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Free-radical scavenging capacity and reducing power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity

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Abstract

The antioxidant properties of two wild edible mushroom species from the northeast of Portugal, *Lactarius deliciosus* (L.) Gray and *Tricholoma portentosum* (Fr.) Quél., were evaluated. Methanolic extracts from the entire mushroom, the cap and the stipe, separately, were screened for their reducing power and free radical scavenging capacity by chemical assays. The total phenolic content was determined, in order to assess its effect on the extract's antioxidant activity. Both two species showed antioxidant potential; but *L. deliciosus* proved to be more active. The portion of the mushroom used had an influence on the results obtained, with the cap methanolic extracts exhibiting the greatest antioxidant effect.
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Keywords: Wild edible mushrooms; *Lactarius deliciosus*; *Tricholoma portentosum*; Scavenging effect; Reducing power; Total phenols

1. Introduction

Reactive oxygen species are formed during normal cellular metabolism, but when present in high concentration they become toxic. Mammalian cells possess intracellular defences such as superoxide dismutase, catalase or glutathione peroxidase, in order to protect the cells against excessive levels of free radicals. Also exogenous addition of compounds such as vitamins (A, E, β-carotene), minerals (selenium, zinc), or proteins (transferrin, ceruloplasmin, albumin) can provide additional protection (Ostrovidov et al., 2000). These natural antioxidants or other compounds that can neutralize free radicals may be of central importance in the prevention of vascular diseases, some forms of cancer (Halliwell, 1997; Nakayama, Yamada, Osawa, & Kawakishi, 1993) and oxidative stress responsible for DNA, protein and membrane damage. Superoxide, hydrogen peroxide and hydroxyl radicals, which are mutagens produced by radiation, are also by-products of normal metabolism. Lipid peroxidation is also a major cause of food deterioration, affecting colour, flavour, texture and nutritional value (Halliwell & Gutteridge, 1999). Even though it is unclear whether active compounds are active against free radicals after being absorbed and metabolized by cells in the body, radical-scavenging assays have gained acceptance for their capacity to rapidly screen materials of interest.

The search for new products with antioxidative properties is a very active domain of research. Mushrooms have been used as food and food-flavouring material in soups and sauces for centuries, due to their unique and subtle flavour. Recently, they have become attractive as functional foods and as a source of physiologically beneficial medicines, while being devoid of undesirable side-effects (Sagakami, Aohi, Simpson, & Tanuma, 1991). Mushrooms were also found to be medically active in several therapies, such as antitumour, antibacterial, antiviral, haematological and immunomodulating treatments. In particular, mushrooms useful against cancers of the stomach, esophagus, lungs, etc., are known in China, Japan, Korea, Russia, the United States and Canada. Both cellular components and secondary metabolites of a large number of mushrooms have been shown to affect the immune system of the host and therefore could be used to treat a variety of diseases (Wasser

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& Weis, 1999). The antioxidative and free radical scavenging properties of the phenolic content of mushroom methanolic extracts have been reported, suggesting possible protective roles of these compounds, due to their ability to capture metals, inhibit lipoxygenase and scavenge free radicals (Mau, Chang, Huang, & Chen, 2004). Recently, Valentão et al. (2005a) identified the presence of six phenolic compounds (3-, 4- and 5-*O*-caffeoylquinic acid, caffeic acid, *p*-coumaric acid and rutin) and five organic acids (citric, ascorbic, malic, shikimic and fumaric acids) in wild edible mushroom *Cantharellus cibarius* Fr., kept under four different conditions (dried, frozen, preserved in olive oil and in vinegar). The organic acids citric, ketoglutaric, malic, succinic, oxalic, ascorbic, quinic, shikimic and fumaric were also found in the edible mushrooms *Amanita caesarea* (Scop.) Pers., *Boletus edulis* Bull., *Gyroporus castaneus* (Bull.) Quél., *Lactarius deliciosus* (L.) Gray, *Suillus collinitus* (Fr.) Kuntze and *Xerocomus chrysenteron* (Bull.) Quél. (Valentão et al., 2005b).

The northeast of Portugal, due to its climatic conditions and flora diversity, is one of the European regions with a high diversity of wild edible mushrooms, some of them with great gastronomic relevance. Within the local edible species, *L. deliciosus* (L.) Gray, *B. edulis* Bull., *Hydnum rufescens* Pers. and *Cantharellus cibarius* Fr. are the most important because of their high consumption by the rural population and their economic value in the markets, of France and Spain (Baptista, Branco, & Martins, 2003; Martins, Baptista, Sousa, Meireles, & Pais, 2002).

Herein, we report chemical assays on the antioxidant activity of two wild edible mushroom species, *L. deliciosus* (L.) Gray and *Tricholoma portentosum* (Fr.) Quél., from northeast Portugal. For the first time, the entire mushroom, the cap and the stipe individually were studied in order to compare their antioxidant properties.

2. Materials and methods

2.1. Standards and reagents

Standards BHA (2-*tert*-butyl-4-methoxyphenol), α-tocopherol and gallic acid were purchased from Sigma (St. Louis, MO, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Methanol was obtained from Pronalab (Lisbon, Portugal). Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, USA).

2.2. Samples

Samples of *L. deliciosus* and *T. portentosum* were collected under live pine trees (*Pinus* sp.), in Bragança (northeast of Portugal), in autumn 2004. After collection, the mushrooms were grouped by taxon and were air-dried in a liophylizator (Ly-8-FM-ULE, Snijders, Holland) before analysis. Taxonomic identification was made according to several authors (Bon, 1988; Courtecuisse, 1999; Courtecuisse & Duhem, 1995; Marchand, 1971–1986; Moser, 1983) and representative voucher specimens were deposited at the herbarium of Escola Superior Agrária of Instituto Politécnico de Bragança.

2.3. Sample preparation

All the assays were performed using either the entire mushroom, the cap or the stipe separately.

A fine dried mushroom powder (20 mesh) sample (10 g) was extracted by stirring with 100 ml of methanol at 25 °C at 150 rpm for 24 h and filtered through Whatman No. 4 paper. The residue was then extracted with two additional 100 ml portions of methanol, as described earlier. The combined methanolic extracts were evaporated at 40 °C to dryness and redissolved in methanol at a concentration of 50 mg/ml, and stored at 4 °C until further use.

2.4. Determination of total phenolic content

Phenolic compounds concentration in the mushroom methanolic extracts was estimated by a colorimetric assay, based on procedures described by Singleton and Rossi with some modifications (Singleton & Rossi, 1965). Briefly, 1 ml of sample was mixed with 1 ml of Folin and Ciocalteu's phenol reagent. After 3 min, 1 ml of saturated sodium carbonate solution was added to the mixture and adjusted to 10 ml with distilled water. The reaction was kept in the dark for 90 min, after which the absorbance was read at 725 nm (Analytikijena 200-2004 spectrophotometer). Gallic acid was used to calculate the standard curve (0.01–0.4 mM). Estimation of the phenolic compounds was carried out in triplicate. The results were mean values ± standard deviations and expressed as mg of gallic acid equivalents/g of extract (GAEs).

2.5. Reducing power assay

The reducing power was determined according to the method of Oyaizu (1986). Various concentrations of mushroom methanolic extracts (2.5 ml) were mixed with 2.5 ml of 200 mmol/l sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 650 rpm for 10 min. The upper layer (5 ml) was mixed with 5 ml deionised water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured at 700 nm: higher absorbance indicates higher reducing power. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm against extract concentration. BHA and α-tocopherol were used as standards.

2.6. Radical-scavenging activity (RSA) assay

The capacity to scavenge the "stable" free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) was monitored according to the method of Hatano, Kagawa, Yasuhara, and Okuda (1988). Various concentrations of methanolic extracts from mushrooms (0.3 ml) were mixed with 2.7 ml of methanolic solution containing DPPH radicals (6×10^{-5} mol/l). The mixture was shaken vigorously and left to stand for 60 min in the dark (until stable absorption values were obtained). The reduction of the DPPH radical was measured by monitoring continuously the decrease of absorption at 517 nm. The radical-scavenging activity (RSA) was calculated as a percentage of DPPH discolouration using the equation: $\%\text{RSA} = 100 \times (1 - A_c/A_D)$, where A_c is the absorbance of the solution when the extract has been added at a particular level, and A_D is the absorbance of the DPPH solution. The assays were carried out in triplicate and the results are expressed as mean values ± standard deviations. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of RSA percentage against extract concentration. BHA and α-tocopherol were used as standards.

3. Results and discussion

Two wild edible mushroom species (*L. deliciosus*, *T. portentosum*) from the northeast of Portugal were evaluated for their content in total phenols, reducing power and free-radical scavenging capacity. Total phenolic content was determined using Folin and Ciocalteu's phenol reagent. The reducing power was evaluated measuring absorbance at 700 nm after mixing the samples with ferric compounds; higher absorbance indicates higher reducing power. The scavenging effects on DPPH radicals were determined measuring the decay in absorbance at 517 nm due to the DPPH radical reduction, indicating the antioxidant activity of the compounds in a short time (Amarowicz, Pegg, Rahimi-Moghaddam, Barl, & Weil, 2004; Cheung, Cheung, & Ooi, 2003; Mau, Lin, & Song, 2002; Yen & Hung, 2000). All the assays were carried out using the entire mushroom, the cap and the stipe separately.

3.1. Determination of total phenols

It had been reported that the antioxidant activity of plant materials is well correlated with the content of their phenolic compounds (Velioglu, Mazza, Gao, & Oomah, 1998). In fact, phenols such as BHT (butylated hydroxytoluene) and gallate, are known to be effective antioxidants. Table 1 shows the phenols concentration in the mushroom extracts, expressed as mg of gallic acid equivalents (GAEs) per g of extract.

The contents of total phenols in methanolic extracts from *L. deliciosus* were 17.25 ± 0.65 mg/g while in *T. portentosum* extracts contained only 10.80 ± 0.47 mg/g. The portion of the mushroom used influenced the results obtained. As expected, the extracts from the entire mushroom showed higher phenolic content than either the cap or the stipe. The amount of phenolic compounds in the cap methanolic extracts (10.66 ± 0.52 mg/g for *L. deliciosus* and 6,57 ± 0.31 mg/g for *T. portentosum*) was higher than the amount found in stipe extracts (6.31 ± 0.29 mg/g for *L. deliciosus* and 3,91 ± 0.17 mg/g for *T. portentosum*).

The higher content of total phenols in the *L. deliciosus* extracts might account for the better results found in their reducing power and radical scavenging effect on DPPH radicals.

3.2. Reducing power assay

Figs. 1 and 2 show the reducing power of mushrooms methanolic extracts as a function of their concentration. In this assay, the yellow colour of the test solution changes to various shades of green and blue, depending on the reducing power of each compound. The presence of reducers (i.e. antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to the ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+}concentration.

The reducing power of the mushroom methanolic extracts increased with concentration. The reducing power

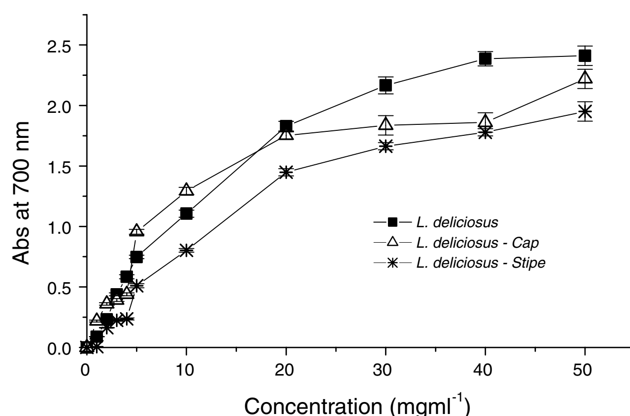

Fig. 1. Reducing power of methanolic extracts from *L. deliciosus*. Each value is expressed as mean ± standard deviation ($n = 3$).

Table 1
Contents of total phenols in methanolic extracts from two wild edible mushrooms

Content[a] (mg/g)					
Lactarius deliciosus	*L. deliciosus* (cap)	*L. deliciosus* (stipe)	*Tricholoma portentosum*	*T. portentosum* (cap)	*T. portentosum* (stipe)
17.25 ± 0.65	10.66 ± 0.52	6.31 ± 0.29	10.80 ± 0.47	6.57 ± 0.31	3.91 ± 0.17

[a] Each value is expressed as mean ± standard deviation ($n = 3$).

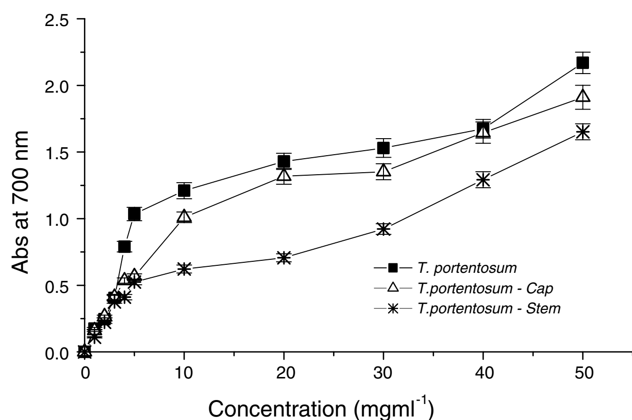

Fig. 2. Reducing power of methanolic extracts from *T. portentosum*. Each value is expressed as mean ± standard deviation ($n = 3$).

of both species was excellent (Figs. 1 and 2); at 50 mg/ml the reducing power was higher than 1.65 and in the order *L. deliciosus* > *L. deliciosus* cap > *T. portentosum* > *L. deliciosus* stipe > *T. portentosum* cap >> *T. portentosum* stipe. At 5 mg/ml, the reducing powers of the methanolic extracts from wild edible mushrooms were 0.52–0.96, and at 1 mg/ml were 0.006–0.22. Reducing power of BHA at 3.6 mg/ml and α-tocopherol at 8.6 mg/ml was only 0.12 and 0.13, respectively. Methanolic extracts from *T. portentosum* showed slightly lower reducing power values (Fig. 2) than *L. deliciosus* (Fig. 1). The extracts obtained from the mushroom cap proved to be a better source of antioxidants than extracts from the mushroom stipe. The use of the entire mushroom increased the reducing power (at 50 mg/ml – 2.41 for *L. deliciosus* and 2.17 for *T. portentosum*).

It was reported that the reducing power of mushrooms might be due to their hydrogen-donating ability (Shimada, Fujikawa, Yahara, & Nakamura, 1992). Accordingly, *L. deliciosus* might contain higher amounts of reductone, which could react with free radicals to stabilise and block radical chain reactions.

3.3. Radical-scavenging activity (RSA) assay

The RSA of mushrooms extracts was tested using a methanolic solution of the "stable" free radical, DPPH. Unlike laboratory-generated free radicals such as the hydroxyl radical and superoxide anion, DPPH has the advantage of being unaffected by certain side reactions, such as metal ion chelation and enzyme inhibition (Amarowicz et al., 2004). A freshly prepared DPPH solution exhibits a deep purple colour with absorption maximum at 517 nm. This purple colour generally fades/disappears when an antioxidant is present in the medium. Thus, antioxidant molecules can quench DPPH free radicals (i.e., by providing hydrogen atoms or by electron donation, conceivably via a free-radical attack on the DPPH molecule) and convert them to a colourless/bleached product (i.e., 2,2-diphenyl-1-hydrazine, or a substituted analogous hydrazine), resulting in a decrease in absorbance at 517 nm. Hence, the more rapidly the absorbance decreases, the more potent the antioxidant activity of the extract. Free radical scavenging is one of the known mechanisms by which antioxidants inhibit lipid oxidation. This test is a commonly employed assay in antioxidant studies of specific compounds or extracts across a short time scale.

The RSA values of methanolic extracts from *L. deliciosus* and *T. portentosum* were examined and compared against one another (Figs. 3 and 4). Results are expressed as the ratio percentage of sample absorbance decrease and the absorbance of DPPH solution in the absence of extract at 517 nm.

From the analysis of Figs. 3 and 4, we can conclude that the scavenging effects of mushrooms methanolic extracts on DPPH radicals increased with the concentration increase and were high (79.1–84.3% at 50 mg/ml) for *L. deliciosus*. Methanolic extracts from *T. portentosum* presented moderate RSA values (30.4–65.0% at 50 mg/ml) and lower than the other species. However, the scavenging effects of BHA (3.6 mg/ml) and α-tocopherol (8.6 mg/ml) were 96% and 95%, respectively. The extracts obtained from the mushroom cap scavenged DPPH radicals by higher per-

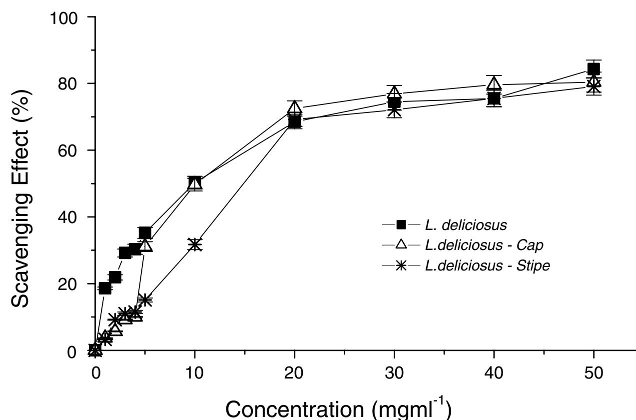

Fig. 3. Scavenging activity (%) on DPPH radicals of methanolic extracts from *L. deliciosus*. Each value is expressed as mean ± standard deviation ($n = 3$).

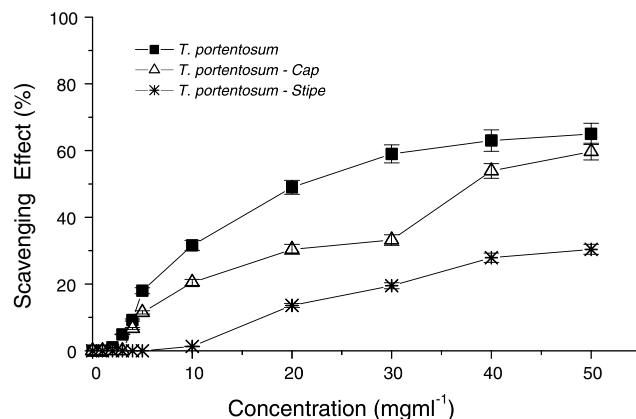

Fig. 4. Scavenging activity (%) on DPPH radicals of methanolic extracts from *T. portentosum*. Each value is expressed as mean ± standard deviation ($n = 3$).

Table 2
EC_{50} values[a,b] (mg/ml) of mushrooms extracts in reducing power and DPPH scavenging assays

Samples	Reducing power (EC_{50}[a])	DPPH (EC_{50}[b])
Lactarius deliciosus	3.42	8.52
L. deliciosus (cap)	3.58	11.9
L. deliciosus (stipe)	6.69	15.1
Tricholoma portentosum	3.12	22.9
T. portentosum (cap)	3.69	40.2
T. portentosum (stipe)	4.82	>50

[a] EC_{50} (mg/ml): effective concentration at which the absorbance is 0.5.
[b] EC_{50} (mg/ml): effective concentration at which 50% of DPPH radicals are scavenged.

centages (80.4% for *L. deliciosus* and 59.7% for *T. portentosum* at 50 mg/ml) than extracts from the stipe mushroom (79.1% for *L. deliciosus* and 30.4% for *T. portentosum* at 50 mg/ml). The use of the entire mushroom increased the RSA values (at 50 mg/ml-80.4% for *L. deliciosus* and 65.0% for *T. portentosum*).

In Table 2, we present the EC_{50} values for reducing power and DPPH scavenging effects obtained from each mushroom methanolic extract.

Overall, *L. deliciosus* revealed better antioxidant properties than *T. portentosum* (lower EC_{50} values), which is in agreement with the higher content of phenols found in the first species. This was much more evident in EC_{50} values for DPPH scavenging effect (8.52 mg/ml for *L. deliciosus* versus 22.9 mg/ml for*T. portentosum*). The use of the entire mushroom is recommended and the mushroom cap proved to be better than the stipe; particularly for *T. portentosum* the DPPH scavenging effects decreased drastically when the stipe was used. The EC_{50} values obtained for reducing power were better than for RSA. Searching wild sources may bring new natural products into the food industry with safer and better antioxidants that provide good protection against the oxidative damage, which occurs both in the body and our daily foods. Therefore, new wild edible mushrooms, as natural sources, could be introduced for this purpose. As far as our literature survey could ascertain, little information was available on the in vitro antioxidative activities of European wild mushrooms and it is the first time that Portuguese wild edible mushrooms were submitted to these studies.

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