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Research Article

Antioxidant and Cytotoxic Activities of Three Edible Fungi (*Tricholoma* spp.) on Tumor Cells

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Abstract

The presence of medicinal properties increases the food and agricultural interest of wild species. In this sense, based on backgrounds, the aim of this study was to elucidate the antioxidant capacities and cytotoxicity as potential anticancer activity of three mushroom species of the genus *Tricholoma* (Higher fungi; bot.: Basidiomycota) present in Europe, *Tricholoma terreum, Tricholoma gausapatum* and *Tricholoma fracticum* which are edible and consumed in the world. Antioxidant capacity of methanolic extracts from fruiting bodies was assayed as well as their cytotoxicity against two different cell lines on HT-29 (Human Caucasian colon adenocarcinoma) and HeLa (Human cervix carcinoma). The extract of *T. terreum* showed the highest radical scavenging activity and exhibited an interesting cytotoxicity on HeLa cell line according to NCI criteria. Additional studies are warranted to characterize compounds responsible for these biological activities.

Keywords: *Antioxidant; Cytotoxicity; Anticancer; Tricholoma*

Introduction

Mushrooms had been widely used since ancient times because of their food value and flavor. As Tel., *et al.* [1] commented, the flavor of wild edible mushrooms attracts consumer attention and many researchers opt for study them, in order to achieve medicinal and functional purposes that increase the food interest of wild mushrooms species, and favours the cultivation and control of wild collection to avoid negative effects on the environment. On the one hand, regarding food source, they contain high amount of proteins, sugars, glycogen, lipids, vitamins, amino acids and fiber. In this context, mushrooms are among the best sources of essential nutrients and their nutritional value is comparable with that of meat, eggs and milk. On the other hand, medicinal values of edible mushrooms have been recognized all over the world, and mainly Basidiomycetes fungi present substances with interesting biological activities, which give them potential medicinal properties. Thus, low molecular mass substances and polysaccharides were isolated and recognized as active substances [2]. In this sense, it was demonstrated that lectins, polysaccharides, polysaccharide-peptide and polysaccharide-protein complex, lanostane-type triterpenoids, and phenolic and flavonoid structured compounds obtained from edible mushroom species have anticancer, antioxidant, antitumor, antiviral, antibacterial, antifungal, anti-cataractogenic, anti-inflammatory, immunomodulator activities, antibiotic effects, and cholesterol-lowering properties. Among mushroom properties, antioxidant and cytotoxic activities have been highlighted through compounds isolation from both fruiting body and mycelium [3].

Oxidation is essential to many living organisms for the production of energy to fuel biological processes, but oxygen-centered free radicals and other reactive oxygen species that are continuously produced in vivo result in cell death and tissue damage. So free radicals are involved in the pathophysiology of a large number of diseases, especially reactive oxygen species. When they are excessively produced, or when antioxidants are depleted, free radicals could damage lipids, proteins, or DNA. The intracellular reduction-oxidation imbalance, called oxidative stress, can subsequently contribute to the development and/or progression of diseases such as cancer, cardiovascular disease, atherosclerosis, ischemia/reperfusion injury, diabetes mellitus, ophthalmic and neurodegenerative diseases, rheumatoid arthritis and ageing. Thus, it is essential to know new natural antioxidants that can protect the human body from free radicals and many chronic diseases. In this regard, antioxidants from diet act as protective agents against oxidative damage, and mushrooms might be an excellent resource because backgrounds show their antioxidant activity.

There are studies emphasizing that free radicals contribute to the development of many diseases such as tumor promotion and carcinogenesis, this is a reason for jointly researching anticancer and antioxidant activities. A relationship between cytotoxicity and anticancer activities exists. The term, "cytotoxic" means causing toxicity to cells, and many anticancer agents aim to kill cancer cells. A new concept related to anticancer properties and mushrooms has arisen, Mycotherapy of cancer, which is a relatively novel and promising scientific field that deals with anticancerogenic agents derived from mushrooms, as Popovic., *et al.* [3] defined. A great variety of compounds and complex fractions were isolated and/or purified from medicinal and edible mushrooms, with special importance regarding anticancer and cancer preventive activity. These substances are contained in many mushroom species [4].

A genus with edible species is *Tricholoma* (Basidiomycota), widely distributed worldwide and generally growing in woodlands. *Tricholoma* is a large genus of gilled mushrooms with white spore prints, fleshy stems, and gills attached to stem. The genus comprises both edible and toxic species. Among medicinal mushrooms, *Tricholoma matsutake* is one species widely cited due to its possible utilities on humans such as lowering cholesterol levels, immunomodulation, antioxidant and antitumor effects [5]. Another interesting species is *Tricholoma populinum*, which showed high inhibitory xanthine oxidase activity, being an edible *Tricholoma* species with a promising antioxidant potential. On the other hand, entire fungi and fungi extracts are used in traditional Chinese medicine for the treatment of cancer, and used as adjuvants to surgery, radiotherapy or chemotherapy. Moreover, an inverse correlation between the risk of developing certain cancers (gastric or breast cancer) and mushroom consumption has been demonstrated [6]. However, not only these species have been studied, but also *Tricholoma acerbum* were evaluated in order to assess their products as sources of nutrients and nutraceuticals due to its antioxidant activity [7]. *Tricholoma mongolicum* showed antioxidant and cytotoxic effects [5]. Antioxidant, immunomodulation and antitumor activities were assessed from extracts and molecules provided by *Tricholoma lobayense* [8]. The radical scavenging capacity (RSC) and anticancer activity for methanolic extracts of both basidiocarps and mycelia of *Tricholoma giganteum* were compared, and both possessed antioxidant property and antitumor activity against human cell lines of cervix, liver and ductal carcinoma.

In Europe, there is a very long tradition on wild mushrooms collection and some species are sold in markets for human consumption. Among them three species of the genus *Tricholoma* (Tricholomateceae) are mainly interesting due to the high wild collection and the consumption level. These species are *Tricholoma terreum S*., *T. fracticum B*., and *T. gausapatum F*. and are considered high class of edible mushrooms because of their fruiting bodies. The food value of these mushrooms has now increased because research results determined that they are low in calories and high in minerals, essential amino acids, vitamins and fibers [9]. Information on their cytotoxicity and antioxidant activities also exists but it is not extensive. Tel., *et al*. [1] published an article where antioxidant and cholinesterase inhibition activities were reflected of three *Tricholoma* species, including *T. fracticum* and *T. terreum*. Later, Yin., *et al*. [10] evaluated the cytotoxicity of *T. terreum* against five human cancer cell lines.

In this study, authors provide data from research results on cytotoxic and antioxidant activity of methanolic extracts from Tricholoma species collected in Spain (Europe), such as *T. terreum, T. fracticum* and *T. gausapatum*. The main aim of this work is to search functional or medicinal properties that can increase the food value in these mushrooms and their agricultural interest. Some molecules involved in these activities could react and easily change into other substances during storage, with partial or complete loss of biological properties [11]. Therefore, the stability of antioxidant and cytotoxic capacity of extracts during storage was also analyzed.

Materials and Methods

Fungi material

The studied species were collected in Teruel (Spain), and fruiting bodies of basidiocarps were used to obtain methanolic extracts. Descriptions of each species were realized based on dichotomous keys for fungi identification and fungal flora of Iberian Peninsula.

Fungi extracts

Fresh collected fungi were dried in a food dehydrator (Back to basics Inc.) for 10 hours. Basidocarps with larvae were isolated for further preparations. Dehydrated material was powdered, extracted and preserved from light with methanol. 100 mL of methanol were added to 10g of mushroom and kept at room temperature for 48h Extracts were periodically agitated, after filtration. Then, in order to obtain a second extract, fresh methanol was added to fungi residue and the same extraction procedure was followed. Both extracts were mixed and concentrated to dryness at 50°C under reduced pressure. Extracts were kept at - 10°C until use. Stock solutions were prepared by dissolving the dried residue in dimethyl sulphoxide (DMSO) (0.1 g/mL).

Four extracts were used to determine their cytotoxic activity: one from *T. gausapatum;* one from *T. fracticum*; and two from *T. terreum:* healthy basidiocarps, labeled as *T. terreum*, and healthy basidiocarps, but extracted 11 months before, in order to study the effect of storage, labeled as *T. terreum*-O.

DPPH free radical scavenging activity assay

Scavenging free radical activity was tested in a DPPH methanol solution [11]. Solution decoloration degree indicates the scavenging efficiency of the sample. Extracts were dissolved in methanol at different concentrations, 100 µL of each dilution was added to 100 µL of DPPH 1 mM in methanol. After 30 minutes, absorbance was measured at 517 nm in a microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA). A blank was prepared with 100 µL of methanol plus 100 µL of DPPH. Ascorbic acid was used as reference compound. The inhibition activity percentage was calculated as [(A0-A1)/A0] x 100, where A0 was the absorbance of the blank and A1 was the absorbance of the extract. Tests were performed in triplicate.

In vitro cytotoxicity assay

Human cervix carcinoma cell line (HeLa), and human Caucasian colon adenocarcinoma (HT-29), obtained from the European Collection of Cell Cultures (ECACC, Ref. 93021013, and 91072201, respectively) were used. HeLa cell line was cultured in EMEM (Minimum Essential Medium Eagle's (Earle's Balanced Salt Solution) supplemented with 2 mM glutamine, 1% nonessential amino acids, 10% foetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. HT-29 cell line was grown on McCoy's 5a containing 2 mM glutamine, 10% foetal bovine serum and 100 units/mL penicillin and 100 µg/mL streptomycin. Cells were incubated at 37°C in an atmosphere of 5% CO2 in air (95% humidity). Cells in log phase of their grown cycle were plated in 96-well plates at a density of 2500 cells/well. After 24h of incubation, cells were treated with different concentrations of each extract. Final DMSO concentration in the assay did not exceed 0.1%. PBS was used as control. After 72h of incubation, fungus cytotoxicity was evaluated using MTT assay. The effective dose that inhibits 50% growth after the incubation period (IC_{50}) was then calculated for each extract [12]. Each experiment was performed in triplicate.

Statistical analysis

Data obtained in DPPH assay were analyzed for statistical significance ($P < 005$) by ANOVA followed by Bonferroni test. Levene test was used to perform variance homogeneity test.

Quantitative values obtained per treatment in the cytotoxicity assay were converted to percentage inhibition. Regression analysis was used to compute the inhibition concentration required to produce a 50% reduction in cell viability (IC_{50}). Results were expressed as the mean ± SD of values obtained in triplicate from three independent experiments. Treatments were compared using one-way analysis of variances (ANOVA) followed by Bonferroni test and considered significantly different where probability values were found to be equal to or less than 0.05.

Results and Discussion

Botanical description of used material

Tricholoma fracticum (Britzelm.) Kreisel has a distinctive, two-colored stem that is white near the apex and orangish brown. Other distinguishing features include the reddish brown, sticky (when young) cap and the strongly bitter taste. It is found in coniferous woodlands, particularly pine forest, in Europe, growing scattered or gregariously, sometimes in clusters. The cap is 3 - 10 cm; broadly convex

or nearly flat, and slimy or sticky when fresh but soon dry to reddish or orangish brown. It is smooth or with a few appressed fibers over the center, with the margin initially rolled under somewhat. The stalk is whitish near the apex, sometimes discoloring a little brownish, 2 - 8 cm long and 1 - 2.5 cm thick, more or less equal or tapering to the base and orangish brown below, with a small but clearly defined bracelet of flimsy and gelatinous tissue between the two zones (the result of a cortina-like partial veil that covers the young gills), solid and fleshy. The flesh is white, not changing on exposure, and has taste strongly bitter and odor, not distinctive or slightly mealy. The gills are attached to the stalk, often by means of a notch, and are close and whitish to very pale orange, developing brownish discolorations.

Tricholoma gausapatum (Fr.) Quél., commonly known as mice, is similar to *T. terreum* and *T. myomyces*. It is found in coniferous woodlands and broadleaf forest in Europe. The cap is up to 8 cm wide, conical-convex to flat with a slight boss and covered in fine cuticle that is dry, separable, prune-like or pilose, with radial arrangement. The stalk is gray-white, up to 7 cm high, cylindrical or somewhat thickened at the base, sometimes with a slight embellishment as a curtain. The whitish flesh is consistent and has mealy smell. The gills are whitish to gray with a notch.

Tricholoma terreum (Schaeff.) P. Kumm., commonly known as the grey knight or dirty *Tricholoma*, is a grey-capped mushroom of the genus Tricholoma. It is found in coniferous woodlands in Europe. The cap is 4 - 7 cm wide and covered in fine grey scales, convex with a slight boss which is broadly conical in shape. The stalk is whitish, 3 - 8 cm high and 1.5 cm wide and has no ring. There is no ring or volva. The whitish flesh is thin, easily broken, and has pleasant mild smell and taste, not mealy. The gills are widely spaced, uneven and free (unattached to the stalk).

Extract yields are shown in table 1.

*T. gausapatu*m	*T. fracticum*	*T. terreum*	*T. terreum*-O
17.04 %	21.42 %	19,34 %	19,16 %

Table 1: *Extracts yields (% w/w).*

DPPH free radical scavenging activity assay

Dietary antioxidants are supplements that can exert positive pharmacological effects on specific human diseases by neutralizing the negative effects of free radicals, because these are directly responsible for cell oxidative stress, thus contribute to chronic disease development [13]. Antioxidant compounds combat free radicals by intervening at their mediated oxidative process [14]. In consequence, antioxidant dietary intake is highly recommended in order to prevent cell damage and to preserve health. Synthetic antioxidants have been reported to be dangerous for human health. Thus, the search for effective, nontoxic natural compounds with antioxidative activity has been intensified in recent years [15].

DPPH scavenging activity of the extracts appears in figure 1 showing a dose dependent scavenging activity.

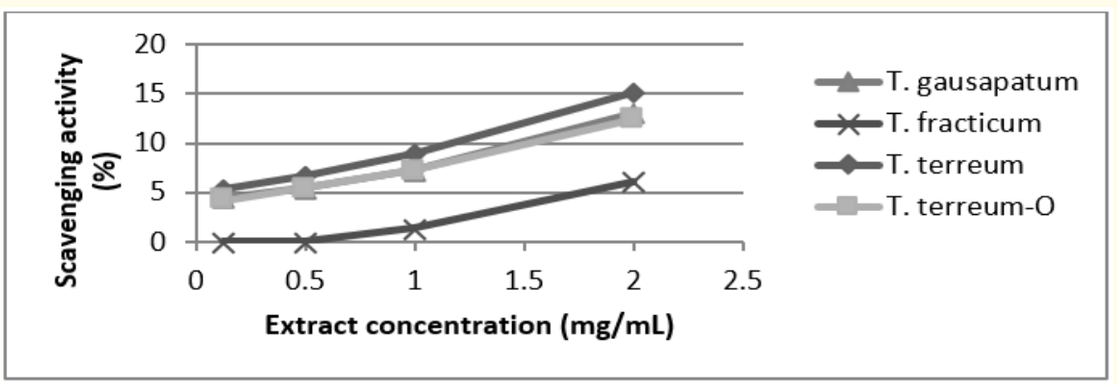

Figure 1: *DPPH scavenging activity (%) of the extracts at different concentrations.*

Except for *T. fracticum* all the extracts showed similar scavenging abilities. An antioxidant capacity decay of the *T. terreum* extract after 11 months of storage is detected. Because of chemical complexity of extracts, where compounds with different polarity, functional groups and chemical behavior are present, alterations and degradation of those chemical compounds are expected during storage. Extracts were kept at low temperatures (8 - 10°C), which has been suggested to preserve bioactive compounds and antioxidant capacity [16]. However, a significant decrease in bioactivity has been detected (Figure 1 and 2).

Although all treatments showed similar trends, there are significant differences between treatments at the highest concentration, as it is shown in figure 2.

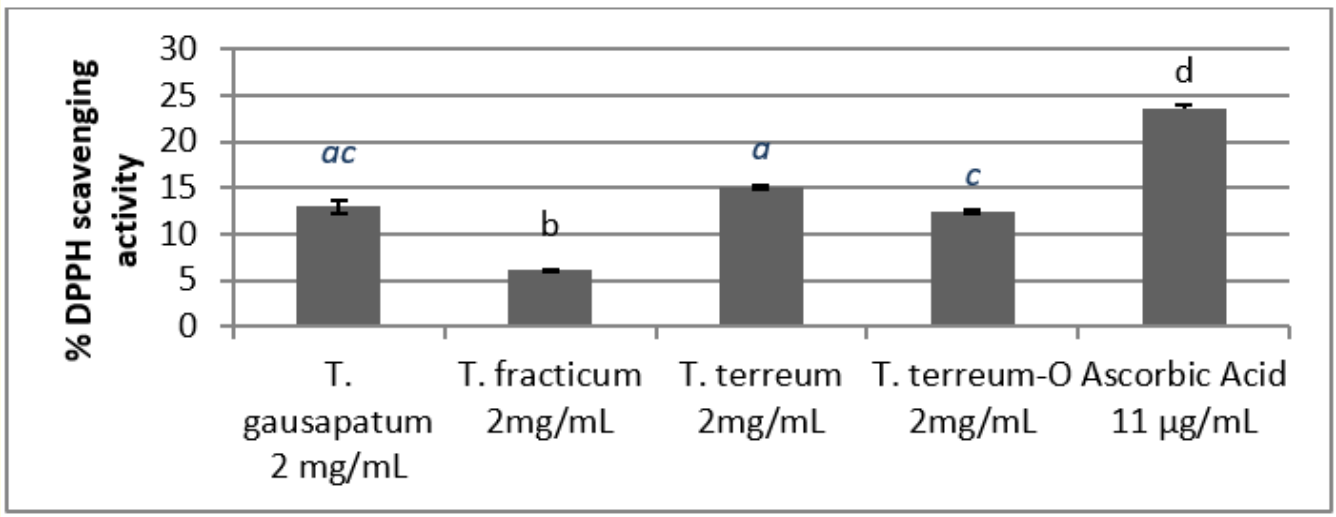

Figure 2: *% of DPPH scavenging activity of extracts at 2 mg/mL concentration and ascorbic acid (reference compound) at 11 µg/mL. Different letters indicate statistical differences (ANOVA-Bonferroni, P < 0,05).*

These results did not agree with those obtained by Tel., *et al*. [1] who found highest antioxidant activities for both *T. terreum* and *T. fracticum*. In addition, contrary to our results these authors found that *T. fracticum* methanolic extract had better DPPH scavenging activity that *T. terreum*. These authors found that their DPPH assay results differed from the other antioxidant assays they tested. This was explained by the fact that in the case of some bulky compounds, some antioxidants give incorrect results in the DPPH assay due to the steric hindrance. This could be also an explanation for the discrepancy between their results and ours. Otherwise, it was reported that cultivated and wild samples of the same species have different chemical composition [17].

The presence of phenolic compounds has been reported for mushrooms [18]. These kinds of compounds are primarily responsible for antioxidant activity [19]. The DPPH scavenging activity of antioxidants are related to their capability to donate their hydrogens, reducing DPPH radical to the non-radical form DPPH-H. In this sense, mushrooms are regarded as non-sources of flavonoids, and their antioxidant activity could be related mainly to other compounds. Diterpenoids [20], triterpenoids [21], steroids [22], phenolic monoterpenoids [23], meroterpenoids [24], indole derivatives and polysaccharides have been isolated [25] and some have demonstrated biological activities.

Cytotoxicity assay

Cytotoxicity of methanolic extract from studied Tricholoma species over HeLa and HT-29 cell lines, were expressed as IC50 values and appear in figure 3.

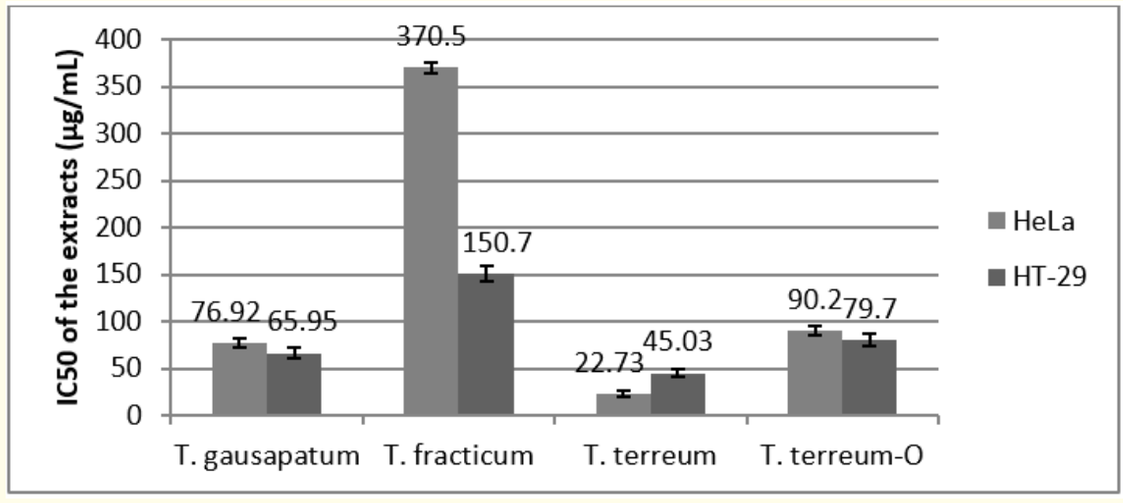

Figure 3: *Concentration of the different Tricholoma methanolic extracts that inhibited 50% cell growth (IC50) in different cancer cell lines (µg/mL). Values are expressed in µg/mL as means ± SD of triplicate experiments. Different letters indicate statistical differences between treatments for each cell line independently (ANOVA-Bonferroni, P < 0,05).*

Results showed that *T. fracticum* and *T. gausapatum* have little or no toxicity over the studied cell lines. Interestingly, *T. terreum* extracts showed lower IC_{50} value related to the concentration IC50 < 30 µg/mL for HeLa cell line. The criteria of cytotoxicity activity for crude extracts is an IC_{50} < 30 µg/mL in the preliminary assay, according to the American National Cancer Institute (NCI). However, when *T. terreum* extract is stored during 11 months, decay in activity is noticeable. HT-29 *T. terreum* treatment did not present a potent cell growth inhibition. This selectivity and an IC_{50} for HeLa near 20 µg/mL let us to consider methanolic extract of *T. terreum* as a potent extract and to identify it as prime targets for further screening. Recently [10,26] isolated four rare meroterpenoids from *T. terreum*. These compounds are probably responsible for the myotoxicity suspected for these mushrooms. These substances also showed cytotoxic activity on five human cancer lines, HL-60, SMMMC-77212, A-549, MCF-7 and SW480 with values comparable with those of cisplatin. Having into account that we have tested a crude extract and not an isolated molecule, further studies with these species are warranted. This cytotoxic property might be added to nutritional value of *T. terreum* therefore it could be considered a potential functional food, defining functional food as those containing various factors to ensure or enhance health.

Conclusion

The results presented in this study showed a significant cytotoxic activity of *T. terreum* methanolic extract on HeLa cell line. It is also noticeable a selectivity toward cervical cancer cell line in comparison with colon adenocarcinoma cell line. Further studies on the isolation and identification of the bioactive constituents are needed. On the other hand, none remarkable DPPH scavenging activity of the methanolic extracts tested was observed. Other assay must be tested in order to know the possible implication of other antioxidant mechanisms of action. In any case, *T. terreum* might be considered a potential functional food due to its cytotoxic activity on several human cancer cell lines which adds to its nutritional characteristics.

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