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Effect of extraction solvent on chemical composition, physicochemical and biological properties of edible mushrooms extracts

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Abstract

Edible mushroom has been described as an important source of biological compounds able to reduce the load of pathogenic bacteria's and the free radical's levels in foods. In this study, chemical proximate analysis of edible mushrooms powder (*Agaricus brasiliensis*, *Ganoderma lucidum* and *Pleurotus ostreatus*) was determined. Also, it was determined the effect of the solvent (water, ethanol, and a mixture of water-ethanol) on the physicochemical properties, phenolic content, antimicrobial, and antioxidant activity of edible mushrooms extracts. The results indicate that *G. lucidum* and *P. ostreatus* powders showed ($p < 0.05$) the lowest moisture (< 4%), lipids (< 2%), the highest carbohydrates contents (> 80%), and *P. ostreatus* the lowest ash and the highest proteins contents ($p < 0.05$). The mushroom water extracts presented the highest lightness and TSS (total soluble solids) values ($p < 0.05$). *P. ostreatus* extracts showed the highest extraction yield (> 40%), pH, redness, yellowness, phenolic and flavonoids contents (p < 0.05). Moreover, *G. lucidum* and *P. ostreatus* ethanol and water-ethanol extracts a highest antimicrobial effect against *Staphylococcus aureus* > *Listeria innocua* > *Escherichia coli* > *Salmonella typhimurium* ($p < 0.05$). Regard antioxidant activity, *P. ostreatus* extracts showed the highest reducing power and antiradical activity, while *G. lucidum* and *P. ostreatus* ethanol and water-ethanol extracts a highest lipid oxidation inhibition ($p < 0.05$). The extracts of edible mushrooms evaluated could be used as antimicrobials and antioxidants ingredients for food industry.
Keywords: antimicrobial, antioxidant, edible mushroom, natural extract, extraction solvent.

Efecto del solvente de extracción sobre la composición química, propiedades fisicoquímicas y biológicas de extractos de hongos comestibles

Resumen

Los hongos comestibles han sido descritos como una fuente importante de compuestos biológicos capaces de reducir la carga de bacterias patógenas y los niveles de radicales libres en los alimentos. En este estudio, se determinó el análisis químico proximal de las harinas de hongos comestibles (*Agaricus brasiliensis*, *Ganoderma lucidum* y *Pleurotus ostreatus*). Asimismo, se determinó el efecto del solvente (agua, etanol y una mezcla de agua-etanol) sobre las propiedades fisicoquímicas, contenido de fenoles, actividad antimicrobiana y antioxidante de los extractos de hongos comestibles. Los resultados indican que las harinas de *G. lucidum* y *P. ostreatus* mostraron ($p < 0.05$) el contenido más bajo de humedad (< 4%), lípidos (< 2%), el contenido más alto de carbohidratos (> 80%), y *P. ostreatus* el contenido más bajo de ceniza y el más alto en proteínas ($p < 0.05$). Los extractos acuosos de los hongos presentaron los valores más altos de luminosidad y TSS (total soluble solids, por sus siglas en inglés) ($p < 0.05$). Los extractos de *P. ostreatus* presentaron alto rendimiento de extracción (> 40%), pH, color rojo, color amarillo y contenido de fenoles y flavonoides ($p < 0.05$). Por otra parte, los extractos etanólicos y acuoso-etanólicos de *G. lucidum* y *P. ostreatus* con un alto efecto inhibidor sobre *Staphylococcus aureus* > *Listeria innocua* > *Escherichia coli* > *Salmonella typhimurium* ($p < 0.05$). Respecto a la actividad antioxidante, los extractos de *P. ostreatus* presentaron el mayor poder reductor y actividad antirradical; mientras que, los extractos etanólicos y acuoso-etanólicos de *G. lucidum* y *P. ostreatus* la mayor inhibición en la oxidación de lípidos ($p < 0.05$). Los extractos de hongos comestibles evaluados podrían utilizarse como ingredientes antimicrobianos y antioxidantes para la industria alimentaria.
Palabras clave: antioxidante, antimicrobiano, extracto natural, hongos comestibles, solvente de extracción.

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Introduction

Food spoilage bacteria and oxidative process are the major contributors to decreasing the nutritional and sensory quality of meat and meat products, which consequently reduce consumers´ acceptability (Papuc, Goran, Predescu, Nicorescu & Stefan, 2017). For this reason, the use of antimicrobial and antioxidant agents are widely used for inhibiting or delay the growth of microorganism and oxidation reactions (Kumarasamy *et al*., 2010; Papuc *et al*., 2017; Poljsak, Šuput & Milisav, 2013). However, there are evidence of a growing number of microorganisms that are resistant to antibiotics, mainly caused by their uncontrolled use, and consequently causes a public health problem (Kumarasamy *et al*., 2010). In addition, the utilization of synthetic antioxidants (i.e., butylated hydroxyanisole, BHA; butylated hydroxytoluene, BHT; among others) have been also limited due to several studies have provided evidence of the potential risk in human health (Poljsak *et al.*, 2013).

In order to avoid the uncontrolled use of antimicrobial drugs and solve the disadvantages of synthetic antioxidant, several investigations have been reported in natural sources (edible and wild mushrooms, green tea, grape, apple, bee products and olive) the presence of antibacterial and antioxidant compounds. Polyphenols like catechin, ellagic acid, epicatechin, epicatechin gallate, gallic acid, tannic acid, caffeic acid, ferulic acid, protocatechuic acid, quercetin, among others, are commonly found in the above mentioned sources (Carneiro *et al*., 2013; Khatun, Mahtab, Khanam, Sayeed & Khan, 2007; Mazzutti *et al*., 2012; Moure *et al*., 2001; Papuc *et al*., 2017; Soares *et al*., 2009). Recently, edible mushroom has been proposed as an important source of bioactive compound to be employed as potential functional ingredient for food industry (Ma *et al*., 2018).

In this context, the food industry requires the use of appropriate and standard methods to extract active compounds from natural sources, i.e., using conventional (Soxhlet, maceration or hydrodistillation extraction) and non-conventional methods (ultrasound-assisted, pulsed-electric field, enzyme and microwave, pressurized liquid and supercritical fluid extraction) (Azmir *et al*., 2013). However, several factors such input parameters like temperature, pressure, time, and solvent used can affect the efficiency of the extraction process (Azmir *et al*., 2013; Cowan, 1999).

Therefore, the aim of this study was to determine the effect of the solvent extraction used on the chemical composition, physicochemical properties, as well as its antioxidant and antimicrobial activity of edible mushrooms extracts (*Agaricus brasiliensis*, *Ganoderma lucidum* and *Pleurotus ostreatus*).

Materials and methods

Materials and chemicals

The edible mushrooms powder (AB, *Agaricus brasiliensis*; GL, *Ganoderma lucidum*; and PO, *Pleurotus ostreatus*) were obtained from Aloha Medicinals, Inc. (USA). All chemicals were of analytical grade. Folin-Ciocalteu's reagent, sodium carbonate (Na_2CO_3), potassium persulfate ($K_2S_2O_8$), aluminum chloride ($AlCl_3$), methanol, ethanol, 1,1-diphenyl-2-picrylhydrazyl ($DPPH^•$), 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid ($ABTS^{•+}$), ammonium persulfate [$(NH_4)_2S_2O_8$], trichloroacetic acid ($C_2HCl_3O_2$), iron chloride ($FeCl_3$), brain hearth infusion agar (BHI, Difco TM), and Mueller Hinton agar were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Proximate composition

Edible mushrooms powder was analyzed for proximate composition (AOAC, 2005). Moisture content was obtained in dried samples (Yamato DX402 drying oven, Tokyo, Japan) at 105 °C for 12 h (method 934.01). Total protein content was determined by Kjeldahl method, using a conversion factor of N x 4.38 (method 968.06). The crude fat content was extracted with petroleum ether in a Goldfish apparatus (method 985.01), while ash content was determined by incineration in dried samples (method 920.153) at 550 °C for 5 h (Felisa muffle furnace FE-361, Jalisco, Mexico). The total carbohydrate content was estimated in percentage as follow: 100 - [moisture content (%) + protein content (%) + crude fat content (%) + ash content (%)].

Obtaining extracts

The bioactive compounds from edible mushrooms powder (20 g) were obtained with 180 mL of a polar (water) and reduced polar (ethanol and water-ethanol, 1:1 ratio) as solvent, using an ultrasound-assisted equipment (Branson 3800 Ultrasonic bath, Brason, Germany) at 25 °C/40 KHz for 1 h. The resultant mixture was filtered (Whatman #1 filter paper) under vacuum (MVP 6 vacuum pump, Jeju, Korea) and concentrated in a rotary-evaporator at 65 °C (Yamato RE301BW, Tokyo, Japan). The edible mushrooms extracts (EME) were lyophilized (Yamato DC401 Freeze Dryer, Tokyo, Japan) and stored at -20 °C in the dark, until analysis (Soares *et al*., 2009).

Physicochemical properties

The pH value of EME (50 mL, 5 mg/mL) was measured using a potentiometer (Hanna Multiparameter HI3512, Rhode Island, USA) with automatic temperature compensation (method 943.02) (AOAC, 2005). The total solids content of EME were determined according to the AOAC, 2005. An aliquot of each EME (5 mg/mL) was placed in a refractometer equipment and the results were expressed as °Brix (%) (method 932.14). The color evaluation of EME was carried out according to the CIElab parameters (L*, a*, and b*) (Robertson *et al*., 1977).

Briefly, EME (5 mg/mL) were placed in a 40 mL quartz cell, and determined with a spectrophotometer (Konica Minolta, CM508d, Tokyo, Japan), using D65 illuminant with 10° observer angle.

Phenolic composition

The total phenolic content (TPC) was determined by the Folin-Ciocalteu method (Ainsworth & Gillespie, 2007), with slight modifications. Briefly, each EME (50 µL, 5 mg/mL) was homogenized with 800 µL of distilled water, 200 µL of Folin-Ciocalteu's reagent (0.25 N), and 300 µL Na_2CO_3 (7%, w/v). The reaction mixture was incubated at 25 °C for 1 h, under dark, and the absorbance was measured at 750 nm in a spectrophotometer (Thermo Scientific Multiskan FC UV-Vis, Vantaa, Finland). The results were expressed as mg of gallic acid equivalents/g of dried extract (mg GAE/g).

The total flavonoid content (TFC) was determined based on aluminum chloride complex formation (Popova *et al*., 2004). Each EME (50 µL, 5 mg/mL) was homogenized with 650 µL of methanol and 50 µL of $AlCl_3$ (2.5%, w/v). The reaction mixture was incubated at 25 °C for 30 min, under dark, and the absorbance was measured at 412 nm. The results were expressed as mg quercetin equivalents/g (mg QE/g).

Antimicrobial activity

The antimicrobial activity was evaluated according to the disc diffusion method (Wiegand, Hilpert & Hancock, 2008). *Staphylococcus aureus* ATCC 29213B, *Listeria innocua*, *Salmonella typhimurium* ATCC 14028 and *Escherichia coli* ATCC 25922 were inoculated in BHI agar and incubated at 37 °C for 24 h. Afterwards, the cellular suspension (McFarland 0.5, approximately 1.5 x 10^8 UFC/mL) was cultivated on Muller-Hinton agar plates and incubated at 37 °C for 24 h. After, each EME (5 µL, 100 µg/mL) was added to the surface of the discs, using ethanol, water, a mixture of water-ethanol, and gentamicin as controls. The results were expressed as halo inhibition zones (mm).

Antioxidant activities

Reducing power and antiradical activity

The reducing power ability (RPA) was determined based on ferricyanide/prussian blue method (Berker, Güçlü, Tor, Demirata & Apak, 2010). Briefly, each EME (200 µL, 100 µg/mL) was homogenized with 500 µL of phosphate buffer (50 mM, pH 7.0) and 500 µL of potassium ferrocyanide (1%, w/v). The reaction mixture was incubated at 50 °C for 20 min (Yamato BM500 water bath, Tokyo, Japan), mixed with 250 µL of trichloroacetic acid (10%, w/v), and centrifuged at 3,900 g for 10 min (Thermo Fisher Scientific Sorvall ST16R, IL, USA). After, 500 µL of supernatant was homogenized with 100 µL of distilled water and 100 µL $FeCl_3$ (0.1%, w/v). The absorbance was measured at 700 nm and results expressed as absorbance at the same wavelength.

Antiradical activity was measured by the $DPPH^{\bullet}$ radical scavenging method (Molyneux, 2004). All EME (500 µL, at 100 µg/mL) were homogenized with 500 µL of $DPPH^{\bullet}$ solution (300 µmol) and incubated at 25 °C for 30 min, under dark. The absorbance was measured at 517 nm. The results were expressed as inhibition (%) = [1 - Abs(S)/Abs(0)] x 100, where Abs(S) is the absorbance of the antioxidants at 30 min, and Abs(0) the absorbance of control at 0 min.

Antiradical activity was also measured by the $ABTS^{\bullet+}$ radical cation scavenging method (Re *et al*., 1999). Each EME (10 µL, 100 µg/mL) was homogenized with 990 µL of $ABTS^{\bullet+}$ solution (adjusted to an absorbance of 0.8 nm). The samples were incubated at 25 °C for 30 min, under dark. The absorbance was measured at 730 nm. The results were expressed as inhibition (%) = [Abs(0) - Abs(S)/Abs(0)] x 100, where Abs(S) is the absorbance of the antioxidants at 30 min, and Abs(0) the absorbance of control at 0 min.

Antioxidant activity in a meat system

Lipid oxidation was measured by the TBARS method (Pfalzgraf, Frigg & Steinhart, 1995), with slight modifications. The meat extract was obtained homogenizing (4,500 rpm/5 °C/1 min) pork meat with distilled water (1:10, w/v) and the respective antioxidants at 500 ppm (50 µL of BHT or 1 mL of EME), using an Ultraturrax (T25, IKA®, Staufen, Germany). The resultant solution was incubated (37 °C, at different periods for 16 h) in a water bath. Then, meat homogenates (0.5 mL) were mixed with 1 mL of TCA solution (10%, w/v) in a vortex mixer at 10,000 rpm for 1 min (Analog vortex mixer, Fisher Scientific TM, Nueva Jersey, USA). After, 1 mL of the resultant filtered solution (Whatman 4 filter paper) was homogenized with 1 mL of TBA solution (0.02 M) and placed in a water bath (97 °C for 20 min), and subsequently cooled. The absorbance was measured at 531 nm and results expressed as mg of malondialdehyde per kg of meat (mg MDA/kg).

Statistical analysis

All measurements were conducted in triplicate with at least three independent experiments, and the results were expressed as mean ± standard deviation. An analysis of variance (ANOVA) was used to analyze the data, and the Tukey-Kramer method was used to compare the averages ($p < 0.05$). Furthermore, a principal component analysis (PCA) was performed to evaluate the relationships among the analyzed variables (SPSS, version 21).

Results and discussion

Proximate composition of edible mushrooms

The results showed significant differences ($p < 0.05$) between the carbohydrates > protein > ash > moisture > fat content of all analyzed mushrooms (Table I). According to these results, *G. lucidum* and *P. ostreatus* powders presented ($p < 0.05$) the

lowest moisture (< 4%) and lipids contents (< 2%), and the highest carbohydrates contents (> 80%). Also, *P. ostreatus* powder showed the lowest ash and the highest protein contents ($p < 0.05$). In agree with our results, it has been reported an ash and carbohydrates content between 2.8-8.9% and 78.2-81.5%, respectively, for *G. lucidum* (Stojkovic *et al*., 2013). However, in the same study were reported highest values for protein (9.9-11.3%) and fat content (2.8-8.9%). In another work (Vargas-Sánchez *et al*., 2018), using *P. ostreatus* was found a protein (12.3%), fat (3.3%), and carbohydrate content (64.4%), results in disagree with our study. Similarly, it has been reported for *A. brasiliensis* a different proximate composition with protein (31.3%), fat (1.8%), ash (7.5%), and carbohydrates (59.4%) content (Carneiro *et al*., 2013).

In addition, it has been reported that edible mushrooms composition varies between species which can also be associated with several factors such as maturation stage, the content of exopolysaccharides in the fruiting body, cultivation method (solid or liquid medium and substrate), environmental conditions, and conservation treatment (pH and temperature) (Carneiro *et al*., 2013; Lin & Sung, 2006; Stojkovic *et al*., 2013). Based on proximate composition, the carbohydrates and crude protein are the two main components (Kalač, 2009); thus, a variation of protein and carbohydrate content can affect the physicochemical and biological activity of the mushrooms (Pan, Jiang, Liu, Miao & Zhong, 2013; Yan *et al*., 2019).

Solvent effect on physicochemical properties

The results showed significant differences ($p < 0.05$) between the extraction yield, pH, and color of the analyzed samples by solvent effect (Table II). The highest extraction yields (> 40%) were obtained by *P. ostreatus* water extract, followed by ethanolic and aqueous-ethanolic extracts ($p < 0.05$). This higher extraction yield indicates a high solubility of the mushroom powder compounds, which is improved by increasing the solvent polarity (Azmir *et al*., 2013; Cheung, Cheung & Ooi, 2003). In agreement with our findings, it was reported an extraction yield of *Pleurotus eous* extract was higher (> 40%) using a polar solvent extraction (water > methanol > ethyl acetate) (Sudha, Vadivukkarasi, Shree & Lakshmanan, 2012). In addition, a similar extraction yield (34.2%) was showed for *P. ostreatus* water extract (Vargas-Sánchez *et al*., 2018). In disagree with our work, it has been reported higher extraction yields for *A. brasiliensis* water extract (45.2-46.6%) and *G. lucidum* water extract (26.4-28.8%) (Kozarski *et al*., 2011).

Moreover, the highest pH value (> 5.0) was obtained in *P. ostreatus* ethanol and aqueous-ethanolic extracts, while the lowest pH values were found in *A. brasiliensis* water and aqueous-ethanolic extracts ($p < 0.05$). No significant differences ($p > 0.05$) were found in pH values of *G. lucidum* extracts, when compared between extraction solvents. The results also showed highest °Brix values (>16) in all samples extracted with the polar solvent; however, no significant differences ($p > 0.05$)

Table I. Proximate composition of the edible mushroom powders (%).

Mushroom	Moisture	Protein	Fat	Ash	Carbohydrates
A. brasiliensis	4.6 ± 0.1^b	6.4 ± 0.1^b	2.9 ± 0.3^c	6.7 ± 0.1^b	79.4 ± 0.2^a
G. lucidum	3.4 ± 0.1^a	5.9 ± 0.1^a	1.4 ± 0.1^a	7.8 ± 0.1^c	81.5 ± 0.3^b
P. ostreatus	3.3 ± 0.3^a	8.4 ± 0.1^c	1.7 ± 0.3^a	5.2 ± 0.1^a	81.3 ± 0.1^b

Data are mean ± standard deviation (n = 6). Different superscripts (a-c) in each column indicate significant differences between samples ($p < 0.05$).

Table II. Effect of solvent extraction on physicochemical properties of EME.

Mushroom	Solvent	Yield	pH	TSS	L*	a*	b*
A. brasiliensis	Water	19.5 ± 0.3^a	4.3 ± 0.01^a	17.0 ± 0.5^b	29.1 ± 0.1^d	0.1 ± 0.0^a	2.7 ± 0.1^b
	Ethanol	25.9 ± 0.7^b	4.1 ± 0.01^c	7.9 ± 0.3^a	27.4 ± 0.3^b	0.2 ± 0.0^b	2.3 ± 0.1^a
	1:1	26.3 ± 0.5^b	4.1 ± 0.01^c	8.0 ± 1.0^a	27.1 ± 0.1^b	0.2 ± 0.0^b	2.2 ± 0.1^a
G. lucidum	Water	18.9 ± 0.5^a	4.2 ± 0.01^b	17.2 ± 1.0^b	29.5 ± 0.1^e	0.1 ± 0.0^a	5.0 ± 0.1^c
	Ethanol	25.8 ± 0.7^b	4.2 ± 0.01^b	6.1 ± 0.2^a	26.1 ± 0.5^a	0.2 ± 0.0^b	2.1 ± 0.1^a
	1:1	25.0 ± 0.7^b	4.2 ± 0.01^b	6.0 ± 1.0^a	26.6 ± 0.0^a	0.2 ± 0.0^b	2.0 ± 0.1^a
P. ostreatus	Water	46.9 ± 0.5^d	4.4 ± 0.02^e	16.4 ± 1.0^b	27.7 ± 0.1^c	2.1 ± 0.1^d	5.6 ± 0.1^d
	Ethanol	34.0 ± 0.3^c	5.5 ± 0.10^d	6.1 ± 0.1^a	28.1 ± 0.1^b	1.7 ± 0.1^c	6.0 ± 0.4^d
	1:1	34.2 ± 0.9^c	5.5 ± 0.02^d	6.0 ± 1.0^a	28.0 ± 0.2^b	1.7 ± 0.1^c	5.8 ± 0.2^d

Data are mean ± standard deviation (n = 6). TSS, total soluble solids (°Brix). Different superscripts (a-e) in each row indicate significant differences between samples ($p < 0.05$).

were found between EME obtained with the same solvent. In addition, it has been reported that acidic compounds presented in EME can reduce the pH values; while an increase in total soluble solid (TSS) is associated with the maturity stage of the wall of the mushrooms, and consequently, with the increase of the °Brix value (Jafri, Jha, Bunkar & Ram, 2013).

Regard color measurements, the highest L* values were obtained in all water extracts, and *G. lucidum* ethanol and aqueous-ethanolic extracts showed the lowest L* value ($p < 0.05$). *P. ostreatus* water extract presented the highest a* values, and the lowest a* values were found in *A. brasiliensis* and *G. lucidum* water extract ($p < 0.05$). The highest b* values was obtained in *P. ostreatus* extracts, and the lowest values were found in *A. brasiliensis* and *G. lucidum* ethanol and aqueous-ethanolic extracts ($p < 0.05$). The color is an important factor that influences the quality of edible mushrooms, which can be affected by the maturity or substrate supplementation received during the production, and by the presence of compounds such as polysaccharides and polyphenols (Mami, Peyvast, Ziaie, Ghasemnezhad & Salmanpour, 2014; Pan *et al.*, 2013). It has been demonstrated that the color of edible mushrooms can be influenced by the cooking method (steaming, pressure cooking, microwave, frying or boiling), as well as by the method used to obtain the powder (sun or oven dried) (Sun, Bai & Zhuang, 2014). In addition, phytochemicals as polysaccharides and phenolic compounds are responsible for the brown color of EME, and an increase in the dark-brown color can be associated with high levels of these compounds (Siu, Chen & Wu, 2014).

Solvent effect on phenolic composition

Phenolic compounds are secondary metabolites widely distributed in plants and mushrooms, which can be used as nutraceutical ingredients (Papuc *et al.*, 2017). The results of TPC and TFC of EME analyzed are display in Table III. The highest TPC (> 30 mg GAE/g) was showed in *P. ostreatus* ethanol and aqueous-ethanolic extracts ($p < 0.05$), and the lowest values ($p < 0.05$) were found in *A. brasiliensis* and *G. lucidum* water extract. In addition, the highest TFC (mg QE/g) value ($p < 0.05$) was found in *P. ostreatus* water extract, and lowest values ($p < 0.05$) were reported in *A. brasiliensis* ethanol and aqueous-ethanolic extracts. Regardless the mushroom species, the TFC was enhanced by the polar solvent.

In agreement with our work, a study reported that the highest TPC was obtained with the polar reduced solvent (ethanol > water) for *Ganoderma* spp. extracts. While, in another work, it was demonstrated that highest TPC were obtained with ethanol > water for *P. ostreatus* extracts (Vamanu, 2013). Also, it has been reported that the highest TFC was reported for *Agaricus* spp. obtained with the polar solvent (water > ethanol) (Abugri & McElhenney, 2013). On the other hand, in disagree it was reported the highest TPC and TFC in *Agaricus* spp. extracts obtained with the polar (water > 60% water-ethanol) and reduced polar (60% water-ethanol > water) solvent extraction, respectively (Gan, Amira & Asmah, 2013). As well, in another work *Pleurotus eous* showed a high TPC values in polar solvent extraction (water > methanol), while high TFC values were obtained in reduced polar solvent (methanol > water) (Sudha *et al.*, 2012).

Table III. Effect of solvent extraction on chemical composition and antibacterial activity of EME.

Mushroom	Solvent	TPC	TFC	Gram-positive		Gram-negative	
				S. aureus	*L. innocua*	*E. coli*	*S. typhimurium*
A. brasiliensis	Water	19.4 ± 0.5^a	13.8 ± 0.3^b	++	++	+	-
	Ethanol	21.7 ± 0.3^b	11.3 ± 0.1^a	++	++	++	-
	1:1	21.1 ± 0.4^b	11.0 ± 0.6^a	+	++	++	-
G. lucidum	Water	18.3 ± 1.2^a	19.1 ± 0.3^d	+	++	+	++
	Ethanol	22.8 ± 0.1^b	13.1 ± 0.2^b	++	+++	+	++
	1:1	22.6 ± 0.4^b	13.0 ± 0.1^b	++	+++	+	++
P. ostreatus	Water	31.7 ± 0.4^c	17.0 ± 1.4^d	++	++	+	+
	Ethanol	35.1 ± 0.1^d	15.3 ± 0.3^c	++	++	++	++
	1:1	34.8 ± 1.4^d	15.5 ± 0.2^c	++	++	++	++
Ethanol				-	-	-	-
Water				-	-	-	-
1:1				-	-	-	-
Gentamicin				+++	+++	+++	+++

(-), no inhibition (0-5 mm); + (< 8 mm), ++ (> 8 mm to < 12 mm), +++ (> 12 mm).

Solvent effect on antimicrobial activity

Furthermore, data of antimicrobial activity (Table III) indicate that EME showed different rates of bacterial inhibition. Regardless of the extraction solvent, the higher effect was obtained in Gram-positive (*L. innocua* > *S. aureus*) than Gram-negative bacteria (*E. coli* > S. *typhimurium*). Also, *G. lucidum* and *P. ostreatus* ethanol and aqueous-ethanolic extracts showed the highest inhibition of Gram-positive and Gram-negative bacteria than water extracts ($p < 0.05$). In addition, water, ethanol, and a solvent mixture (1:1) were used as controls and results indicate that both solvents showed lowest or negative antimicrobial effect (< 5 mm), while the standard gentamicin exert the highest antimicrobial effect. In agree with our results, *G. lucidum* extracts showed the high inhibition zone against *S. aureus*, *E. coli* and *S. typhi* in methanol > ethanol > water (Quereshi, Pandey & Sandhu, 2010). Also, it has been reported that *Pleurotus* spp. extracts obtained with the polar reduced solvent (ethanol), showed the highest inhibition against *S. aureus* > *E. coli* > *S. typhimurium* (Kalyoncu, Oskay & Kayalar, 2010).

In disagree, it has been reported that *Pleurotus squarrosolus* water extract showed highest antimicrobial activity against *S. aureus*, when compared with the polar reduced extract (ethanol) (Nwachukwu & Uzoeto, 2010). Also, it has been reported that *A. brasiliensis* aqueous-ethanolic extracts did not exert antimicrobial activity against *S. aureus* and *E. coli* (Ye & Lin, 2001). It has been demonstrated that solvent polarity is a key factor in the type of compound to be extracted from natural sources, e.g., in aqueous extracts compounds such as polyphenols (phenolic acids and flavonoids), tannins, saponins and terpenoids can be extracted; while in ethanolic extracts flavonoids, tannins, terpenoids and alkaloids can be extracted (Abugri & McElhenney, 2013; Papuc *et al*., 2017). Whereas, in methanolic extracts has been found anthocyanins and flavonols, terpenoids, saponins and tannins (Azmir *et al*., 2013).

In addition, the antimicrobial activity of EME can be associated with the specie and differences in their phenolic constituents (Nowacka *et al*., 2014). These compounds can follow different action mechanism: (1) inhibition of cellular division; (2) destruction of the cytoplasm and cellular membrane causing cellular components exit and create changes in the fatty acids and phospholipids of the membrane; (3) bacteriolysis; (4) inhibition of protein synthesis and finally; (5) inhibition of DNA and RNA synthesis (Martínez & Sánchez, 2007).

Solvent effect on antioxidant activities

Regardless of the extraction solvent, the highest antioxidant values (Table IV), i.e., RPA (> 0.40 abs) and antiradical $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ activity (> 80 and 50%, respectively), were showed in *P. ostreatus* extracts. In all analyzed EME, a high RPA was found in samples obtained with water ($p < 0.05$). While a high $DPPH^{\bullet}$ inhibition was observed in treatments obtained with ethanol and aqueous-ethanol than water. The results of $ABTS^{\bullet+}$ inhibition showed no significant differences ($p < 0.05$) between extractions solvent analyzed in *A. brasiliensis*, *G. lucidum* and *P. ostreatus* extracts. In addition, according to the Figure 1 the highest MDA formation inhibition (45.1%) was found in meat samples stored (at 37 °C during 16 h), and treated with *G. lucidum* and *P. ostreatus* ethanolic and aqueous-ethanolic extracts ($p < 0.05$).

In agree with our results, it has been reported a highest reducing power activity for *Agaricus* spp. extracts obtained with water > 60% water-ethanol. While the highest $DPPH^{\bullet}$ inhibition was found in the extract obtained with the 60% water-ethanol > water (Gan *et al.*, 2013). Also, reported the

Table IV. Effect of solvent extraction on antioxidant activity of EME.

Mushroom	Solvent	RPA	$DPPH^{\bullet}$	$ABTS^{\bullet+}$
A. brasiliensis	Water	0.20 ± 0.04^{c}	82.6 ± 0.4^{c}	52.3 ± 0.3^{a}
	Ethanol	0.10 ± 0.01^{a}	84.8 ± 0.4^{d}	53.5 ± 0.1^{b}
	1:1	0.10 ± 0.02^{a}	84.4 ± 0.2^{d}	53.4 ± 0.8^{b}
G. lucidum	Water	0.18 ± 0.03^{c}	80.9 ± 1.5^{b}	53.0 ± 1.1^{ab}
	Ethanol	0.16 ± 0.01^{bc}	82.2 ± 0.3^{c}	52.6 ± 0.1^{b}
	1:1	0.16 ± 0.02^{bc}	82.1 ± 0.2^{c}	52.4 ± 1.7^{ab}
P. ostreatus	Water	0.43 ± 0.02^{d}	89.1 ± 1.1^{e}	56.4 ± 0.6^{c}
	Ethanol	0.37 ± 0.01^{d}	91.4 ± 0.2^{e}	55.9 ± 0.1^{c}
	1:1	0.37 ± 0.02^{d}	91.1 ± 0.8^{e}	55.7 ± 0.9^{c}
BHT		0.70 ± 0.10^{e}	71.2 ± 0.8^{a}	65.0 ± 1.0^{d}

Data are mean ± standard deviation (n = 6). TPC, total phenolic content (mg GAE/g); TFC, total flavonoid content (mg QE/g); RPA, reducing power ability (absorbance at 700 nm); antiradical assays are expressed as inhibition percentage. Different superscripts (a-e) in each row indicate significant differences between samples ($p < 0.05$).

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highest RPA for *A. brasiliensis* extracts obtained with water > ethanol (Tsai, Tsai & Mau, 2007). On the other hand, it was reported the highest DPPH$^•$ inhibition for *G. lucidum* was showed in extract obtained with water > ethanol (Kalyoncu *et al.*, 2010). In another work *Pleurotus eous* showed the highest DPPH$^•$ and ABTS$^{•+}$ inhibition using methanol > water (Sudha *et al.*, 2012). Additionally, it has been demonstrated the use of edible mushrooms in the development of meat products to reduce lipid oxidation process (Montes, Rangel-Vargas, Lorenzo, Romero & Santos, 2020).

Multivariate analysis

A principal component analysis was carried out to evaluate the differences between treatments and analyzed variables (Figure 2). The first and second component showed a variance of 49.4 and 21.9%, respectively; thus, an accumulative 71.3% of the total variation was explained by the two components. Also, the results showed a separation of analyzed treatment and biological activity ($p < 0.05$); for example, *P. ostreatus* ethanol and aqueous-ethanolic extracts showed the highest antioxidant and antimicrobial activity, which was associated

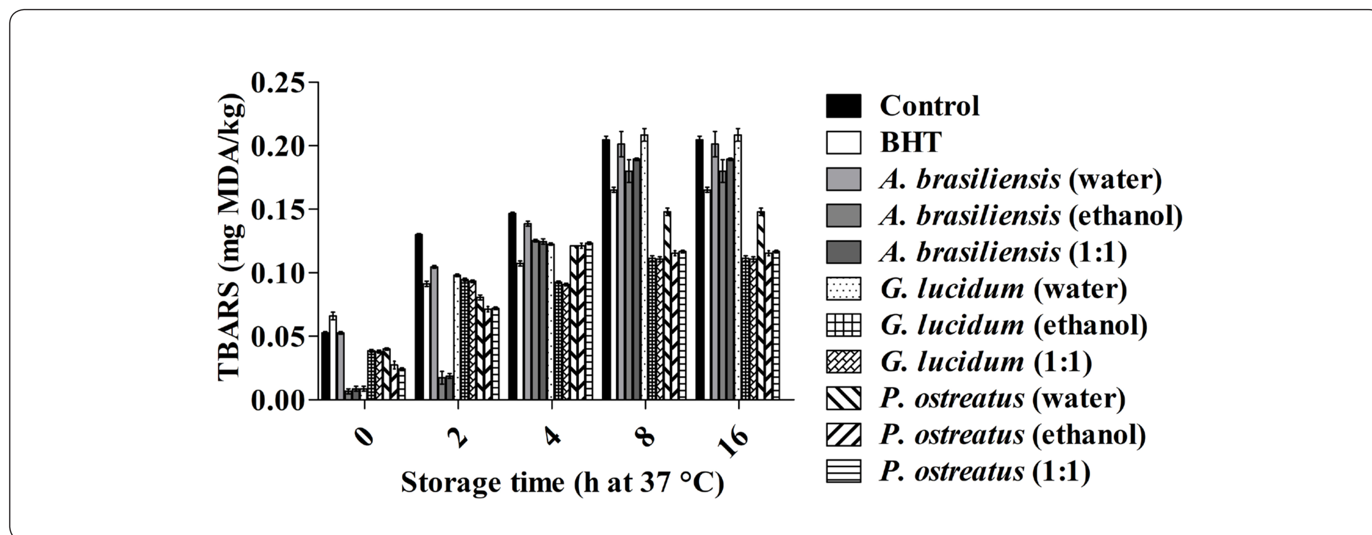

Figure 1. Lipid oxidation of raw meat homogenates during storage time (h at 37 °C).

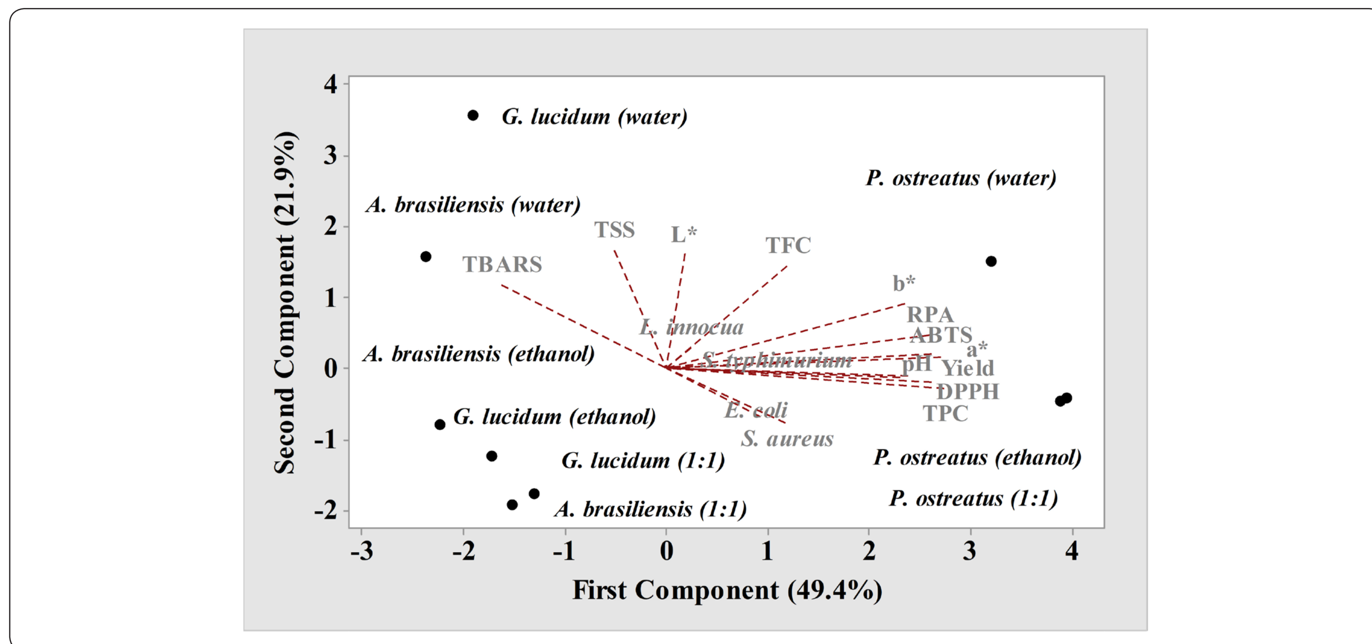

Figure 2. Analysis of the principal components of EME and evaluated parameters.

to the solvent extraction, yield, color (a* y b*), TPC, TFC and pH of the samples. The above suggest that principal component analysis could be used as a good predictive for antimicrobial and antioxidant activity of EME for their application in food industry.

Conclusions

Edible mushrooms (*A. brasiliensis*, *G. lucidum* and *P. ostreatus*) are an important source of nutriments (protein, fat, ash, and carbohydrates). However, the solvent used for obtaining edible mushrooms extracts exert an effect on phenolic components (flavonoids content) and physicochemical properties (extraction yield, pH, TSS and color). Respect antibacterial activity, *G. lucidum* and *P. ostreatus* ethanol and water-ethanol extracts showed the highest antimicrobial effect against *Staphylococcus aureus* > *Listeria innocua* > *Escherichia coli* > *Salmonella typhimurium*. While antioxidant assays revealed that *P. ostreatus* extracts showed the highest reducing power and antiradical activity, and *G. lucidum* and *P. ostreatus* ethanol and water-ethanol extracts showed the highest lipid oxidation inhibition. Therefore, EME can be used to reduce the growth of pathogenic microorganisms and as stabilizers of oxidative reaction.

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References

Abugri, D. A. & McElhenney, W. H. (2013). Extraction of total phenolic and flavonoids from edible wild and cultivated medicinal mushrooms as affected by different solvents. *Journal of Natural Product and Plant Resources*, **3(3)**, 37-42.

Ainsworth, E. A. & Gillespie, K. M. (2007). Estimation of total phenolic content and other oxidation substrates in plant tissues using Folin-Ciocalteu reagent. *Nature Protocols*, **2(4)**, 875. DOI: 10.1038/nprot.2007.102

Association of Official Analytical Chemists (AOAC). (2005). Official Methods of Analysis, 18th ed., Gaitherburg, USA.

Azmir, J., Zaidul, I. S. M., Rahman, M. M., Sharif, K. M., Mohamed, A., Sahena, F., Jahurul, M. H. A., Ghafoor, K., Norulaini, N. A. N. & Omar, A. K. M. (2013). Techniques for extraction of bioactive compounds from plant materials: a review. *Journal of Food Engineering*, **117(4)**, 426-436. DOI: 10.1016/j.jfoodeng.2013.01.014

Berker, K. I., Güçlü, K., Tor, İ., Demirata, B. & Apak, R. (2010). Total antioxidant capacity assay using optimized ferricyanide/prussian blue method. *Food Analytical Methods*, **3(3)**, 154-168. DOI: 10.1007/s12161-009-9117-9

Carneiro, A. A., Ferreira, I. C., Dueñas, M., Barros, L., Da Silva, R., Gomes, E. & Santos-Buelga, C. (2013). Chemical composition and antioxidant activity of dried powder formulations of *Agaricus blazei* and *Lentinus edodes*. *Food Chemistry*, **138(4)**, 2168-2173. DOI: 10.1016/j.foodchem.2012.12.036

Cheung, L. M., Cheung, P. C. & Ooi, V. E. (2003). Antioxidant activity and total phenolics of edible mushroom extracts. *Food Chemistry*, **81(2)**, 249-255. DOI: 10.1016/S0308-8146(02)00419-3

Cowan, M. M. (1999). Plant products as antimicrobial agents. *Clinical Microbiology Reviews*, **12(4)**, 564-582. DOI: 10.1128/CMR.12.4.564

Gan, C. H., Amira, N. B. & Asmah, R. (2013). Antioxidant analysis of different types of edible mushrooms (*Agaricus bisporous* and *Agaricus brasiliensis*). *International Food Research Journal*, **20(3)**, 1095.

Jafri, M., Jha, A., Bunkar, D. S. & Ram, R. C. (2013). Quality retention of oyster mushrooms (*Pleurotus florida*) by a combination of chemical treatments and modified atmosphere packaging. *Postharvest Biology and Technology*, **76**, 112-118. DOI: 10.1016/j.postharvbio.2012.10.002

Kalač, P. (2009). Chemical composition and nutritional value of European species of wild growing mushrooms: A review. *Food Chemistry*, **113(1)**, 9-16. DOI: 10.1016/j.foodchem.2008.07.077

Kalyoncu, F., Oskay, M. & Kayalar, H. (2010). Antioxidant activity of the mycelium of 21 wild mushroom species. *Mycology*, **1(3)**, 195-199. DOI: 10.1080/21501203.2010.511292

Khatun, K., Mahtab, H., Khanam, P. A., Sayeed, M. A. & Khan, K. A. (2007). Oyster mushroom reduced blood glucose and cholesterol in diabetic subjects. *Mymensingh Medical Journal*, **16(1)**, 94-99. DOI: 10.3329/mmj.v16i1.261

Kozarski, M., Klaus, A., Niksic, M., Jakovljevic, D., Helsper, J. P. & Van Griensven, L. J. (2011). Antioxidative and immunomodulating activities of polysaccharide extracts of the medicinal mushrooms *Agaricus bisporus*, *Agaricus brasiliensis*, *Ganoderma lucidum* and *Phellinus linteus*. *Food Chemistry*, **129(4)**, 1667-1675. DOI: 10.1016/j.foodchem.2011.06.029

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., Chaudhary, U., Doumith, M., Giske, C. G., Irfan, S., Krishnan, P., Kumar, A. V., Maharjan, S., Mushtaq, S., Noorie, T., Paterson, D. L., Pearson, A., Perry, C., Pike, R., Rao, B., Ray, U., Sarma, J. B., Sharma, M., Sheridan, E., Thirunarayan, M. A., Turton, J., Upadhyay, S., Warner, M., Welfare, W., Livermore, D. M. & Woodford, N. (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *The Lancet Infectious Diseases*, **10(9)**, 597-602. DOI: 10.1016/S1473-3099(10)70143-2

Lin, E. S. & Sung, S. C. (2006). Cultivating conditions influence exopolysaccharide production by the edible Basidiomycete *Antrodia cinnamomea* in submerged culture. *International*

Journal of Food Microbiology, **108(2)**, 182-187. DOI: 10.1016/j.ijfoodmicro.2005.11.010

Ma, G., Yang, W., Zhao, L., Pei, F., Fang, D. & Hu, Q. (2018). A critical review on the health promoting effects of mushrooms nutraceuticals. *Food Science and Human Wellness*, **7(2)**, 125-133. DOI: 10.1016/j.fshw.2018.05.002

Mami, Y., Peyvast, G., Ziaie, F., Ghasemnezhad, M. & Salmanpour, V. (2014). Improvement of shelf life and postharvest quality of white button mushroom by electron beam irradiation. *Journal of Food Processing and Preservation*, **38(4)**, 1673-1681. DOI: 10.1111/jfpp.12129

Martínez, J. A. & Sánchez F. (2007). Mecanismo de acción de los antibióticos. *Jano: Medicina y Humanidades*, **1660,** 28-34.

Mazzutti, S., Ferreira, S. R., Riehl, C.A., Smania, A., Smania, F.A. & Martínez, J. (2012). Supercritical fluid extraction of *Agaricus brasiliensis*: antioxidant and antimicrobial activities. *The Journal of Supercritical Fluids*, **70**, 48-56. DOI: 10.1016/j.supflu.2012.06.010

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology*, **26(2)**, 211-219.

Montes, A. P., Rangel-Vargas, E., Lorenzo, J. M., Romero, L., & Santos, E. M. (2020). Edible mushrooms as a novel trend in the development of healthier meat products. *Current Opinion in Food Science*, **37**, 118-124. DOI: 10.1016/j.cofs.2020.10.004

Moure, A., Cruz, J. M., Franco, D., Domínguez, J. M., Sineiro, J., Domínguez, H., Núñez, M. J. & Parajó, J. C. (2001). Natural antioxidants from residual sources. *Food Chemistry*, **72(2)**, 145-171. DOI: 10.1016/S0308-8146(00)00223-5

Nowacka, N., Nowak, R., Drozd, M., Olech, M., Los, R. & Malm, A. (2014). Analysis of phenolic constituents, antiradical and antimicrobial activity of edible mushrooms growing wild in Poland. *LWT-Food Science and Technology*, **59(2)**, 689-694. DOI: https://doi.org/10.1016/j.lwt.2014.05.041

Nwachukwu, E. & Uzoeto, H. O. (2010). Antimicrobial activity of some local mushrooms on pathogenic isolates. *Journal of Medicinal Plant Research*, **4(23)**, 2460-2465. DOI: 10.5897/JMPR10.154

Pan, K., Jiang, Q., Liu, G., Miao, X. & Zhong, D. (2013). Optimization extraction of *Ganoderma lucidum* polysaccharides and its immunity and antioxidant activities. *International Journal of Biological Macromolecules*, **55**, 301-306. DOI: 10.1016/j.ijbiomac.2013.01.022

Papuc, C., Goran, G. V., Predescu, C. N., Nicorescu, V. & Stefan, G. (2017). Plant polyphenols as antioxidant and antibacterial agents for shelf-life extension of meat and meat products: Classification, structures, sources, and action mechanisms. *Comprehensive Reviews in Food Science and Food Safety*, **16(6)**, 1243-1268. DOI: 10.1111/1541-4337.12298

Pfalzgraf, A., Frigg, M. & Steinhart, H. (1995). Alpha-tocopherol contents and lipid oxidation in pork muscle and adipose tissue during storage. *Journal of Agricultural and Food Chemistry*, **43**, 1339-1342. DOI: 10.1021/jf00053a039

Poljsak, B., Šuput, D. & Milisav, I. (2013). Achieving the balance between ROS and antioxidants: when to use the synthetic antioxidants. *Oxidative Medicine and Cellular Longevity*, **956792**, 1-12. DOI: https://doi.org/10.1155/2013/956792

Popova, M., Bankova, V., Butovska, D., Petkov, V., Nikolova-Damyanova, B., Sabatini, A. G., Marcazzan, G. L. & Bogdanov, S. (2004). Validated methods for the quantification of biologically active constituents of poplar-type propolis. *Phytochemical Analysis*, **15(4)**, 235-240. DOI: 10.1002/pca.777

Quereshi, S., Pandey, A. K. & Sandhu, S. S. (2010). Evaluation of antibacterial activity of different *Ganoderma lucidum* extracts. *People´s Journal of Science Research*, **3(1)**, 9-13.

Re, R., Pellegrini, N., Proteggente, A., Pannala, A., Yang, M. & Rice-Evans, C. (1999). Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radical Biology and Medicine*, **26(9-10)**, 1231-1237. DOI: 10.1016/S0891-5849(98)00315-3

Robertson, A. R., Lozano, R. D., Alman, D. H., Orchard, S. E., Keitch, J. A., Connely, R., Graham, L. A., Acree, W. L., John, R. S. & Hoban, R. F. (1977). CIE Recommendations on uniform color spaces, color-difference equations, and metric color terms. *Color Research and Application*, **2**, 5-6.

Siu, K. C., Chen, X. & Wu, J. Y. (2014). Constituents actually responsible for the antioxidant activities of crude polysaccharides isolated from mushrooms. *Journal of Functional Foods*, **11**, 548-556. DOI: 10.1016/j.jff.2014.08.012

Soares, A. A., de Souza, C. G. M., Daniel, F. M., Ferrari, G. P., da Costa, S. M. G. & Peralta, R. M. (2009). Antioxidant activity and total phenolic content of *Agaricus brasiliensis* (*Agaricus blazei* Murril) in two stages of maturity. *Food Chemistry*, **112(4)**, 775-781. DOI: 10.1016/j.foodchem.2008.05.117

Stojkovic, D. S., Barros, L., Calhelha, R. C., Glamoclija, J., Ciric, A., van Griensven, L. J., Sokovic, M. & Ferreira, I. C. (2013). A detailed comparative study between chemical and bioactive properties of *Ganoderma lucidum* from different origins. *International Journal of Food Science and Nutrition*, **65(1)**, 42-47. DOI: 10.3109/09637486.2013.832173

Sudha, G., Vadivukkarasi, S., Shree, R. B. I. & Lakshmanan, P. (2012). Antioxidant activity of various extracts from an edible mushroom *Pleurotus eous*. *Food Science and Biotechnology*, **21(3)**, 661-668. DOI: 10.1007/s10068-012-0086-1

Sun, L., Bai, X. & Zhuang, Y. (2014). Effect of different cooking methods on total phenolic contents and antioxidant activities of four Boletus mushrooms. *Journal of Food Science and Technology*, **51(11)**, 3362-3368. DOI: 10.1007/s13197-012-0827-4

Tsai, S. Y., Tsai, H. L. & Mau, J. L. (2007). Antioxidant properties of *Agaricus blazei*, *Agrocybe cylindracea*, and *Boletus edulis*. *LWT-Food Science and Technology*, **40(8)**,

1392-1402. DOI: 10.1016/j.lwt.2006.10.001

Vamanu, E. (2013). Antioxidant properties and chemical compositions of various extracts of the edible commercial mushroom, *Pleurotus ostreatus*. *Revista de Chimie*, **64(1)**, 49-54.

Vargas-Sánchez, R. D., Torrescano-Urrutia, G. R., Ibarra-Arias, F. J., Portillo-Loera, J. J., Ríos-Rincón, F. G. & Sánchez-Escalante, A. (2018). Effect of dietary supplementation with *Pleurotus ostreatus* on growth performance and meat quality of Japanese quail. *Livestock Science*, **207**, 117-125. DOI: 10.1016/j.livsci.2017.11.015

Yan, J., Zhu, L., Qu, Y., Qu, X., Mu, M., Zhang, M., Muneer, G., Zhou, Y. & Sun, L. (2019). Analyses of active antioxidant polysaccharides from four edible mushrooms. *International Journal of Biological Macromolecules*, **123**, 945-956. DOI: 10.1016/j.ijbiomac.2018.11.079

Ye, Z. & Lin, Y. (2001). Study on antibacterial effects of *Agaricus blazei*. *Food Science*, **22(4)**, 82-84.

Wiegand, I., Hilpert, K. & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, **3(2)**, 163-175. DOI: 10.1038/nprot.2007.521