactive oxygen species (ROSs, as most important oxidants involved in many human diseases) include various free radical particles such as O•− (superoxide), ONOO•− (peroxynitrite), and OH• (hydroxyl), as well as non-radicals such as H_2O_2 (hydrogen peroxide) [10]. In general, oxidative stress is closely related to many pathological outcomes such as endothelial dysfunction, neurodegenerative conditions, and vascular complications. Oxidant accumulation progress (as an age-related process) constitutes an important part of risk factors in the development of chronic neuro-cardiovascular systems diseases. These age-related diseases are a consequence of the accumulation of cellular damage and the reduced response of oxidative stress protection pathways [11]. Many animal models as well as clinical trials on dietary compositions such as phenolic compounds have reported slowed progression of many oxidative stress-related diseases by improving lipid profile, reducing the generation of ROSs, and by enhancing the body's antioxidant capacity.

Tricholoma is one of the largest genera of Tricholomataceae family which is spread worldwide in coniferous woodlands[12]. The edible uses of some Tricholoma species as part of the common diet are well known for their pleasant flavor and taste. Some of the well-known edible mushrooms can be found among this genus especially in East Asia and North

America [13]. In this study, the methanolic extract of *T. virgatum* (formerly known as *Agaricus virgatus* Fr. a common basidiomycete of European, Eastern European and North American coniferous forest popularly known "Ashen knight") was assessed for DNA protection using with pBR 322 supercoiled DNA assay. Free radical scavenging, total oxidant status (TOS), oxidative stress index (OSI), and total anti-oxidant status (TAS) were examined. The antimicrobial activity (based on the minimum inhibitory concentration) of *T. virgatum* phenolic extract was tested against *S. aureus, E. faecalis, E. coli, P. aeruginosa, C. albicans*, and *C. tropicalis* microorganisms. Additionally, the phenolic contents of this mushroom were determined using high performance liquid chromatography – diode array detector (HPLC-DAD) in compression with gallic acid, catechin, chlorogenic acid, epicatechin and coumaric acid as phenolic standard.

2. MATERIALS AND METHODS

2.1. Basidiocarp (fruiting body) selection.

The mushroom bodies were collected from Gaziantep province during routine field studies in September. In the field, the morphological aspects such as color, shape, size, odor, and taste were noted. The identified specimens were transported under suitable conditions to the laboratory. The microscopic characteristics of the specimens were observed by mounting in 3% KOH solution where the features of spores, basidia, and pileipellis structures were analyzed by a light microscope (Leica DM 750). The specimen was identified morphologically using macroscopic, microscopic, and ecological features with references and it kept in Akadeniz University biology department fungarium (Şen 1560) [14]. They were then dried and powdered using an automated grinder. The powdered mushroom samples were extracted with methanol in a Soxhlet apparatus at 50°C (Gerhardt EV 14). This methanolic extract was concentrated using rotary evaporator (Heidolph Laborator 4000 Rotary Evaporator) at 40°C and stored at +4°C.

2.2. Phenolic compounds extraction.

A total of 150 g of *T. virgatum* fine powder was extracted using Soxhlet apparatus with methanol for 6 hours at 50 °C. This extract was filtered through Whatman no. 1 paper and concentrated by BUCHI Rotavapor R-144 at 40° C. The dried extract was stored in the freezer at 2 °C for future uses.

2.3. Determination of phenolic contents.

The phenolic content of the *T. virgatum* methanolic extract was measured based on the Caponio et al. method with a high performance liquid chromatography – diode array detector (HPLC-DAD) Shimadzu apparatus [15]. An Agilent Eclipse XDB-C18 (250 × 4.6 mm id 5 µm) was used as column for chromatographic separation with an isocratic system. A solvent system consisting of 3% acetic acid in methanol at a flow rate of 0.8 mL/min and the injection volume 20 µL was used. The phenolic constitutions of the samples were detected at 280 nm. Also, the retention time of the constitutions was used for comparison with the standard compounds.

2.4. Antimicrobial activity tests.

The agar dilution method was used for *T. virgatum* methanolic extract antimicrobial potential determination [16]. Two fungal strains *C. albicans* ATCC10231, *C. tropicalis* ATCC 13803 and four bacterial strains *S. aureus* ATCC 29213, *E. faecalis* ATCC 29212, *E. coli* ATCC 25922, and *P. aeruginosa* ATCC27853 were used as the tested microorganisms. Muller Hinton Broth medium and RPMI 1640 Broth medium were employed for pre-cultured of bacterial and fungal strains, respectively. Turbidity based on McFarland 0.5 scale was considered for standard inoculum. Concentrations 800-12.5 µg/mL (in distilled water) of the extract were assessed for possible antimicrobial activities. Ciprofloxacin, ampicillin, and fluconazole were used as antimicrobial reference compounds for bacterial and fungal microorganisms. For the minimum inhibitory concentration (MIC) determination, 9 mL of dissolved Müller Hinton Agar medium was distributed into 15 mL sterile tubes after sterilization. Then, 1 mL samples were added to these tubes and mixed with the media after which these mixtures were transferred to Petri dishes. Seven dilutions of all samples (concentrations 800-12.5 µg/mL) were prepared for each plate. The lowest dilution that prevented the propagation of microorganisms was considered as MIC [17].

2.5. Determination of TAS, TOS and OSI.

Rel Assay commercial kits were used for determining the *T. virgatum* methanol extract TAS, TOS and OSI values [18]. Hydrogen peroxide (H_2O_2) and trolox were used as the calibrators in the TOS and TAS analysis. The values are presented as mean ± S.D, while µmoL H_2O_2 equiv. /L and mmoL trolox equiv. /L were used for tests values report, respectively. Experiments were performed in triplicate.

For OSI (AU: Arbitrary Unit) percentage report, we adopted the calculation method of Erel et al. [19], who presented the following formula

OSI (AU) = [(TOS, µmol H_2O_2 equiv. /L) / (TAS, mmol Trolox equiv. /L)] × 10

2.6. DNA protective activity.

For the *T. virgatum* methanol extract, Lee et al. DNA protection potential model were used based on the DNA supercoiled with plasmid pBR 322 [20]. Methanol extract solutions were prepared with 100 and 200 µg/mL. Next, 10 µL of each concentration was added to the Eppendorf tubes with 0.5 µg plasmid pBR322 and supercoiled DNA. After the addition of 10 µL Fenton agent (30 mM H_2O_2, 50 µM ascorbic acid and 80 µM $FeCl_3$), these prepared solutions were incubated at 25 ° C for 10 min. Then, all samples mixture volumes were adjusted to 20 mL and incubated again for 10 minutes at 37 °C. The electrophoresis on a 1% agarose gel with ethidium bromide was used for analysis possible DNA changes.

3. RESULTS

3.1. Phenolic content.

Gallic acid was reported as an antioxidant, antimicrobial, antitumor, and anti-inflammatory agent [21]. Similar activities such as antioxidant, anti-mutagenic, antimicrobial, and anticancer effects were also reported for catechin and epicatechin [22-23]. Another phenolic compound, chlorogenic acid, has shown several biological activities such as anti-oxidant and anti-inflammatory effects, as well as regulation of glucose and lipid metabolisms,

plus anti-diabetic, anti-carcinogenic, anti-inflammatory, and anti-obesity properties in various investigations [24]. Similarly, diverse biological activities including antioxidant, anti-cancer, antimicrobial, antiviral, anti-inflammatory, and anti-thrombocyte aggregation have been observed from coumaric acid [25]. In this study, six phenolic components of *T. virgatum* methanolic extract were analyzed and compared with several phenolic compounds as standards by HPLC/DAD method. Based on our result, the most prevalent compound was chlorogenic acid in *T. virgatum* methanolic extract (Table 1). Other constitutions were identified as catechin, gallic acid, epicatechin, and coumaric acid. In conclusion, it was determined that *T. virgatum* mushroom is a suitable source of some phenolic compounds including chlorogenic acid, catechin, and gallic acid. The presence of considerable antioxidant contents in mushrooms may confer protection against many chronic diseases such as cardiovascular disease (CVD), neurodegenerative disease, and other oxidative stress related diseases [26-28]. Their health promotion effects on oxidative stress-related diseases such as CVD are often attributed to their antioxidant capabilities, in particular their free radical scavenging ability plus metal ion homoeostasis via chelating activities.[29-31]

3.2. DPPH radical scavenging activity.

The antioxidant capacity of the methanol extract of our mushroom showed a concentration dependent trend (Table 2). The best outcome was observed at the concentration of 2 mg/mL extract (DPPH radical inhibition 56.49±2.64%). *T. virgatum* methanol extract showed a good potential at 2 mg/mL which is comparable with the caffeic acid antioxidant potential. Although previous work on *Tricholoma* species exhibited that these mushrooms have a good antioxidant activity, sparse studies have been done on the chemical compounds (especially phenolic constituents) of this genus. A water-soluble heteroglycans polysaccharide of *T. matsutake* showed antioxidant activity in different models including DPPH radical, superoxide anion, and hydroxyl radicals scavenging methods [32]. In another work on the antioxidant potential and identification of compositions of *Tricholoma* species, several polysaccharides were isolated from *Tricholoma mongolicum Imai*. These isolated polysaccharides exhibited suitable activities in ferric reducing antioxidant power (FRAP), DPPH radical, and hydroxyl radical scavenging assessment[33] . Similar investigations on the methanol extract of edible mushroom, *T. giganteum massee*, showed antioxidant activities against DPPH radicals with a half-maximal effective concentration (EC_{50}) 0.7mg/mL [34].

3.3. TAS, TOS, and OSI values.

Superoxide anion radical, hydrogen peroxide, hydroxyl radical, singlet oxygen, and peroxyl radicals are the major free oxygen radicals. Note that some amounts of free oxygen radicals occur in all eukaryotic cells throughout the course of a regular physiologic metabolism [35]. Cellular damage results from oxidative stress through impairing the antioxidant/oxidant balance and production of free radicals/reactive oxygen species that attack macromolecules (DNA, lipids, proteins, carbohydrates). These damages often cause progressive diseases or conditions such as senescence, cardiovascular diseases, cancer, renal diseases, and neurological diseases. The harmful free radicals, which constantly occur in the body due to physiological conditions, are detoxified via inhibitive antioxidant mechanisms. These detrimental effects are caused by imbalanced oxidant/antioxidant contents and accumulation of oxidants which finally result in oxidative stress. Indeed, the accumulation of these harmful reactive metabolites leads to oxidative stress and has a destructive impact on organisms. If the antioxidant content is not adequate to neutralize the oxidants, oxidant and antioxidant imbalance occurs favoring the oxidants[36]. It is well known that the oxidant / antioxidant balance is cleared by higher antioxidant contents in the body. Note that free oxygen radicals and antioxidants are produced continuously in live tissues in a controlled manner. Excessive free oxygen radicals are neutralized by neutralizing systems such as endogenous and exogenous antioxidants [35]. The imbalance favoring the oxidants induces tissue damage, which is mentioned in the literature as oxidative stress. Oxidative tissue damage is caused by reactions between oxidant molecules and the cellular building stones such as proteins, lipids, carbohydrates, nucleic acid, and enzymes.

In this study, *T. virgatum* methanol extract showed TAS, TOS, and OSI with 3.754 ±0.088 mmol /L, 8.362 ±0.085 µmol /L, and 0.223 ±0.007, respectively. Literature review revealed no data on the TAS, TOS, and OSI values of *T. virgatum* mushroom. A previous investigation on Anatolian region mushrooms *T. terreum* found 0.38 mmol/L, 16.76 µmol/L, and 4.41 for TAS, TOS, and OSI values, respectively[37]. The higher TAS value of *T. virgatum* compared to *T. terreum* probably is due to the better ability of *T. virgatum* for producing more antioxidant constituents. Considering the lower TOS and OSI values of *T. virgatum*, probably *T. virgatum* produced less effective antioxidant compounds compared to *T. terreum*. Similar results were found about *Cyclocybe cylindracea* mushroom, where the TAS, TOS and OSI values of this mushroom were 4.325 mmol/L, 21.109 µmol/L, and 0.488, respectively. These values showed comparable findings of the quantity and quality of *T. virgatum* antioxidant compounds [38].

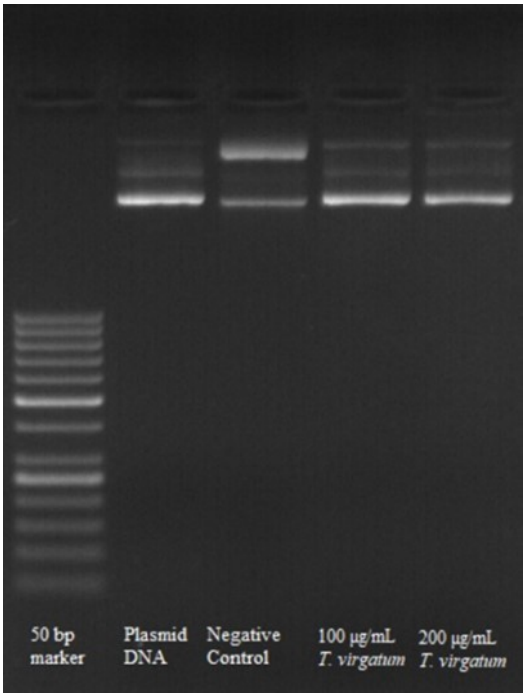

Figure 1. DNA protection activity of *T. virgatum* methanol extract with 100, and 200 µg/mL. A protection effect was observed against DNA damage compared with the plasmid DNA control and negative control.

3.4. Antimicrobial activity.

T. virgatum methanol extract showed an inhibitory effect on *S. aureus*, *E. faecalis*, *E. coli* with 400 µg/mL and 200 µg/mL, for *P. aeruginosa*, *C. albicans*, *C. tropicalis* in agar dilution method, respectively (Table 3). Previous works on *T. portentosum* mushroom showed that this species was effective against *B. cereus*, *B. subtilis*, *E. coli* , *P. aeruginosa*, *C. albicans* and *C. neoformans* within 100-300 mg/mL (MIC) [39]. Other investigations on *T. equestre* and *T. portentosum* have also indicated that these mushrooms have a growth inhibitory potential on *Listeria monocytogenes* [40-41].

The same observation was reported about the antimicrobial activity of *T. lobayensis* extract on *B. cereus*, *E. coli*, *K. pneumoniae*, *P.vulgaris*, and *S. aureus* [42]. In another investigation on *Tricholoma* species, different fractions of *T. fracticum* were examined for antibacterial activity. Chloroform fraction could reveal a suitable inhibitory potential on *P. aeruginosa* (ATCC 27853) and *S. aureus* (ATCC 25923) growth [43]. Our findings suggest that *T. virgatum* methanol extract could be presented as an effective complementary antimicrobial agent (Table 3).

3.5. DNA protection activities.

Many studies have reported the anti-tumor potential of different mushrooms. Concerning the traditional uses, the anti-tumor properties of some mushrooms were obtained based on various mechanisms including antioxidant and immunoregulatory activities. Shameem et al. work on the two Kashmir Himalaya edible mushrooms *Verpa bohemica* and *Morchella esculenta* showed that *M. esculenta* could offer significant protection against the hydroxyl radical-produced DNA damage [44].

Investigation on another mushroom, *Russula virescens* "green brittlegill", a folk remedy in traditional Chinese medicine showed that water and ethanol extracts of this mushroom could present DNA damage prevention activity[45]. Similar results have also been reported from *Ganoderma lucidum* (Curtis) P. Karst. "Reishi", *Helvella lacunosa* (Afzel.) "slate grey saddle", *Laetiporus sulphureus* (Bull.) Murrill., *Inonotus obliquus* "Chaga", and *Agaricus blazei* Murrill "Himematsutake" as edible mushrooms [46-48].

Presence of the phenolic compounds in mushrooms could neutralize hydroxyl radical produced from Fenton reagent. This distractive reaction is based on the breakage of the DNA strand by hydroxyl radical generated from Fenton reagent- pBR322 plasmid DNA reaction. *T. virgatum* methanol extract (100, 200 µg/mL) showed a protective effect against DNA damage compared with the plasmid DNA control (Lane 2 of Figure 1) and negative DNA damage control (Lane 3 of Figure 1).

The hydroxyl radical generated from Fenton reaction converted supercoiled DNA to liner. When DNA was treated with 100 and 200 µg/mL of the *T. virgatum* ethanol extract, the supercoiled form was restored. The results suggested that methanol extracts of *T. virgatum*, a rich in phenolic constitutions, could have DNA protective antioxidant properties.

Table 1. Antioxidant activity of *T. virgatum* (% inhibition) methanol extract in comparison with caffeic acid, rosmarinic acid, and ascorbic acid as standards.

Standards & sample	Concentration			
	2 mg/mL	**1 mg/mL**	**0.50 mg/mL**	**0.25 mg/mL**
Caffeic acid	54.47±0.05	38.39±0.66	21.34±0.66	8.62±0.91
Rosmarinic acid	61.92±0.15	35.09±7.96	7.00±0.41	6.03±0.15
Ascorbic acid	95.96±1.96	93.72±1.39	91.40±0.70	89.45±1.44
T. virgatum	56.49±2.64	46.96±2.23	33.87±1.32	24.90±2.99

Values are presented as mean±S.D.; n=6 (Experiments were made as 3 parallel)

Table 2. Phenolic compounds of *T. virgatum* methanol extract in comparison with gallic acid, catechin, chlorogenic acid, epicatechin, coumaric acid as standards.

	Gallic acid	**Catechin**	**Chlorogenic acid**	**Epicatechin**	**Coumaric acid**
T. virgatum	10.88 ppm	54.16 ppm	56.85 ppm	3.80 ppm	2.02 ppm

Table 3. Antimicrobial activity of *T. virgatum* in comparison with ampicillin, ciprofloxacin, and fluconazole as antimicrobial standards.

	***S. aureus* (µg/mL)**	***E. faecalis* (µg/mL)**	***E. coli* (µg/mL)**	***P. aeruginosa* (µg/mL)**	***C. albicans* (µg/mL)**	***C. tropicalis* (µg/mL)**
T. virgatum	400	400	400	200	200	200
Fluconazole	-	-	-	-	1.56	3.12
Ampicillin	3.12	1.56	3.12	-	-	-
Ciprofloxacin	0.78	0.78	1.56	3.12	-	-

* 400, and 200 (µg/mL) indicate concentrations of extract affecting microorganisms.

4. CONCLUSIONS

Although there are diverse mushrooms used as food, the number of these mushrooms with evident antioxidant/ anticancer effects is limited. In this regard, these findings suggested a potential role for mushroom phenolic compounds and other kinds of chemicals as a possible effective antioxidant/ anticancer remedies. Also, *T. virgatum* possessed acceptable in vitro efficacy and selectivity suggesting that further in vivo studies, as well as preclinical and clinical trials should be conducted to assure the potential of *T. virgatum* for therapeutic indication. It is anticipated that more comprehensive data will provide a new path for researchers towards authentication and establishment of this natural functional food such as antioxidants, DNA protection and antimicrobial potential as a felicitous pharmacological drug in the years to come.

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6. ACKNOWLEDGEMENTS

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