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Insights in the antioxidant synergistic effects of combined edible mushrooms: phenolic and polysaccharidic extracts of *Boletus edulis* and *Marasmius oreades*

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Summary

In a previous work, we reported the presence of *Marasmius oreades* in mixtures with antioxidant synergistic effects, and the mixture *Boletus edulis* and *Marasmius oreades* (50% of each) as having the highest antioxidant activity, but without synergism among the phenolic extracts. Herein, phenolic and polysaccharidic extracts from both species were combined in different proportions (12.5, 25, 50, 75 and 87.5%) and compared to controls (individual samples), in order to give insight in the contribution of each species to antioxidant synergistic effects. The antioxidant activity of the individual or combined extracts obtained from both edible mushrooms was evaluated through their 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical-scavenging activity, reducing power, β-carotene bleaching inhibition and lipid peroxidation inhibition in brain homogenates using thiobarbituric acid reactive substances (TBARS) assay. Synergism was the main observed effect among the combined extracts, either phenolic or polysaccharidic. However, the effect of each *B. edulis* and *M. oreades* proportion was not the same for all antioxidant activity assays. In fact, the same mixture exhibited sometimes opposite behaviours according with the performed antioxidant activity assay.

Keywords

edible mushrooms; phenolics; polysaccharides; antioxidant activity; synergism

Mushrooms have been described as a good source of digestible proteins, saccharides, fibres and vitamins, while having low fat contents [1–4]. They are consumed as a special food particularly for their specific aroma and texture, being culinary-processed in different manners, or used as food-flavoring material in soups and sauces [5].

Besides their nutritional quality, mushrooms demonstrated health-promoting properties, proving anti-inflammatory, antitumour, antibacterial, antiviral, showing also antiallergic, antiatherogenic, hypoglycemic and hematological properties, being as well included in immunomodulating therapies [6, 7]. Some of the mentioned properties involve antioxidant mechanisms, and mushrooms are also rich sources of antioxidant compounds, mainly phenolic compounds [8]. These compounds can act as free radical inhibitors (chain breakers), peroxide decomposers, metal inactivators or oxygen scavengers [9]. Despite their non-nutritive nature, when included in diet, phenolic compounds may provide a health advantage associated with reduced risk of chronic diseases related to oxidative stress [10].

Mushroom polysaccharides also have radical-scavenging activity, which is related to an increase in the activities of the antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) [11]. Furthermore, biological properties of mushroom polysaccharides and their protein complexes have been extensively described, including their antitumour and immunomodulatory effects [12, 13].

Details on the antioxidant effects of individual mushroom extracts have been published. In a previous work, our research group evaluated the anti-

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oxidant properties of combined edible mushrooms (50% of each extract), in order to analyse synergistic effects between mushroom species [14]. *Marasmius oreades* was present in the mixtures with higher antioxidant properties and synergistic effects. The mixture *Boletus edulis* and *Marasmius oreades* (50% each) exhibited the highest antioxidant activity, but without synergism.

In the present work, phenolic and polysaccharidic extracts from both species were combined in different proportions (12.5, 25, 50, 75 and 87.5%) and compared to controls (individual samples), in order to give insight in the contribution of each species to antioxidant synergistic effects.

MATERIALS AND METHODS

Standards and reagents

Gallic acid, starch and Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) were purchased from Sigma (St. Louis, Missouri, USA). 2,2-Diphenyl-1-picrylhydrazyl (DPPH) was obtained from Alfa Aesar (Ward Hill, Massachusetts, USA). All other chemicals and solvents were of analytical grade and purchased from common sources. Water was treated in a Milli-Q water purification system (TGI Pure Water Systems, Greenville, South Carolina, USA).

Mushroom species and sample preparation

Boletus edulis Fr. (Be) and *Marasmius oreades* (Bolt. ex Fr.) Fr. (Mo) were commercial dried (dehydrated) samples obtained in local supermarkets (Bragança, Northeast Portugal). For extraction, the mushroom samples were combined in different proportions: 0% (1.5 g Be), 12.5% (0.1875 g Mo + 1.3125 g Be), 25% (0.375 g Mo + 1.125 g Be), 50% (0.75 g Mo + 0.75 g Be), 75% (1.125 g Mo + 0.375 g Be) and 87.5% (1.3125 g Mo + 0.1875 g Be) and 100% (1.5 g Mo).

Preparation of the phenolic extracts

The mushrooms combined in different proportions (1.5 g) were extracted with methanol (30 ml) at 25 °C, 2.5 Hz (Velp Are magnetic stirrer; VELP Scientifica, Usmate, Italy) for 1 h and subsequently filtered through Whatman No. 4 paper (Whatman, Maidstone, United Kingdom). The residue was then extracted with two additional 30 ml portions of methanol. Combined extracts were evaporated under reduced pressure (rotary evaporator Büchi R-210; Büchi, Flawil, Switzerland) and redissolved in methanol at a known concentration (20 mg·ml^{-1}) for further analyses.

Preparation of the polysaccharidic extracts

The mushrooms combined in different proportions (1.5 g) were extracted with water at boiling temperature (50 ml) for 2 h and agitated (2.5 Hz) and subsequently filtered through Whatman No. 4 paper. The residue was then extracted with two more portions of boiling water, in a total of 6 h of extraction. The combined extracts were lyophilized, and then 95% ethanol (10 ml) was added and polysaccharides were precipitated overnight at 4 °C. The precipitated polysaccharides were collected after centrifugation (Centurion K24OR refrigerated centrifuge, Centurion Scientific, Stoughton, United Kingdom) at 3 100 ×*g* for 40 min followed by filtration, and then were lyophilized, resulting in a crude polysaccharidic sample [15]. The crude polysaccharidic samples were re-dissolved in water at a known concentration (20 mg·ml^{-1}) for further analyses.

Quantification of antioxidants

Total phenolics

The extract solution (1 ml) was mixed with Folin-Ciocalteu reagent (5 ml, previously diluted with water 1 : 10, v/v) and sodium carbonate (75 g·l^{-1}, 4 ml). The tubes were vortex-mixed for 15 s and allowed to stand for 30 min at 40 °C for colour development [16]. Absorbance was then measured at 765 nm (Analytik Jena spectrophotometer, Analytik Jena, Jena, Germany). Gallic acid was used to construct the standard curve (0.0094–0.15 mg·ml^{-1}), and the results were expressed as grams of gallic acid equivalents (GAE) per kilogram of extract.

Total polysaccharides

The extract solution (1 ml) was added to 80% phenol (25 μl) and concentrated sulphuric acid (1 ml). The mixture was shaken and allowed to stand at 30 °C for 30 min. The absorbance was measured at 490 nm [17]. Starch (although glycogen is the storage polysaccharide in mushrooms, starch is the most available polysaccharide) was used to construct the standard curve (0.625–40 mg·ml^{-1}), and the results were expressed as grams of polysaccharides equivalents (PE) per kilogram of extract.

Evaluation of antioxidant activity

DPPH radical-scavenging activity

The assay was performed in 96-well microtiter plates using an ELX800 Microplate Reader (Bio-Tek Instruments, Potton, United Kingdom). The reaction mixture in each well consisted of

the extract with various concentrations (30 μl) and methanolic solution containing DPPH radicals (6×10^{-5} mol·l^{-1}, 270 μl). The mixture was left to stand for 30 min in the dark. The reduction of the DPPH radical was determined by measuring the absorption at 515 nm. The radical-scavenging activity (*RSA*) was calculated as a percentage of DPPH discolouration using the equation:

$$RSA = \frac{(A_{\mathrm{DPPH}} - A_{\mathrm{S}})}{A_{\mathrm{DPPH}}} \times 100 \qquad (1)$$

where A_S is the absorbance of the solution when the sample extract was added at a particular level, and A_{DPPH} is the absorbance of the DPPH solution. The extract concentration providing 50% of radical-scavenging activity (EC_{50}) was calculated from the graph of *RSA* percentage against extract concentration. Trolox was used as standard.

Reducing power

Extract with various concentrations (0.5 ml) was mixed with sodium phosphate buffer (200 mmol·l^{-1}, pH = 6.6, 0.5 ml) and potassium ferricyanide (1% w/v, 0.5 ml). The mixture was incubated at 50 °C for 20 min, and trichloroacetic acid (10% w/v, 0.5 ml) was added. The mixture (0.8 ml) was poured into the wells of a 48-well microplate, followed by deionized water (0.8 ml) and ferric chloride (0.1% w/v, 0.16 ml), and the absorbance was measured at 690 nm. The extract concentration providing 50% of absorbance (EC_{50}) was calculated from the graph of absorbance at 690 nm against extract concentration. Trolox was used as standard.

Inhibition of β-carotene bleaching

A solution of β-carotene was prepared by dissolving β-carotene (2 mg) in chloroform (10 ml). Two millilitres of this solution were pipetted into a round-bottom flask. Chloroform was removed at 40 °C under vacuum and linoleic acid (40 mg), Tween 80 (400 mg) as emulsifier and distilled water (100 ml) were added to the flask at vigorous shaking. Aliquots (4.8 ml) of this emulsion were transferred into test tubes containing extract with various concentrations (0.2 ml). The tubes were shaken and incubated at 50 °C in a water bath. At the addition of the emulsion to the tube, the zero-time absorbance was measured at 470 nm. β-Carotene bleaching inhibition was calculated using the following equation:

$$\frac{P_2}{P_0} \times 100 \qquad (2)$$

where P_2 is β-carotene content after 2 h of assay and P_0 is the initial β-carotene content.

The extract concentration providing 50% antioxidant activity (EC_{50}) was calculated by interpolation from the graph of β-carotene bleaching inhibition percentage against extract concentration. Trolox was used as standard.

Inhibition of lipid peroxidation using thiobarbituric acid reactive substances (TBARS)

Brains were obtained from porcine (*Sus scrofa*), dissected and homogenized with a Polytron instrument (Capitol Scientific, Austin, Texas, USA) in ice-cold Tris-HCl buffer (20 mmol·l^{-1}, pH = 7.4) to produce a 1 : 2 w/v brain tissue homogenate, which was consequently centrifuged at 3 000 ×*g* for 10 min. An aliquot (0.1 ml) of the supernatant was incubated with the extract with various concentrations (0.2 ml) in the presence of $FeSO_4$ (10 mmol·l^{-1}; 0.1 ml) and ascorbic acid (0.1 mmol·l^{-1}, 0.1 ml) at 37 °C for 1 h. The reaction was stopped by the addition of trichloroacetic acid (28% w/v, 0.5 ml), followed by thiobarbituric acid (TBA, 2%, w/v, 0.38 ml), and the mixture was then heated at 80 °C for 20 min. After centrifugation at 3 000 ×*g* for 10 min to remove the precipitated protein, the colour intensity of the malondialdehyde (MDA)-TBA complex in the supernatant was quantified based on its absorbance at 532 nm. The inhibition ratio *I* (in percent) was calculated using the following formula:

$$I = \frac{(A - B)}{A} \times 100 \qquad (3)$$

where *A* and *B* were the absorbances of the control and the sample solution, respectively. Trolox was used as standard.

Statistical analysis

All dependent variables were analysed using 2-way ANOVA, with the factors "extract (E)" (phenolic or polysaccharidic) and the "*M. oreades* percentage (Mo)" (0, 12.5, 25, 50, 75, 87.5 and 100%). The 2-way ANOVA, with Type III sums of squares, was performed using the General Linear Model (GLM) procedure of the SPSS software, version 18.0 (SPSS, New York, New York, USA). Since a statistically significant interaction effect ("E × Mo") was found in all tests for 2-way ANOVA, the two factors were evaluated simultaneously by plotting the estimated marginal means for all levels of each factor.

RESULTS AND DISCUSSION

B. edulis, king bolete, is a popular edible mushroom in Europe (in Portugal is among the most

Tab. 1. Antioxidant activity as EC_{50} values of the combined mushroom samples.

		Radical-scavenging activity [mg·ml^{-1}]	Reducing power [mg·ml^{-1}]	β-Carotene bleaching inhibition [mg·ml^{-1}]	TBARS inhibition [mg·ml^{-1}]
M. oreades percentage (Mo)	0%	2.7 ± 0.1	1.6 ± 0.4	1.3 ± 0.4	3 ± 2
	12.5%	3 ± 1	1.1 ± 0.5	0.8 ± 0.4	1 ± 1
	25%	3 ± 1	0.8 ± 0.2	0.4 ± 0.2	1 ± 1
	50%	6 ± 1	1.2 ± 0.4	0.3 ± 0.3	0.5 ± 0.4
	75%	3 ± 1	1.0 ± 0.3	0.5 ± 0.1	0.17 ± 0.03
	87.5%	4 ± 1	1.2 ± 0.2	0.4 ± 0.3	1 ± 1
	100%	2.7 ± 0.1	1.4 ± 0.5	1.1 ± 0.5	0.5 ± 0.3
	p-value	< 0.001	< 0.001	< 0.001	< 0.001
Type of extract (E)	Phenolic	4 ± 1	1.6 ± 0.3	1.0 ± 0.5	0.4 ± 0.2
	Polyssacharidic	3 ± 1	0.8 ± 0.2	0.4 ± 0.3	2 ± 1
	p-value	< 0.001	< 0.001	< 0.001	< 0.001
Mo × E	*p*-value	< 0.001	< 0.001	< 0.001	< 0.001

Results are reported as a mean value of *M. oreades* percentage (Mo) in both types of extracts (E) as well as mean value of both E within each Mo. Therefore, standard deviation reflects values in those samples (under different Mo or E). Presenting results in this way allows a more suitable evaluation of the effects of both factors (Mo and E) independently of each other.
Number of samples $n = 18$, for each *M. oreades* percentage (Mo); $n = 63$ for each type of extract (E).
Mo × E – interaction effect among *M. oreades* percentage and the type of extract.

appreciated), North America and Asia [2, 18]. In previous studies, we reported its nutritional and nutraceutical properties [1], as well as its strong antioxidant activity [14], related to phenolic compounds previously described by our research group such as protocatechuic (2.02 mg·kg^{-1} of dry weight, dw), *p*-hydroxybenzoic (6.55 mg·kg^{-1} dw) and *p*-coumaric (1.17 mg·kg^{-1} dw) acids and a related compound (cinnamic acid- 3.72 mg·kg^{-1} dw) [2]. Information about *M. oreades* is scarcer, however, it was reported to have antioxidant and antimicrobial activities, and to contain bioactive compounds, such as tocopherols, phenolics, flavonoids, carotenoids and ascorbic acid [1, 19]. In view of the chemical complexity observed among natural matrixes, combining the extracts of different species often results in synergistic effects regarding their bioactivity. However, when the extracts of these two mushrooms were mixed in a 50:50 ratio, that effect was not observed [14]. From Tab. 1 in relation to the type of extract, it is possible to conclude that the proportions of each mushroom extract used in the mixture resulted in different antioxidant activities, with a higher discrepancy

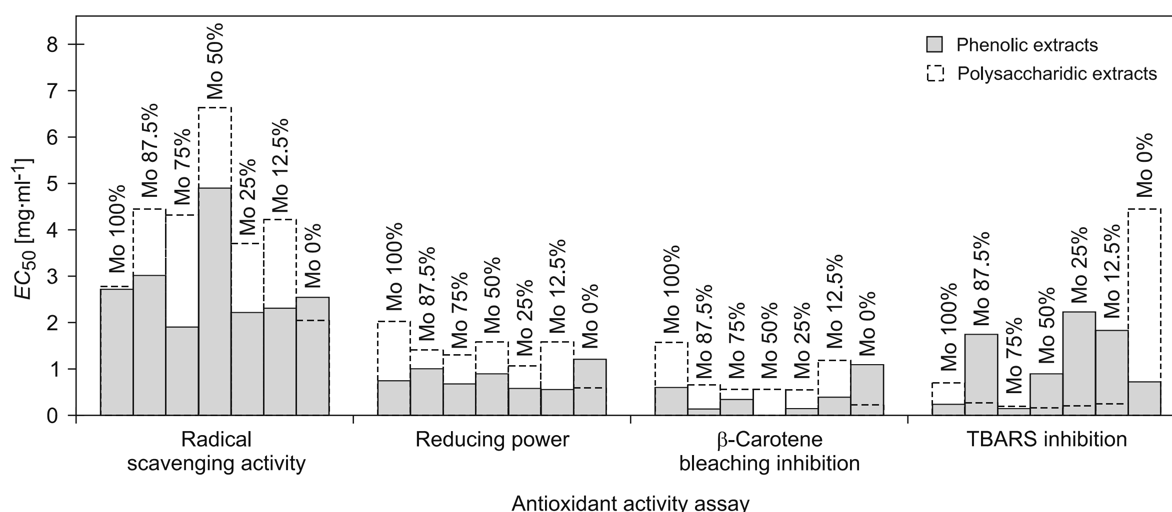

Fig. 1. Comparison of the antioxidant activity assays by EC_{50} values obtained for phenolic and polysaccharidic extracts.

for TBARS assay. These differences could not be statistically classified since a significant interaction effect (“E × Mo”) was found in all tests. It is not possible to infer which of the studied factors contributes most to the observed differences. Nevertheless, the former tendencies may be observed in Fig. 1, where the graph indicates a slight disparity for the diverse assayed proportions. These differences were also observed among the contents of bioactive compounds (Fig. 2). However, the obtained values were not linearly correlated ($R^2 < 0.6$) with any of the measured antioxidant activities.

Another interesting finding is that, independently of the assayed proportions, the polysaccharidic extracts showed higher antioxidant activities than the phenolic extracts (except for TBARS assay). In fact, polysaccharides extracted from *B. edulis* were reported to have many biological functions such as anticancer, antioxidant, antidiabetic and anti-inflammatory effects [20], showing also potential to be employed as ingredients in healthy and functional food to alleviate the oxidative stress [21].

In order to define which proportions of each mushroom allow the best antioxidant activity, the type of observed interactions (synergism, additive or negative synergism effects) were assessed (Tab. 2). The assayed combinations were more

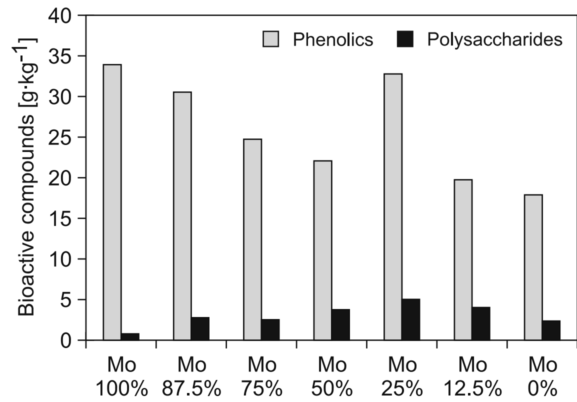

Fig. 2. Contents of phenolics and polysaccharides in the mushroom mixtures.

Contents of phenolics are expressed as grams of gallic acid equivalents per kilogram of extract. Contents of polysaccharides are expressed as grams of polysaccharide equivalents per kilogram of extract.

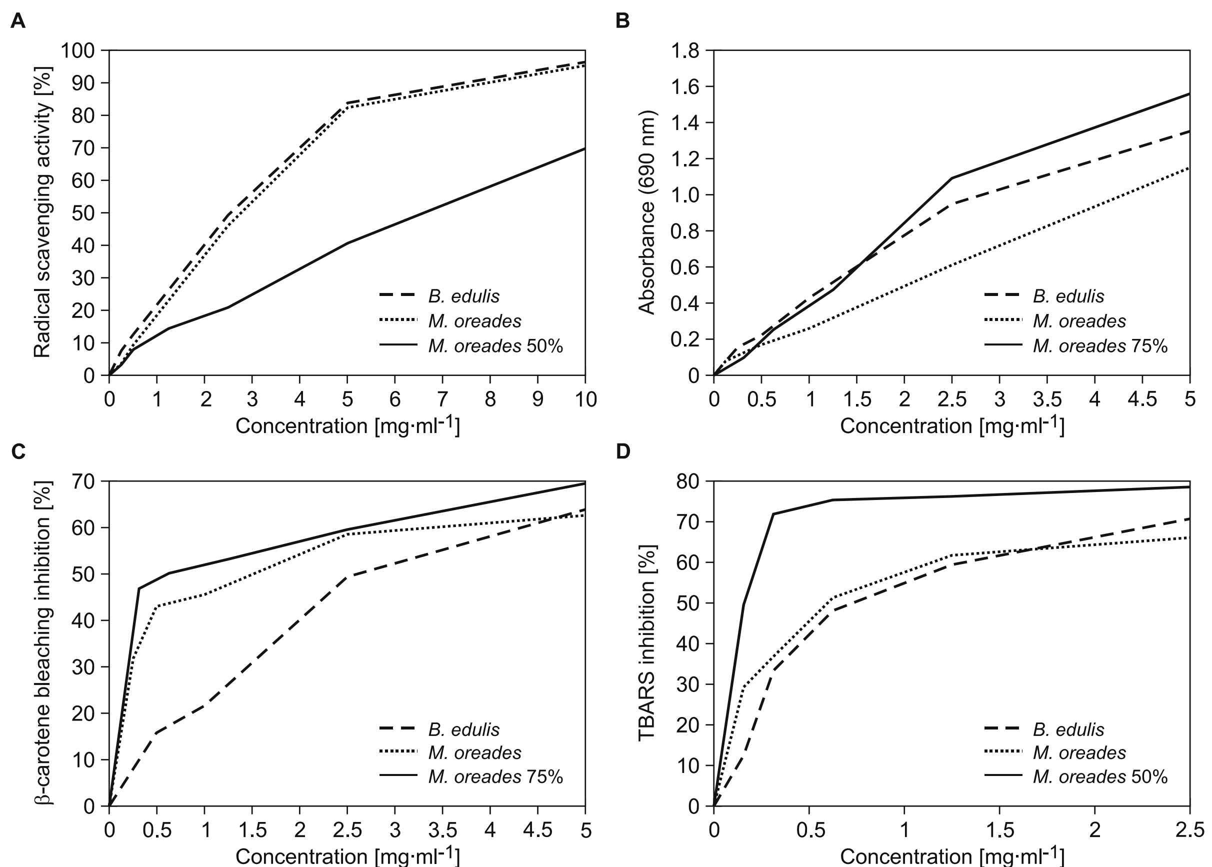

Fig. 3. Effects of combined phenolic extracts on DPPH scavenging inhibition (A), reducing power (B), β-carotene bleaching inhibition (C) and TBARS inhibition (D).

Tab. 2. Theoretical versus experimental values of antioxidant activity EC_{50} of the combined mushroom samples.

Extracts	Proportions	Radical scavenging activity [mg·ml⁻¹]			Reducing power [mg·ml⁻¹]			β-Carotene bleaching inhibition [mg·ml⁻¹]			TBARS inhibition [mg·ml⁻¹]		
		Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect	Theoretical	Experimental	Effect
Phenolic	Mo (0%)*	–	2.5 ± 0.1	–	–	1.21 ± 0.04	–	–	1.1 ± 0.3	–	–	0.72 ± 0.04	–
	Mo (12.5%)	2.6 ± 0.1	4.2 ± 0.2	NS	1.312 ± 0.004	1.58 ± 0.01	NS	1.2 ± 0.3	1.2 ± 0.3	A	0.7 ± 0.1	0.25 ± 0.01	S
	Mo (25%)	2.60 ± 0.05	3.7 ± 0.1	NS	1.413 ± 0.004	1.06 ± 0.01	S	1.2 ± 0.3	0.55 ± 0.01	S	0.7 ± 0.1	0.21 ± 0.04	S
	Mo (50%)**	2.66 ± 0.04	6.6 ± 0.2	NS	1.617 ± 0.003	1.6 ± 0.1	A	1.3 ± 0.3	0.6 ± 0.1	S	0.7 ± 0.1	0.159 ± 0.001	S
	Mo (75%)	2.72 ± 0.02	4.3 ± 0.1	NS	1.821 ± 0.003	1.30 ± 0.01	S	1.5 ± 0.3	0.56 ± 0.04	S	0.7 ± 0.1	0.196 ± 0.002	S
	Mo (87.5%)	2.75 ± 0.02	4.4 ± 0.1	NS	1.922 ± 0.002	1.41 ± 0.03	S	1.5 ± 0.3	0.7 ± 0.1	S	0.7 ± 0.2	0.272 ± 0.001	S
	Mo (100%)	–	2.78 ± 0.02	–	–	2.02 ± 0.01	–	–	1.6 ± 0.4	–	–	0.7 ± 0.1	–
Poly-saccharidic	Mo (0%)*	–	2.0 ± 0.1	–	–	0.59 ± 0.01	–	–	0.22 ± 0.04	–	–	4.4 ± 0.4	–
	Mo (12.5%)	2.1 ± 0.1	2.3 ± 0.2	NS	0.61 ± 0.01	0.56 ± 0.01	S	0.27 ± 0.01	0.39 ± 0.02	NS	3.9 ± 0.3	1.8 ± 0.1	S
	Mo (25%)	2.22 ± 0.05	2.2 ± 0.1	A	0.63 ± 0.01	0.58 ± 0.01	S	0.32 ± 0.01	0.145 ± 0.001	S	3.4 ± 0.3	2.2 ± 0.2	S
	Mo (50%)	2.38 ± 0.02	4.9 ± 0.1	NS	0.67 ± 0.05	0.89 ± 0.01	NS	0.41 ± 0.05	0.055 ± 0.001	S	2.3 ± 0.2	0.9 ± 0.1	S
	Mo (75%)	2.55 ± 0.04	1.9 ± 0.3	S	0.708 ± 0.002	0.68 ± 0.01	A	0.5 ± 0.1	0.34 ± 0.01	S	1.3 ± 0.1	0.147 ± 0.004	S
	Mo (87.5%)	2.6 ± 0.1	3.2 ± 0.2	NS	0.728 ± 0.003	1.01 ± 0.01	NS	0.6 ± 0.1	0.138 ± 0.001	S	0.77 ± 0.03	1.7 ± 0.1	NS
	Mo (100%)	–	2.7 ± 0.1	–	–	0.75 ± 0.01	–	–	0.6 ± 0.1	–	–	0.24 ± 0.01	–

Theoretical values were calculated as weighted mean EC_{50} values (considering additive contributions of individual species in each percentage).
A – additive effect: theoretical and experimental values reveal differences lower than 5%, S – synergistic effect: experimental values are more than 5% lower for EC_{50} when compared with theoretical values, NS – negative synergistic (antagonistic) effect: experimental values are more than 5% higher for EC_{50} when compared with theoretical values.
* – Mo (0%) corresponds to *B. edulis* (100%), ** – Reference [14].

effective in the lipid peroxidation inhibition assays (β-carotene bleaching and TBARS assays), in which synergism was the predominant effect (80% of the cases for β-carotene bleaching inhibition and 90% of the cases for TBARS assay). In fact, the free radical scavengers are located in different sites (intracellular and extracellular), acting in a cooperative way, and some antioxidant compounds inhibit the oxidation of biological molecules interacting with different compounds [22]. In this way, the relative similarity of β-carotene bleaching and (specially) TBARS inhibition assays with some specific in vivo conditions might have exerted a positive influence on the antioxidant activity of the compounds present in the extracts.

The graphs presented in Fig. 3 and Fig. 4 elucidate the most representative mixture (this is, the mixture that revealed the highest percentage of the effect observed in the majority of tested mixtures) for each antioxidant activity assay. In Fig. 3, where the results for the phenolic extracts are represented, the mixture containing 50% of *M. oreades* was the most antagonistic for DPPH scavenging activity, but also the one with the highest synergistic effect for TBARS assay, while the mixture with 75% of *M. oreades* was the most antagonistic for reducing power and the most synergistic for β-carotene bleaching inhibition. Regarding polysaccharidic extracts (Fig. 4), the mixture containing equal proportions of each mushroom was again the most antagonistic for DPPH scavenging activity, but also the one with the highest synergistic effect for β-carotene bleaching inhibition, while the mixture with 87.5% of *M. oreades* was the most antagonistic for reducing power and the most synergistic for TBARS assay.

CONCLUSION

The effect (synergistic, additive and negative synergistic) observed after mixing different percentages of mushroom species showed different behaviour according to the used propor-

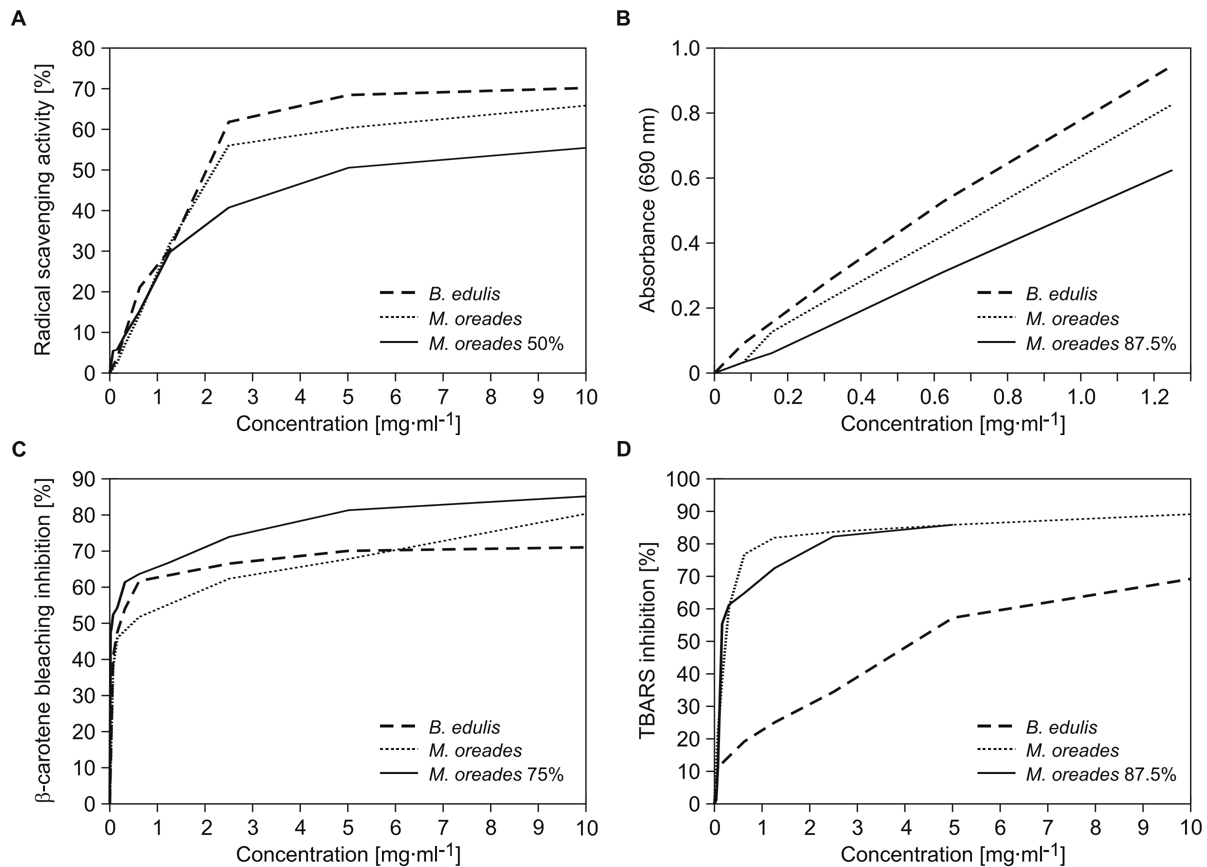

Fig. 4. Effects of combined polysaccharidic extracts on DPPH scavenging inhibition (A), reducing power (B), β-carotene bleaching inhibition (C) and TBARS inhibition (D).

tions. Despite the same solid-to-liquid ratio used in all extractions (1.5 g per 30 ml for methanolic extracts and 1.5 g per 50 ml for aqueous extracts), it is possible that, either for phenolic or polysaccharidic extracts, the different extracted masses resulted in different yields of specific compounds, resulting in different chemical interactions. Furthermore, it became clear that the produced interactions act in a specific manner, giving an effect that depends on the antioxidant activity assay used. Actually, the same mixture was frequently the one that induced the most synergistic effect in an antioxidant activity assay and also the one that provoked the most intensive negative synergistic effect in a different antioxidant activity assay (for example, the mixture containing 50% of *M. oreades*, using phenolic extracts, for DPPH scavenging activity and TBARS inhibition assay). This might indicate different compositions among the antioxidant constituents of each mushroom, showing also that the proportion of each mushroom must be defined considering the specific effect to which it is meant: reducing power, scavenging properties or lipid peroxidation inhibition.

The present study defines the proportion of mushrooms to be used in order to obtain antioxidant effects. Reducing or scavenging properties require different proportions of mushrooms. Combined mushrooms can be incorporated in diets as sources of antioxidants, or explored as sources of bioactive molecules by food and pharmaceutical industries.

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