

1 **ENHANCING ANTIOXIDANT ACTIVITIES OF LIVER PÂTÉ BY**
2 ***BOLETUS EDULIS* SUPPLEMENTATION**

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11 Running title: Antioxidant activities of pâté with added B. edulis
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ABSTRACT

The antioxidant properties of liver pâtés might be enhanced by adding specific bioactive ingredients such as Boletus edulis mushrooms. Water- and methanol-soluble fractions of supplemented pâtés showed higher ABTS and DPPH scavenging activities than control samples. B. edulis supplementation resulted in lower EC₅₀ but only up to 5% (w/w). The high antioxidant activity observed in supplemented pâtés was stable during 30 storage days and decreased after 60 days but still it was higher than control pâtés and did not influence lipid oxidation. Addition of dried water or methanol preparations extracted from the mushroom did not improve the antioxidant activity observed when the complete fruiting body was utilized. Ergosterol- related compounds and ergotioneine were involved, but they were not exclusively responsible of the high antioxidant activity observed, phenolic compounds might be involved too.

PRACTICAL APPLICATION

Antioxidants are thought to exert a potential protective effect against free radical damage preventing cardiovascular diseases (CVD) and tumour formation by inhibiting oxidative reactions on DNA, lipids, etc. Traditional liver pâtés are a good source of bioavailable iron but they are also rich on saturated fatty acids and cholesterol. The frequent consumption of the latter compounds increases the risk of CVD. Moreover, the industrial manufactures usually add synthetic antioxidants to inhibit pâtés lipid oxidation, since their iron content might act as catalyst and accelerate lipid oxidation reactions. Edible fungi are a good source of natural antioxidants and fungal flavours, textures and colours are similar to animal products. Therefore, *B. edulis* fruiting bodies could be added at low

46 concentrations (2.5 – 1% w/w) to liver pâté to design a new functional pâté with
47 higher antioxidant activities without the need of synthetic antioxidants to prevent
48 lipid oxidation up to 30 storage days.

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50 Keywords:

51 Mushroom, DPPH, ABTS, TBARS, ergosterol, ergothioneine, TLC, functional
52 ingredients

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INTRODUCTION

Liver pâtés, spreading pastes commonly consumed worldwide, are generally considered as an added value product with high sensory qualities and nutritional value because of their iron content but with a high amount of fat (approx. 35%) (Estevez *et al.* 2007).

During the pâté preparation, chopping and mincing of liver and other fatty ingredients facilitate oxygen interaction with the paste matrix. Later on, the pasteurization procedure increases the temperature and the high iron levels catalyze oxidative reactions on the free fatty acids provoking hydroperoxides and other degradation products (Kanner *et al.* 1991).

Traditionally, industrial manufactures usually added synthetic antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl, octyl, and dodecyl gallates etc. to inhibit pâté lipid oxidation (Pinho *et al.* 2000).

Actually, other alternatives are being investigated such as the use of sage and rosemary essential oils (Estevez *et al.* 2007). However, edible fungi are also a good source of natural antioxidants and fungal flavours, textures and colours are more similar to animal products than plant extracts (Tsai *et al.* 2007).

Boletus edulis is a widely consumed mushroom with appreciated sensory characteristics and nutritional value. *Boletus* sp. contains high levels of carbohydrates including dietary fibres and proteins and very low fat content (Manzi *et al.* 2001; Ouzouni and Riganakos 2007). They are a good source of vitamins and minerals (Mattila *et al.* 2001) particularly, a high Vitamin D₂ (Mattila *et al.* 2002; Teichmann *et al.* 2007) and selenium contents (8.7 – 32 µg/kg DM) (Falandysz 2003) and a wide range of nutraceuticals (Wasser 2002; Zheng *et al.* 2007). These basidiomycetes contain bioactive compounds with high

antioxidant (Ramirez-Anguiano *et al.* 2007; Tsai *et al.* 2007; Sarikurkcu *et al.* 2008), antimicrobial (Lee *et al.* 1999; Santoyo *et al.* 2009), antitumor and immunomodulating properties (Wasser 2002; Zhen *et al.* 2007).

There is a new trend in the market to functionalize food by adding specific ingredients of natural origin, which might not only avoid the food spoilage but improve consumer's health. Usually, to reach that health beneficial effect, the natural compounds have to be extracted and concentrated using more or less complicated technologies to prepare an active concentrate (Kitzberger *et al.* 2007). The objective of this work is to demonstrate that the antioxidant status of a liver pâté might be improved just by promoting new " recipes" with specific combination of food ingredients, for instance, by adding powdered mushroom fruiting bodies directly to pâtés. Liver pâtés - if consumed in large quantities - could increase the risk of cardiovascular diseases (CVD) because of its high saturated fat and cholesterol levels. Moreover, such a high amount of fat might be easily oxidized (in the absence of antioxidant compounds) during the pâté preparation and/or during storage. In this work *Boletus edulis* fruiting bodies were added to liver pâtés to study whether they were able to inhibit lipid oxidation and enhance the antioxidant properties of the food matrix. The effect of pâté processing and storage on these properties was also investigated.

Therefore, the ABTS and DPPH scavenging activities and TBARS values of traditional and supplemented pâtés were compared. A preliminary study using TLC was carried out to visualize the antioxidants potentially responsible of the observed activities.

MATERIAL AND METHODS

Materials and reagents

The mushroom used in this investigation was a wild strain of *Boletus edulis* (Fries/Bull). Fresh mushroom fruit bodies, Iberian pork bacon, beef liver, pepper, salt, powered garlic and white wine were purchased at a local supermarket.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS^{•+}), 2,2-diphenyl-1-picrylhydrazyl (DPPH[•]), 2-Thiobarbituric acid, and 1,1,3,3 tetraethoxypropane (TEP) were purchased from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). Trichloroacetic acid (TCA) and analytical grade organic solvents were obtained from Panreac Quimica SA 17 (Barcelona, Spain).

Samples preparation

Boletus edulis fruiting bodies were cut in small pieces, frozen at -20 ° C and lyophilized (Unitop 400 SL, Virtis, Gardiner, NY, USA). Dehydrated material was homogenized in a mill (JR MF10basic IKA Laburtechnik, Germany) and sieved (Orto Alresa, Spain) until particle size was smaller than 0.3 mm. The resulting powder was stored at -20 ° C and marked as complete mushroom powder (BC) or submitted to extraction using water and methanol as solvents.

Boletus edulis water fractions (BWF) were prepared by adding 500 mL distilled water to 10 g mushroom powder. The mixture was stirred and submitted to centrifugation at 5 °C, 3500 rpm during 15 min. Supernatant was collected, stored at -20 °C and lyophilized.

Boletus edulis methanol fractions (BMF) were prepared by adding 10 mL methanol to 1 g mushroom powder and shaking in a Vortex during 2 min. The mixture was submitted to centrifugation at 12000 rpm during 2 min. Supernatant was dried in a vacuum concentrator (SpeedVac plus, Savant Instruments Inc. Holbrook, NY, USA).

Liver pâtés were prepared following a domestic recipe. Beef liver (166 g) was cut in pieces, mixed with 333 g bacon pieces and ground in a mixer (Ufesa, 600 W, Spain) until a fine paste was obtained. Pepper (0.42 g), salt (3.33 g), powdered garlic (0.333 g) and 25 mL white wine were added and mixed with the paste. In pâtés supplemented with *B. edulis* preparations, different concentrations were added (BC= 1, 2.5, 5 and 7.5 %, BWF= 0.25 and 1.25% and BMF = 0.12 and 0.62 % w/w) together with these condiments. Crude pâté (90 g) was placed in jars (200 mL) sterilized in an autoclave at 121 °C for 20 min and for storage experiments a few jars were maintained at 4 °C in darkness up to 60 days.

DPPH• scavenging capacity

For DPPH• assays, the antioxidant compounds were extracted following two procedures. In the first procedure, liver pâtés (5 g) were mixed twice with 10 mL hexane, stirred for 4 min to remove fat samples before methanol extraction. Hexane phase was removed using vacuum. Defatted paste was mixed twice with 10 mL (x2) methanol and stirred for 4 min. Supernatants were pooled together and evaporated until dryness in a rotary evaporator (Laborota 4000-efficient. Heidolph Instruments, Germany). Dry extracts were dissolved in methanol for the analysis. In the second procedure, liver pâtés (5 g) were

directly submitted to methanol extraction without fat removal, concentrated and treated as previous samples.

Scavenging effect on DPPH• free radical was monitorized according to Ramirez-Anguiano *et al.* (2007). The EC₅₀ was defined as the concentration of dry extract able to scavenge half of the radical and it was calculated in order to compare the samples antioxidant activity. Lower EC₅₀ values indicate higher antioxidant power.

ABTS•⁺ scavenging capacity

For ABTS•⁺ assays, liver pâtés (5 g) were mixed with 10 mL distilled water, stirred in a Vortex for 4 min and later in an Ultra-Turrax blender (Ika-Werke GmbH & Co., Germany) at 11000 rpm during 10 s. The mixture was filtrated through a Watman paper and the filtrate was immediately analyzed. A filtrate aliquot (1 mL) was dried in an oven at 40 °C overnight to calculate the dry matter content.

ABTS•⁺ radical was chemically generated from ABTS-H using manganese dioxide. ABTS•⁺ scavenging activity was analyzed spectrophotometrically following the procedure described by Ramirez-Anguiano *et al.* (2007).

Lipid oxidation measurement (TBARS value)

Lipid oxidation in pâté with or without *B. edulis* (BC, BWF and BMF) supplementation was measured as thiobarbituric acid reactive substances (TBARS) using the TBA method of Pfalzgraf *et al.* (1995). This method is based on the malondialdehyde (MDA) reaction with 2-thiobarbituric acid (TBA) to

obtain a pink pigment, resulting from the condensation of two molecules of TBA with one molecule of MDA (Sinnhuber *et al.* 1958). The substances that react with TBA are called TBA-reactive substances. TBARS value expressed as μg MDA/g pâté and results were calculated in duplicate from a standard curve of TEP (1,1,3,3 tetraethoxypropane).

Briefly, pâté samples (5 g) were homogenized with 10 mL trichloroacetic acid 10% using the Ultra-Turrax (10 s, 11.000 rpm). The resulting mixtures were centrifuged at 5°C and 3500 rpm during 20 min and supernatants were filtrated through a Watman paper. Afterwards, filtrates (2 mL) were mixed with 2 mL TBA 3 mmol/L and heated on a water bath at 100°C during 20 min. When the samples cooled down absorbance at 531 nm was recorded.

Antioxidants detection by TLC

Compounds present in the water and methanol extracts obtained from *B. edulis* were separated and visualised using TLC silica sheets. Samples were developed and stained with a DPPH• solution as described in Soler-Rivas *et al.* (2000).

Organic antioxidants from *B. edulis* powder (0.1 g) were extracted using 1mL methanol. The mixture was shaken and centrifuged at 14000 rpm during 2 min. Drops (10 μL) of the methanol extract and standard compounds were placed on the silica sheet and a TLC was developed using as mobile phase a mixture of toluene: ethyl acetate: formic acid (50:40:10) (v/v/v). The TLC sheet was dried and stained with the DPPH• solution to detect only those compounds with antioxidant activity.

Aqueous extracts were prepared as above described for methanol fractions but using water as solvent. Afterwards, they were submitted to filtration to separate small molecules from proteins and polysaccharides since the LMW fraction was mainly responsible for the high antioxidant activity as previously observed (Ramirez-Anguiano *et al.* 2007). Two hundred microliters of freshly prepared water extracts (100 mg/mL) were submitted to filtration using Microcom filters (Millipore) with a cut off of 10000 daltons and a microfuge (14000 rpm). Drops (2 µL) of the LMW fraction and standard compounds were placed on silica sheet and TLC was developed using as mobile phase a mixture of methanol: formic acid: acetic acid: water (75:16:8:1) (v/v/v/v).

Statistical analysis

One way analysis of variance (ANOVA) was performed using a Statgraphics® Plus 3.1 for Windows software (Statistical Graphics Corporation, Maryland, USA). The mean comparison test used was Fisher's least significant differences procedure (LSD).

RESULTS AND DISCUSSION

Effect of processing on radical scavenging activities

Two steps are critical during a pâté processing because they might influence the antioxidant levels of a freshly prepared pâté. During mincing of the different ingredients, oxygen get into the mixture and lipid oxidations might take place. The second critical step is the heat treatment applied to the pâté in jars because increase of temperature implies increase of reaction velocity, and in iron-rich

food matrices such as pâté, this metal might act as catalyst and accelerate lipid oxidation reactions.

When the domestic pâté recipe was followed, the minced paste was not left long enough to provoke a significant change in its antioxidants level (results not shown) but the heating process seemed detrimental (Table 1).

The thermal treatment applied to preserve the jars, following a traditional pâté recipe, reduced the antioxidant capacity of water soluble compounds of control pâtés since the EC₅₀ of their ABTS^{•+} scavenging capacity significantly increased after the heating. When the pâté was supplemented with complete *B. edulis* powder (BC), no significant reduction of water soluble antioxidants was observed. Moreover, in both paste and prepared pâté, the EC₅₀ of supplemented preparations were significantly lower than the traditional control samples.

Heating did not significantly affect the methanol-soluble antioxidants present in the traditional pâté or in those supplemented with the mushroom powder (BC).

However, the latter showed higher antioxidant properties since the EC₅₀ was, on average, 0.2 mg/mL while 1.1 mg/mL was established for traditional pâtés.

Optimization of the *B. edulis* supplementation

Pâté paste and mushroom powder (BC) were mixed at several concentrations to design a functional product with a high antioxidant activity but also with adequate sensory properties and economically affordable. An excess of antioxidant compounds could lead to a pro-oxidant effect particularly on a high lipidic matrix such as liver pâté (Barlow 1990).

Boletus edulis additions up to 7.5% (w/w) increased the antioxidant capacity of the water soluble fraction of pâtés (Fig. 1a). The EC₅₀ of the traditional liver pâté (extrapolated by linear regression from the curves) changed from 0.46 mg/mL to 0.26 mg/mL by only 1% *B. edulis* addition. Higher mushroom doses decreased furthermore the EC₅₀ but up to certain extent. Increases of 5% and 7.5% mushroom powder did not significantly change the EC₅₀ (0.15 mg/mL). Before measuring the antioxidant activity of the methanol soluble fractions in the above mentioned pâtés, two extraction methods were compared to detect a possible interference when organic solvents are utilized due to the high fat content of the samples. Nevertheless, no significant differences on the antioxidant activity were found between the methanol extracts submitted to the hexane pre-treatment to remove the fat and those without the washing step (Table 2). Results were also independent of the type of sample (control and supplemented) and apparently no synergistic effects occurred between the antioxidants present in the fatty matrix (soluble in organic solvents such as chloroform, hexane etc.) and those extracted with methanol. Therefore, the DPPH• scavenging capacity was evaluated without hexane pre-treatment. *Boletus edulis* addition to functionalize liver pâtés resulted in higher antioxidant activity of the methanol-soluble fraction with only 1% addition (Fig. 1b). The EC₅₀ decreased almost half concentration, from 1.18 in control samples to 0.62 mg/mL in supplemented samples (Table 2). As observed for water soluble antioxidants, the EC₅₀ was decreasing with increasing mushroom concentration. However, EC₅₀ values from samples including 5 and 7.5% were more similar than between lower concentrations.

Pâté supplementation with water and methanol fractions extracted from *B. edulis*

Liver pâté pastes were also supplemented with two separate *B. edulis* fractions, a lyophilized fraction containing the water soluble compounds (BWF) and a dried methanol soluble fraction (BMF). Water extracted more compounds since 247.5±4.9 mg/g dw were obtained compared to the 126.5±5.1 mg/g obtained using methanol. BWF fraction was added to pâté at 0.25 and 1.25% and BMF at 0.12 and 0.62% because those were the concentrations corresponding to 1% and 5% complete mushroom powder to compare the effect of the separate fractions with the complete fruiting body addition.

Control pâtés showed higher EC₅₀ than in previous experiments (Table 3).

These changes are usually due to the natural variability between samples preparations, particularly, liver and bacon pieces might differ from batch to batch. But, control and supplemented samples were prepared from the same pâté paste to allow further comparisons.

BWF additions (0.25 and 1.25%) decreased respectively less than 13 and 30% the control EC₅₀. However, samples including the complete mushroom powder (1 and 5% BC) decreased it respectively 43 and 67%. BMF additions (0.12 and 0.62 %) decreased respectively less than 10 and 58% the control EC₅₀ while similar concentrations but including the complete mushroom powder (1 and 5% BC) decreased it respectively 47 and 78%. Therefore, the use of a water or a methanol fraction instead of the complete mushroom as supplement to improve the antioxidant activity was less effective than the use of the complete fruiting body. Perhaps, synergic interactions between BC components might take place or some of the antioxidants were degraded during the extraction procedure.

Thus, it is not encouraged the use of any separated mushroom extract to functionalize liver pâtés.

Effect of storage on antioxidant levels and lipid oxidation

The effect of BC, BWF and BMF supplementation on pâté lipid oxidation was also studied (Fig. 2). No significant differences were observed between the TBARs values of control samples and pâtés supplemented with both BWF concentrations and 1% BC. These values were maintained constant during 15 storage days at 4°C. The TBARs values of samples including BMF (both concentrations) or 5% BC were slightly higher than control however only in those samples including BMF, the values increased after 15 days. Results were not surprising since BMF compounds were extracted with an organic solvent therefore, these compounds had a higher tendency to react with lipid-like compounds than water soluble extracts. Thus, these BMF extracts might have been partially oxidised during the extraction procedure or in case of 5% BC, they might have been added in excess. However, lipid oxidation levels were in all the samples very low and even lower than other pâtés functionalized with plants extracts (Estevez *et al.* 2007).

The antioxidant activity of the BWF and BMF supplemented pâtés were lower than those including the complete fruiting body and the methanol fractions slightly enhanced lipid oxidation thus, none of the separated mushroom extracts was further more utilized to functionalize liver pâtés and the complete *B. edulis* fruiting bodies were added but in concentrations lower than 5% (w/w). Then, control pâtés and pâtés supplemented with 2.5% *Boletus edulis* powder were prepared and stored at room temperature for 60 days to study the lipid

oxidation and the stability of the high antioxidant activity observed in supplemented samples (BC).

Water soluble antioxidants presents in control and supplemented samples were rather stable during 30 days because the ABTS^{•+} scavenging capacity did not significantly changed in samples with 0, 15 and 30 storage days (Fig. 3a).

Control samples showed on average an EC₅₀ of 0.3 mg/mL and supplemented samples maintained their higher antioxidant activity (EC₅₀ 0.17 mg/mL on average). Storage for 60 days provoked a decrease in the radical scavenging activities of both control and supplemented pâtés being the later still higher than the control.

Similarly, the antioxidant activity of the methanol soluble fraction extracted from both control and supplemented pâtés did not change within the 30 storage days (Fig. 3b). Afterwards, a high reduction was noticed after 60 days in control samples while supplemented pâtés showed only a low reduction in their DPPH[•] scavenging capacity after those 60 days.

The lipid oxidation was concomitantly measured and no significant differences were observed from 0 up to 60 storage days in both control pâtés and samples supplemented with *B. edulis* powder (Fig. 3c). The latter samples showed a slightly higher TBARs values than the control although they were still very low indicating a proper stability of the lipid matrix during 60 days if *Boletus edulis* was applied at the selected concentration (2.5% w/w).

Antioxidants separation by TLC

Boletus methanol and water extracts were developed by TLC to separate and detect the type of compounds potentially responsible for the high antioxidant activity observed.

When the methanol extracts were developed, only 2 bands with antioxidant activity were observed (Fig. 4a). One band migrated ($R_f = 0.86$) and the other remained at the application point. The first band could be ergosterol or ergosterol derivatives since the band showed similar R_f value than a commercially available ergosterol used as standard and their UV-spectra coincided when they were scratched from the silica sheet. Ergosterol is a common compound for fungi since it is a cell membrane component (Barajas-Aceves *et al.* 2002) and a vitamin D₂ precursor (Teichmann *et al.* 2007). It is also able to decrease lipid peroxidation and scavenge radicals (Zhang *et al.* 2002; Hu *et al.* 2006; Kobori *et al.* 2007). Moreover, the ergosterol concentration in this *B. edulis* strain was 7.73 mg/g and it was the major compound of the unsaponifiable fraction (Ramirez-Anguiano, 2009).

No ascorbic acid, α -tocopherol, ergocalciferol (vit. D₂) (co-migrated with vit. D₃) and β -carotene ($R_f = 1$) were detected, apparently their contribution to the antioxidant activity of this fraction was insignificant. The vitamin D₂ levels in *B. edulis* were 1000 fold lower concentration than ergosterol (Mattila *et al.* 2002; Teichmann *et al.* 2007) and the highest concentration described for the other compounds were still 2-100 fold lower than ergosterol (Tsai *et al.* 2007; Jaworska and Bernas, 2009), thus their contribution might be below the detection level of the TLC-DPPH assay.

However, the most intense band did not migrate indicating that the antioxidant activity was mostly due to very polar compounds which might be mainly soluble

in water and partially in methanol. Therefore, the water extract was also analyzed by TLC.

The water extract was fractionated in a low molecular weight (LMW) and a high molecular weight (HMW) fractions and only the LMW fraction was applied to TLC sheets since the HMW contained antioxidant polysaccharides bound to proteins (Liu *et al.* 1997) but they contributed minimally to the high antioxidant activity observed in the complete water extract (Ramirez-Anguiano *et al.* 2007).

The TLC development of the water extract yielded three bands (Fig. 4b), a fine band ($R_f = 0.68$) with very low intensity and two other more intense at the application point and at similar migration place than ergothioneine ($R_f = 0.13$).

L-Ergothioneine is water soluble thiol compound (2-thioimidazole betaine) with high antioxidant capacity (Dubost *et al.* 2007) present in high amounts in *B. edulis* fruiting bodies (528 mg/kg fw) (Ey *et al.* 2007) and the UV spectrum of the *B. edulis* band extracted from the silica sheet resembled ergothioneine (Dubost *et al.* 2006).

Other reports mentioned the presence of alkaloids, organic acids and unidentified phenols which could also be present in the applied LMW water fraction. Alkaloids could be partially extracted and with the utilized mobile phase they might migrate, perhaps they could be responsible of the fine band observed ($R_f = 0.68$). *B. edulis* contained oxalic, citric, malic, succinic and fumaric acids being malic acid the compound present in higher amount (Ribeiro *et al.* 2008). These compounds have not interesting antioxidant activities but they influence the pH and acid pHs shift the DPPH absorbance maximum producing false positives when compared with samples with neutral pHs.

Therefore, they might influence the yellow spot visualized at the application

point (because those compounds are not expected to migrate with the utilized mobile phase). However, phenolic compounds and peptides might also be present at the application point and might be involved in the observed antioxidant activity. The LMW fraction contained 36.68 mg/g phenolic compounds and 4.74 mg/g peptides (Ramirez-Anguiano, 2009). Thus, since most of the reports pointed the phenolic fraction as responsible of the antioxidant activity observed at the application point (Soares *et al.* 2009) and they were in high concentration in the LMW extracts they might be the major responsible compounds of the detected activity. Thus, the antioxidant activity of the water extracts might be mainly due to ergothioneine, and also probably due to the phenolic compounds.

Concluding, a functional liver pâté might be easily prepared by direct supplementation with only 1- 2.5% of powdered *B. edulis* fruiting bodies. The antioxidant activity of supplemented pâtés was higher than traditional pâtés, it was maintained during the manufacture processes and pâté storage and did not induce lipid oxidation. The antioxidant activity of the methanol extract might be due to the ergosterol derivatives and polar compounds partially extracted from the water. The water extracts contained ergothioneine but also other still unidentified phenols. Further investigations are directed at the present to identify those compounds.

ACKNOWLEDGMENTS

University of Guadalajara (Jalisco, México) is acknowledged for the financial support granting to A.C. Ramirez-Anguiano with a doctoral fellowship.

This work was also supported by the AGL2004-07227-C02-02 project from the *Ministerio de Ciencia y Tecnología* under the framework of the R+D+I National Program (2004-2007) and ALIBIRD-CM S-0505/AGR-0153 regional program from the *Comunidad de Madrid, Spain*.

REFERENCES

- BARAJAS-ACEVES, M., HASSAN, M., TINOCO, R. and VAZQUEZ-DUHALT, R. 2002. Effect of pollutants on the ergosterol content as indicator of fungal biomass. *J. Microbiol. Meth.* 50, 227-236.
- BARLOW, S.M. 1990. Toxicological aspects of antioxidants used as food additives. *In: Food antioxidants* Ed. HUDSON, B.J.F. Elsevier Applied Science. London and New York.
- DUBOST, N.J., BEELMAN, R.B., PETERSON, D. and ROYSE, D.J. 2006. Identification and quantification of ergothioneine in cultivated mushrooms by liquid chromatography-mass spectroscopy. *Int. J. Med. Mushrooms* 8, 215-222.
- DUBOST, N.J., OU, B. and BEELMAN, R.B. 2007. Quantification of polyphenols and ergothioneine in cultivated mushrooms and correlation to total antioxidant capacity. *Food Chem.* 105, 727-735.
- ESTÉVEZ, M., RAMÍREZ, R., VENTANAS, S. and CAVA, R. 2007. Sage and rosemary essential oils versus BHT for the inhibition of lipid oxidative reactions in liver pâté. *LWT - Food Sci. Technol.* 40, 58-65.
- EY, J., SCHÖMIG, E., and TAUBERT, D. 2007. Dietary sources and antioxidant effects of ergothioneine. *J. Agric. Food Chem.* 55, 6466-6474.

- FALANDYSZ, J. 2003. Selenium in selected species of mushrooms from Poland. *Roczniki Panstwowego Zakladu Higieny* 54, 249-254.
- HU, S.H., LIANG, Z.C., CHIA, Y.C., LIEN, J.L., CHEN, K.S., LEE, M.Y. and WANG, J.C. 2006. Antihyperlipidemic and antioxidants effects of extracts from *Pleurotus citrinopileatus*. *J. Agric. Food Chem.* 54, 2103-2110.
- JAWORSKA, G. and BERNAS, E. 2009. The effect of preliminary processing and period of storage on the quality of frozen *Boletus edulis* (Bull:Fr.) mushrooms. *Food Chem.* 113, 936-943.
- KANNER, J., HAZAN, B. and DOLL, L. 1991. Catalytic 'free' iron ions in muscle foods. *J. Agric. Food Chem.* 36, 412-415.
- KITZBERGER, C.S.G., SMÂNIA, A., CURI PEDROSA, R. and SALVADOR FERREIRA, S.R. 2007. Antioxidant and antimicrobial activities of shiitake (*Lentinula edodes*) extracts obtained by organic solvents and supercritical fluids. *J. Food Eng.* 80, 631-638.
- KOBORI, M., YOSHIDA, M., OHNISHI-KAMEYAMA, M. and SHINMOTO, H. 2007. Ergosterol peroxide from an edible mushroom suppresses inflammatory responses in AW264.7 macrophages and growth of HT29 colon adenocarcinoma cells. *Brit. J. Pharmacol.* 150, 209-219.
- LEE, S.J., YEO, W.H., YUN, B.S. and YOO, I.D. 1999. Isolation and sequence analysis of new peptaibol, boletusin, from *Boletus* spp. *J. Pept. Sci.* 5, 374-378.
- LIU, F., OOI, V.E.C. and CHANG, S.T. 1997. Free radical scavenging activities of mushroom polysaccharide extracts. *Life Sci.* 60, 763-771
- MANZI, P., AGUZZI, A. and PIZZOFERRATO, L. 2001. Nutritional value of mushrooms widely consumed in Italy. *Food Chem.* 73, 321-325.

472 MATTILA, P., KÖNKÖ, K., EUROLA, M., PIHLAVA, J.M., ASTOLA, J.,
 473 VAHTERISTO, L., HIETANIEMI, V., KUMPULAINEN, J., VALTONEN, M.
 474 and PIIRONEN, V. 2001. Contents of vitamins, mineral elements and
 475 some phenolic compounds in cultivated mushrooms. J. Agric. Food Chem.
 476 49, 2343-2348.

477 MATTILA, P., LAMPI, A.M., RONKAINEN, R., TOIVO, J. and PIIRONEN, V.
 478 2002. Sterol and vitamin D₂ contents in some wild and cultivated
 479 mushrooms. Food Chem. 76, 293-298.

480 OUZOUNI, P.K. and RIGANAKOS, K.A. 2007. Nutricional value and metal
 481 content profile of Greek wild edible fungi. Acta Aliment. 36, 99-110.

482 PFALZGRAF, A., FRIGG, M. and STEINHART, H. 1995. α -Tocopherol contents
 483 and lipid oxidation in pork muscle and adipose tissue during storage. J.
 484 Agric. Food Chem. 43, 1339-1342.

485 PINHO, O., FERREIRA, I.M.P.L.V.O., OLIVEIRA, M.B.P.P. and FERREIRA,
 486 M.A. 2000. Quantification of synthetic phenolic antioxidants in liver pâtés.
 487 Food Chem. 68, 353-357.

488 RAMIREZ-ANGUIANO, A.C., SANTOYO, S., REGLERO, G. and SOLER-
 489 RIVAS, C. 2007. Radical scavenging activities, endogenous oxidative
 490 enzymes and total phenols in edible mushrooms commonly consumed in
 491 Europe. J. Sci. Food Agric. 87, 2272-2278.

492 RAMIREZ-ANGUIANO, A.C. 2009. Estudio de las propiedades funcionales de
 493 los hongos comestibles para el diseño de alimentos cárnicos funcionales.
 494 Department of Applied Chemistry-Physic. Faculty of Science. Universidad
 495 Autonoma de Madrid. Ph.D. thesis.

496 RIBEIRO, B., LOPES, R., ANDRADE, P.B., SEABRA, R.M., GONÇALVES,
 497 R.F., BAPTISTA, P., QUELHAS, I. and VALENTÃO, P. 2008. Comparative
 498 study of phytochemicals and antioxidant potential of wild edible mushroom
 499 caps and stipes. Food Chem. 110, 47-56.

500 SANTOYO, S., RAMÍREZ-ANGUIANO, A.C., REGLERO, G. and SOLER-
 501 RIVAS, C. 2009. Improvement of the antimicrobial activity of edible mushrooms
 502 extracts by inhibition of oxidative enzymes. Int. J. Food Sci. Technol. 44, 1057-
 503 1064.

504 SARIKURKCU, C., TEPE, B. and YAMAC, M. 2008. Evaluation of the
 505 antioxidant activity of four edible mushrooms from the Central Anatolia,
 506 Eskisehir – Turkey: *Lactarius deterrimus*, *Suillus collitinus*, *Boletus edulis*,
 507 *Xerocomus chrysenteron*. Bioresource Technol. 99, 6651-6655.

508 SINNHUBER, R.O., YU, I.C. and YU, T.C. 1958. Characterization of the red
 509 pigment formed in the 2-thiobarbituric acid determination of oxidative
 510 rancidity. Food Res. 23, 624-634.

511 SOLER-RIVAS, C., ESPÍN, J.C. and WICHERS, H.J. 2000. An easy and fast
 512 test to compare total free radical scavenger capacity of foodstuffs.
 513 Phytochem Anal 11, 330-338.

514 SOARES, A.A., GIATTI MARQUES DE SOUZA, C., DANIEL, F.M. PEZENTE
 515 FERRARI, G., GOMES DA COSTA, S.M. and PERALTA, R.M. 2009.
 516 Antioxidant activity and total phenolic content of *Agaricus brasiliensis*
 517 (*Agaricus blazei* Murril) in two stages of maturity. Food Chem. 112, 775-
 518 781.

519 TEICHMANN, A., DUTTA, P.C., STAFFAS, A. and JÄGERSTAD, M. 2007.
 520 Sterol and vitamin D2 concentrations in cultivated and wild grown

521 mushrooms: Effects of UV irradiation. LWT - Food Sci. Technol. 40, 815-
522 822.

523 TSAI, S.Y., TSAI, H.L. and MAU, J.L. 2007. Antioxidant properties of *Agaricus*
524 *blazei*, *Agrocybe cylindracea* and *Boletus edulis*. LWT - Food Sci. Technol.
525 40, 1392-1402.

526 WASSER, S.P. 2002. Medicinal mushrooms as a source of antitumor and
527 immunomodulating polysaccharides. Appl. Microbiol. Biot. 60, 258-274.

528 ZHANG, Y., MILLS, G.L. and NAIR, M.G. 2002. Cyclooxygenase inhibitory and
529 antioxidant compounds from the mycelia of the edible mushroom. J. Agric.
530 Food Chem. 50, 7581-7585.

531 ZHENG, S., LI, C., NG, T.B. and WANG, H.X. 2007. A lectin with mitogenic
532 activity from the edible wild mushroom *Boletus edulis*. Process. Biochem.
533 42, 1620-1624.

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TABLES AND FIGURES

TABLE 1.

ABTS^{•+} AND DPPH[•] SCAVENGING ACTIVITIES OF WATER AND
METHANOL EXTRACTS OBTAINED FROM PÂTÉS WITH AND WITHOUT
7.5% *B. EDULIS* ADDITION BEFORE AND AFTER THE THERMAL
TREATMENT.

Samples	EC ₅₀ (ABTS) mg/mL (water soluble compounds)		EC ₅₀ (DPPH) mg/mL (methanol soluble compounds)	
	Before	After	Before	After
Control	0.28±0.01 ¹	0.46±0.02 ³	1.11±0.06 ^a	1.18±0.01 ^a
7.5% <i>B. edulis</i>	0.12±0.01 ²	0.15±0.02 ²	0.20±0.02 ^b	0.24±0.02 ^b

Values are the mean ± SD of three separate experiments

^{1,2,3}Denotes statistically significant differences (P<0.05) among EC₅₀(ABTS) values

^{a,b}Denotes statistically significant differences (P<0.05) among EC₅₀(DPPH) values

TABLE 2.

DPPH• SCAVENGING ACTIVITIES OF METHANOL EXTRACTS OBTAINED
FROM PÂTÉS SUPPLEMENTED WITH SEVERAL *B. EDULIS*
CONCENTRATIONS WITH OR WITHOUT A HEXANE PRE-TREATMENT.

Samples	EC ₅₀ (DPPH) mg/mL	
	With hexane pretreatment	Direct methanol extraction
Control	1.20±0.01 ^a	1.18±0.01 ^a
1% <i>B. edulis</i>	0.59±0.04 ^b	0.62±0.02 ^b
2.5% <i>B. edulis</i>	0.30±0.01 ^c	0.34±0.03 ^c
5% <i>B. edulis</i>	0.26±0.01 ^d	0.26±0.01 ^d
7.5% <i>B. edulis</i>	0.21±0.01 ^f	0.23±0.01

Values are the mean ± SD of three separate experiments

a,b,c,d,e,f Denotes statistically significant differences (P<0.05) among EC₅₀(DPPH)

values

TABLE 3.

ABTS^{•+} SCAVENGING ACTIVITIES OF WATER EXTRACTS OBTAINED
FROM PÂTÉS SUPPLEMENTED WITH *B. EDULIS* AQUEOUS FRACTIONS
(BWF) AND DPPH[•] SCAVENGING ACTIVITIES OF METHANOL EXTRACTS
OBTAINED FROM PÂTÉS SUPPLEMENTED WITH *B. EDULIS* METHANOL
FRACTIONS (BMF).

Aqueous fraction (BWF) (% w/w)	EC ₅₀ (ABTS) (mg/mL)	Methanol fraction (BMF) (% w/w)	EC ₅₀ (DPPH) (mg/mL)
0	1.03±0.01 ¹	0	1.4±0.02 ^a
0.25	0.90±0.00 ²	0.12	1.27±0.01 ^b
1.25	0.73±0.01 ³	0.62	0.59±0.00 ^c

Values are the mean ± SD of three separate experiments

^{1,2,3}Denotes statistically significant differences (P<0.05) among EC₅₀(ABTS) values

^{a,b,c}Denotes statistically significant differences (P<0.05) among EC₅₀(DPPH) values

FIG. 1. A) ABTS^{•+} SCAVENGING CAPACITY OF WATER EXTRACTS AND B) DPPH[•] SCAVENGING CAPACITY OF METHANOL EXTRACTS OBTAINED FROM LIVER PÂTÉS CONTAINING DIFFERENT *B. EDULIS* CONCENTRATIONS.

(□) Control pâté, (▲) 1%, (◆) 2.5%, (●) 5% and (■) 7.5% mushroom addition.

FIG. 2. TBARS VALUES FROM CONTROL PÂTÉS AND PÂTÉS SUPPLEMENTED WITH *B. EDULIS* AQUEOUS FRACTIONS (BWF), WITH *B. EDULIS* METHANOL FRACTIONS (BMF) AND WITH 1 AND 5% OF COMPLETE MUSHROOM. *DENOTES STATISTICALLY SIGNIFICANT DIFFERENCES (P<0.05) BETWEEN SAMPLES INCUBATED 0 AND 15 DAYS

FIG. 3. EVOLUTION DURING THE STORAGE TIME OF A) EC₅₀(ABTS) OF WATER EXTRACTS, B) EC₅₀(DPPH) OF METHANOL EXTRACTS AND C) TBARS VALUES OBTAINED FROM LIVER PÂTÉS WITH AND WITHOUT 2.5% *B. EDULIS* SUPPLEMENTATION. *DENOTES STATISTICALLY SIGNIFICANT DIFFERENCES (P<0.05) BETWEEN CONTROL AND SUPPLEMENTED SAMPLES

FIG. 4. A) METHANOL EXTRACT AND B) LMW FRACTION FROM THE WATER EXTRACT OBTAINED FROM *BOLETUS EDULIS* DEVELOPED BY TLC AND STAINED WITH A DPPH[•] SOLUTION.

BE = *B. edulis* extracts, ERT = ergothionine, ERG = ergosterol, ASC = ascorbic acid, TOC= tocopherol, VitD3= Vitamin D₃.

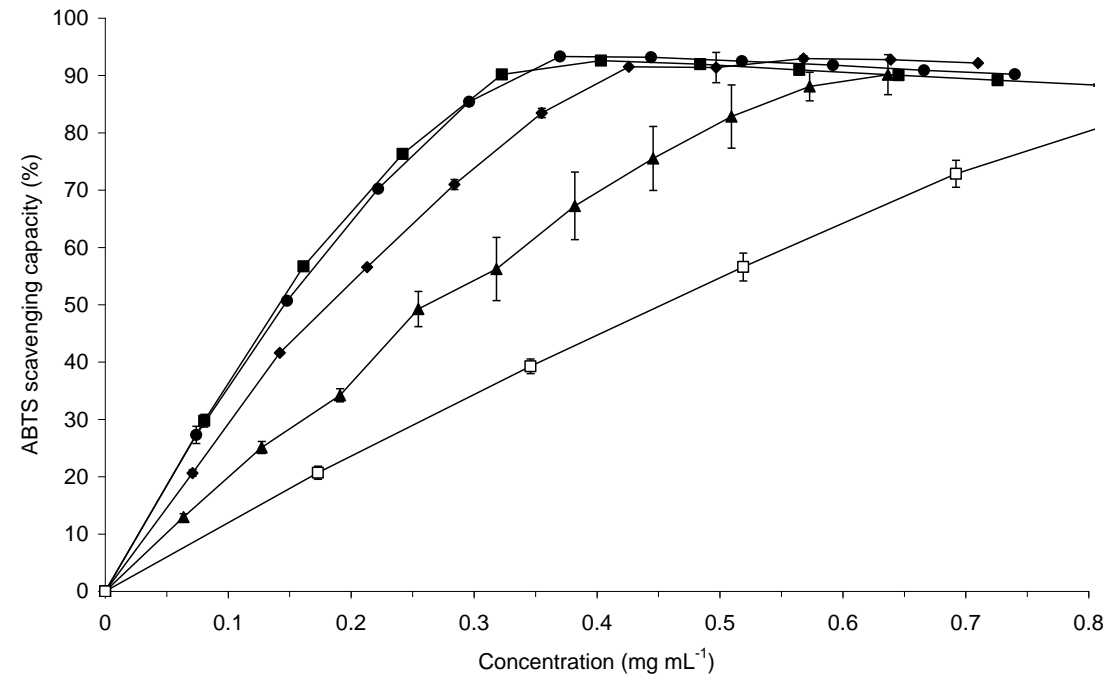
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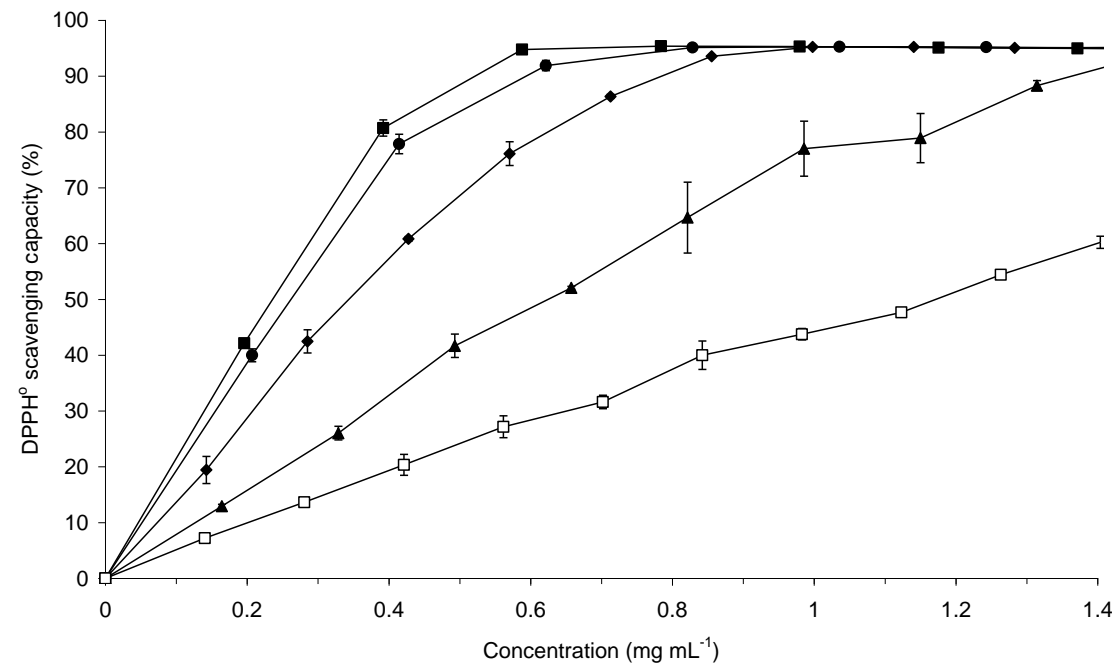
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FIG. 1.

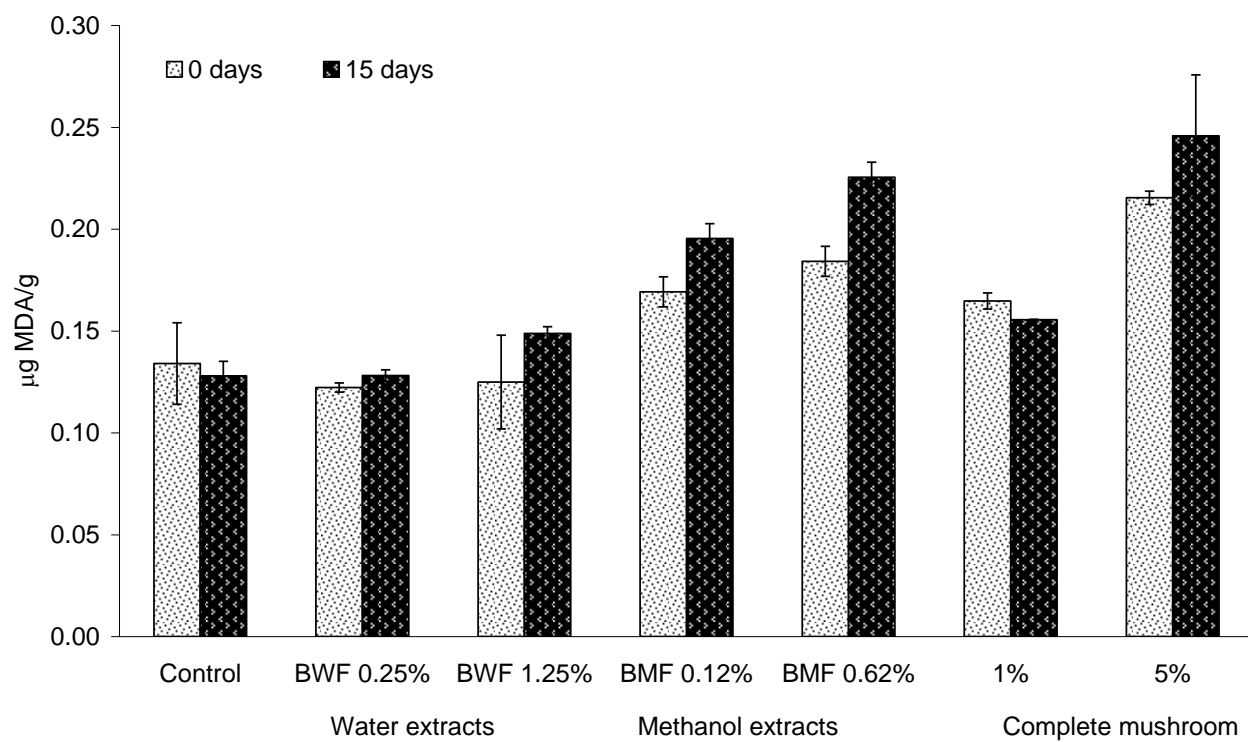
a)



b)



608 FIG. 2.



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610 FIG. 3.

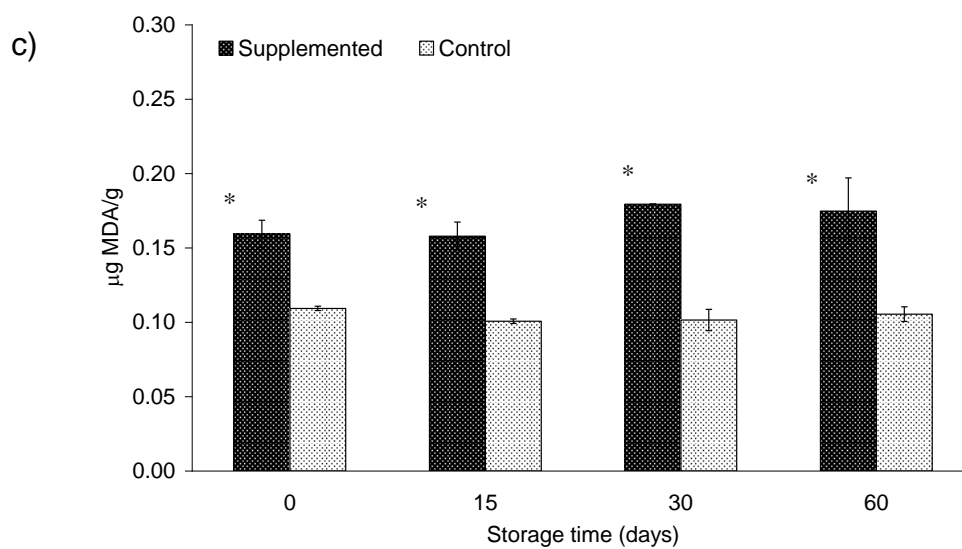
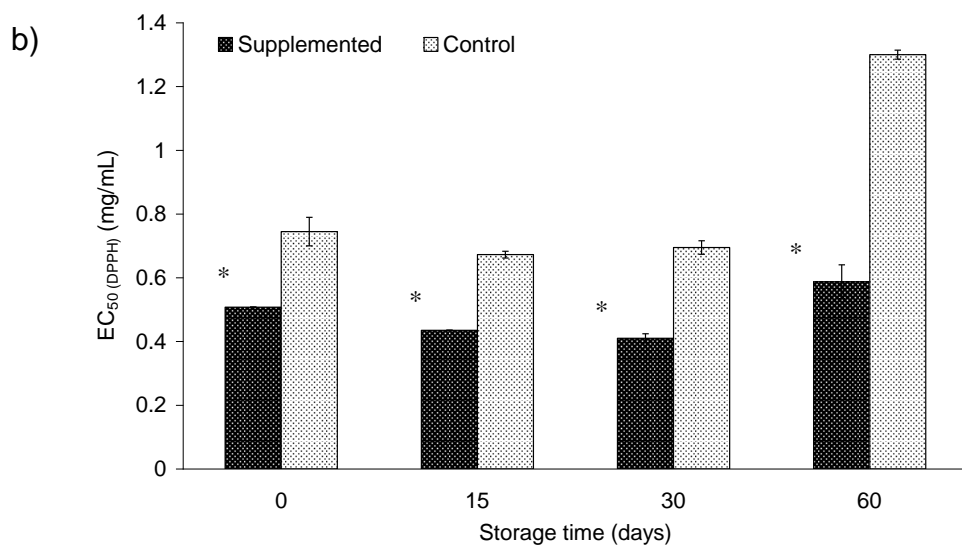
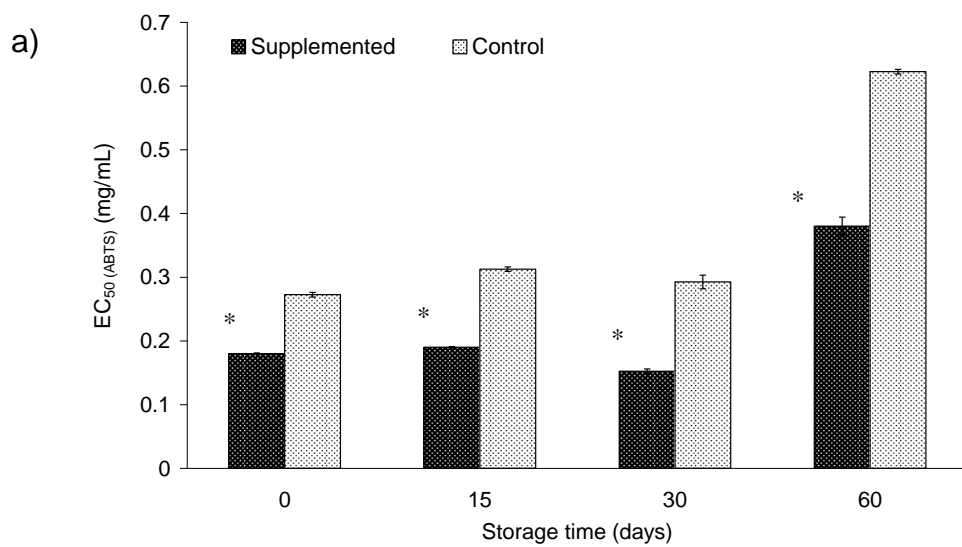
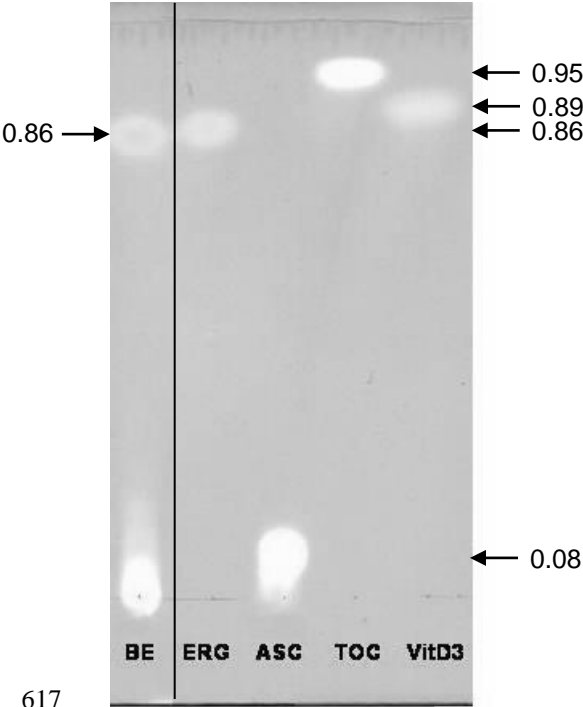


FIG. 4.

a)



b)

