Extracts of white mushroom (*Agaricus bisporus*) protect against breast tumors/cancer and atherosclerosis *in vitro*

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Abstract: Edible mushrooms include many fungal species that are either harvested wild or cultivated, and are consumed by humans for their nutritional and medicinal values. White mushrooms such *Agaricus bisporus*, belong to *Agaricaceae* family, is the most extensively cultivated mushroom in the world including Egypt, accounting for 38% of the world production of cultivated mushrooms. Therefore, the present study aims to investigate the potential protective effects of such white mushroom species extracts against breast tumors/cancer and aarteriosclerosis *in vitro*. White mushroom samples were obtained from the interior areas of Egypt and used for preparation of various media extracts including water, ethanol and methanol. The methanolic extract showed the strongest antioxidant activity (AA, 89.96%) and the highest bioactive compounds (except vitamin c) including total phenolics content (104.69 mg GAE.g^{-1}), flavonoids (17.54 QE.g^{-1}) carotenoids (2.4 mg.g^{-1}) and minerals (Fe, 1.58 mg.g^{-1} and Se, 21.87 mg.g^{-1}) while the water extract showed the lowest activity (AA= 46.43 %) and low concentration of total phenolics 31.64 mg GAE.g^{-1}), flavonoids (4.92 QE.g^{-1}) carotenoids (0.61 mg.g^{-1}) and minerals (Fe, 0.67 mg.g^{-1} and Se, 6.04 mg.g^{-1}). When all different mushroom extracts were included in the statistical analysis, there was a relatively positive and significant ($p \leq 0.01$) relationship between total phenolics (r^2= 86.45), flavonoids (r^2=81.65), carotenoids (r^2= 71.56) and vitamin C (r^2=68.89) and vitamin E (r^2= 62.1) and antioxidant activity. Also, mushroom methanol extract may be a useful chemopreventive agent for breast cancer/ tumor, as they have been shown to decrease in maximal optical density of intact DNA and DNA fragmentation in EAC (Ehrlich Ascites Carcinoma) cell line in all extract tested concentrations (0.50, 1.00, 1.5 and 2.0%). Furthermore, data confirmed a possibility of white mushroom extracts may be more promising in the prevention of atherosclerosis by inhibiting LDL oxidation and scavenging peroxyl radicals forming during oxidation of lipids in oxidative stress. Hence, white mushroom might be useful as antioxidant, anticarcinogenic and Anti-atherosclerosis agents, and its extracts especially the methanol one will probably be used successfully for development of dietary foods, food products and pharmaceutical industry.

Key words: White mushroom, methanol extract, antioxidant, bioactive compounds, minerals, DNA fragmentation, inhibition, LDL oxidation,

Introduction

Mushrooms are the fleshy, spore-bearing fruiting bodies of several species of fungi typically produced above ground on soil or on its food source. They belong to the macrofungi, because their fruiting structures are large enough to be seen with the naked

eye (Chang and Miles, 1989). Over 2,500 different mushrooms grow in the wild around the world. White mushrooms (A. bisporus) is the most extensively cultivated mushroom in the world including Egypt, accounting for 38% of the world production of cultivated mushrooms. Mushrooms share some of the benefits of fruits and vegetables in that they are low in energy, and are virtually free of fat and sodium. A. bisporus mushrooms are a source of fiber and are fairly rich in vitamins and minerals. They contain an especially high amount of minerals, essential amino acids, vitamins and fibers. In addition, they contain other substances that may have potential health benefits and play a role in the prevention of serious diseases (Roupas et al., 2010). Mushrooms are considered a natural source of antioxidants, including phenolics, vitamins and minerals (Beelman et al., 2003 and Sadler, 2003) compounds that can reduce the risk of degenerative diseases caused by oxidative stress, such as cancer, cardiovascular disease etc. (Muzzarelli, 1999 and Roupas et al., 2010).

In additional antioxidant activity, anti-tumor effects have been reported bioactive compounds extracted from various mushrooms (Khatab, 2013). Amongst of those compounds polysaccharides play the central position as anti-tumorigenic effects. The polysaccharides generally belong to the β-glucan family of compounds and appear to exert their anti-tumorigenic effects via enhancement of cellular immunity. Anti-tumor effects of proteoglycan fractions from a variety of mushrooms, including Agaricus bisporus, involve the elevation of natural killer (NK) cell numbers and the stimulation of inducible nitric oxide (NO) synthase gene expression, which is then followed by NO production in macrophages via activation of the transcription factor, NF-kappaB. Activation of NK cells is likely via interferon-gamma and interleukin mediated pathways (Jayakumar et al., 2011). While studies in human cell lines provide supporting evidence, well-designed human clinical trials are required before anti-cancer health outcomes in humans can be validated. In recent years, a number of human trials have been undertaken and these are outlined below (Angeli et al., 2006). The anti-tumor activity of agaritine (from mushrooms) against leukemic cells has reported by Endo et al., (2010). Roupas et al., (2010) also concluded that agaritine from consumption of cultivated A. bisporus mushrooms poses no known toxicological risk to healthy humans. Another in vivo study demonstrated that crude extracts of A. blazei Murrill significantly reduced DNA damage in liver induced by diethylnitrosamine in adult male Wistar rats (Barbisan et al., 2003), while DNA strand breaking by the carbon-centered radical generated from 4-(hydroxymethyl) benzenediazonium salt from A. bisporus has been reported in the mouse (Hiramoto et al., 1995). Vitamin D2 could be one of the protective phytonutrients against breast cancer as mushrooms are rich in ergosterol, generating vitamin D2 when exposed to ultraviolet B (UVB) light and ergocalciferol being bioavailable and increasing serum 25(OH) vitamin D2 levels in humans (Furlanetto, 2009).

Although research has focused mainly on the antioxidant effects of mushrooms, little information is available about the impact of extraction methods on their antioxidant activity. Therefore, one of the objectives of the present study was to determine the antioxidative activity and total phenolic content of the various extracts of the white mushroom (A. bisporus) found on Egypt. Also, the present study aims to investigate the potential protective effects of such white mushroom species extracts against breast tumors/cancer and atherosclerosis in vitro.

Materials and methods

Materials

Mushroom samples: The samples of white mushroom (*A. bisporus*) used in this study were obtained from Horticulture Research Institute, Agricultural Research Center, Ministry of Agriculture, Giza, Egypt.

Chemicals and reagents: Folin-Ciocalteu reagent, o-phosphoric acid, serine borate buffer (SBB), N-1-(pyrenyl) maleimide (NPM), dimethyl sulfoxide (DMSO), Trolox, 2,2-azobis (2-amidinopropane) dihydrochloride (AAPH), standard vitamins, gallic acid, quercetin, agarose, ethylene diaminotetra acetic acid (EDTA), ethidium bromide and tween-80 were obtained from Sigma Chemical Co., St. Louis, Mo. The rest of chemicals, reagents and solvents were of analytical grade and purchased from Al-Gomhoryia Company for Trading Drugs, Chemicals and Medical Instruments, Cairo, Egypt.

Analytical methods

Preparation of mushroom extracts

White mushroom (A. bisporus) was extracted with 2.5 L aqueous methanol for four times (24 h x 4) at room temperature (25 0C), filtered and evaporated to dryness in vacuum. The residue mushroom materials were similarly extracted, filtered and evaporated by using distilled water and aqueous ethanol solvent, successively. The yields of the extracts were given as follow: Aqueous, 2.02 ± 0.28, methanol, 6.68 ± 1.04 and ethanol, 3.11 ± 0.27. Solvent suspension of Mushroom (A.bisporus) extract (ME) was prepared using 1 % (v/v) Tween-80 and used for the treatment.

Bioactive compounds determination in mushroom extracts

Total phenolics, total flavonoids and carotenoids

Total phenolics, total flavonoids and carotenoids in mushroom samples extract were analyzed as follow: mushroom extract samples was extracted with 80% acetone and centrifuged at 10,000g for 15 min. The supernatant obtained from both samples were used for the analysis of total phenolics, total flavonoids, carotenoids and antioxidant activity. Total phenolics were determined using Folin-Ciocalteu reagent (Singleton and Rossi, 1965). In brief, 200 milligrams of sample was extracted for 2 h with 2 mL of 80% MeOH containing 1% hydrochloric acid at room temperature on an orbital shaker set at 200 rpm. The mixture was centrifuged at 1000g for 15 min and the supernatant decanted into 4 mL vials. The pellets were combined and used for total phenolics assay. One hundred microliters of extract was mixed with 0.75 mL of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and allowed to stand at 22 0C for 5 min; 0.75 ml of sodium bicarbonate (60 g/L) solution was added to the mixture after 90 min at 22 0C, absorbance was measured at 725 nm. Results are expressed as gallic acid equivalent (GAE/g extract). The total carotenoids in 80% acetone extract were determined by using the method reported by Litchenthaler (1987). Total flavonoid content in mushroom extracts was determined spectrophotometrically according to the method of Layzon et al., (2015).

Vitamins

All vitamins (A, E and C) were extracted and analyzed by HPLC techniques as follow: Epler *et al*., (1993), Hung *et al*., (1980) and Moeslinger *et al*., (1994), respectively. The chromatographic conditions for vitamins A and E were flow rate, 1.5 ml/min; detection, UV absorption at 265 nm, volume of injection, 20 µl; temperature, room temperature; and the mobile phase composition was an isocratic system of isopropanol : hexane (1:99) while in vitamin C were flow rate, 1 ml/min; detection, UV absorption at 254 nm, volume of injection, 20 µl; temperature, room temperature, and mobile phase composition was an isocratic system of 100 % methanol. Retention times and absorbance ratio against those of standards were used to identify the separated vitamins. Quantitative determination of each vitamin was determined from its respective peak area and corresponding response factor. The percent recoveries of vitamins were also studied by adding each vitamin to serum after sample preparation and HPLC determination. Under such chromatographic conditions, the Mean ±SD values of vitamins A, C and E recoveries were 90.1 ± 5.3, 88.9 ± 4.4, and 84.7 ± 5.2, respectively.

Minerals

Minerals content of mushroom extracts sample were determined according to the method mentioned by Singh et al., (1991) as follow: 0.5 ml of sample were transferred into a digester glass tube of Kjeldahl digestion unit and 6 ml of tri-acids mixture (contaninig nitric acid : percloric acid : sulphuric acid in the ratio of 20 : 4 : 1 v/v respectively) were added to each tube. The tubes content were digested gradually as follow, 30 min at 70 0C; 30 min at 180 0C and 30 min at 220 0C. After digestion, the mixture was cooled, dissolved in distilled water, and the volume was increased to 50 ml in volumetric beaker. After filteration in ashless filter paper, aliquots were analysed for minerals using of atomic absroption spectrophotometer, type Perkin - Elmer, Model 2380.

Antioxidant activity determination in mushroom extracts

Antioxidant activity (AA) of white mushroom extracts and standards (α-tocopherol; Sigma Chemical Co., St. Louis, Mo) was determined according to the β-carotene bleaching method following a modification of the procedure described by Marco (1968). For a typical assay, 1mL of β-carotene (Sigma) solution, 0.2 mg/mL in chloroform, was added to round-bottom flasks (50 mL) containing 0.02 mL of linoleic acid (J.T. Baker Chemical Co., Phillipsburg, NJ) and 0.2 mL of Tween 20 (BDH Chemical Co., Toronto, Canada). Each mixture was then dosed with 0.2 mL of 80% MeOH (as control) or corresponding plant extract or standard. After evaporation to dryness under vacuum at room temperature, oxygenated distilled water (50 ml) was added and the mixture was shaken to form a liposome solution. The samples were then subjected to thermal auto-oxidation at 50 0C for 2 h. The absorbance of the solution at 470 nm was monitored on a spectrophotometer (beckman DU-50) by taking measurements at 10 min intervals, and the rate of bleaching of β-carotene was calculated by fitting linear regression to data over time. All samples were assayed in triplicate. Various concentrations of BHT, BHA, and α-tocopherol in 80% methanol was used as the control. Antioxidant activity was calculated Antioxidant activity (AA)

was all calculated as percent inhibition ralative to control using the following equation **(**Al-Saikhan *et al*., 1995).

$$AA= (R\ control - R\ sample) / R\ control \times 100$$

Where: $R_{control}$ and R_{sample} were the bleaching rates of beta-carotene in reactant mixture without antioxidant and with mushroom extract, respectively.

Effect of white mushroom methanol extract (WMME) on Deoxyribonucleic acid (DNA) of EAC cell line

The DNA extraction method

Dexoyribonucleic acids extracted from Ehrlich Ascites Carcinoma (EAC) cell line according to salting out extraction method of Aljanabi and Martinez (1997) and modification introduced by Hassab El-Nabi (2004). In brief, treated (2×10^6 cell/ml) from EAC cell line which added to different concentration from white mushroom methanol extracts (WMME) 5 µl, 10 µl, 15 µl 20 µl in eppendrof tubes were lysed with 600 µl lysing buffer (50 mM NaCl, 1 mM Na_2EDTA ,0.5% SDS , pH 8.3) and gently shacked. The mixture was incubated over night at 37 ^{0}C then, 200 µl of saturated NaCl was added to the samples, shocked gently and centrifuge at 1200 rpm for 10 min. The supernatant was transferred to another appendrof tubes and then DNA precipitated by 600 µl cold isopropanol. The mixture was inverted several times till fine fiber appears, and then centrifuge at 1200 rpm for 5 min. The supernatant is removed, and the pellets were washed with 500 µl 70% EtOH, centrifuged at 1200 rpm for 5 min. After centrifugation, the alcohol layer was decanted or tipped out and the tubes blotted on whatman paper or clean tissue, till the pellets appeared to be dry. The pellets were resuspended in 50 µl or appropriate volume of TE buffer (10 mM tris, 1mM EDTA, pH 8.3) supplemented with 5 % glycerol. The re-suspended DNA was in incubated for 30-60 min with loading mix (RNase + loading buffer) and then loaded directly into the wells.

Gel preparation and analysis

Gel was prepared according to Sallam and El-Abd, (2005) using 1.8% electrophoretic grade agarose (BRL).The agarose was boiled with tris borate EDTA buffer (1 TBE buffer, 89 mM tris, 89 mM boric acid, 2 mM EDTA, pH 8.3), and then 0.5 µg.ml-1 ethidium bromide was add to agarose mixture at 40 0C. Gel was poured and allowed to solidify at room temperature (24±4 0C) for 1 hour before samples were loaded. Finally, added the mixture (cell line and extracts) to well of electrophoresis. Electrophoresis was run for 1.5 hour at 50-60v. The gel was illuminated with UV light and photographed. The DNA fragments were analyzed by using Gel-Pro software computer program, Chicago, USA.

Determination of peroxyl radical (ROO-)-scavenging activity

Peroxyl radical (ROO-)-scavenging activity was determined by using oxygen radical absorbance capacity (ORAC) assay according to Ou et al., (2001). In brief, the 200 µl of mushroom extract, phosphate buffer (3.5 ml, 75 mM, pH 7.4), and FL (100 µl, 35 nM) were mixed in a test tube and incubated at 37 0C for 5 min before the initial

fluorescence intensity was recorded. The 2,2-azobis [2-amidinopropane dihydrochloride (AAPH), 200 µl, 75 g.l-1] was added to initiate the reaction. Fluorescence readings were recorded every three minutes until zero fluorescence intensity was reached. The excitation (Ex) and emission (Em) wavelengths were 493 and 515 nm, respectively. Trolox was also used as a standard to calibrate the final results.

Determination of inhibition of low density lipoprotein (LDL) oxidation

Inhibition of LDL oxidation was determined such as described by Princen et al., (1992). In brief, adult male white albino rat, Sprague Dawley strain, serum was collected and diluted by phosphate buffer (50 mM, pH 7.4) to the concentration of 0.6%. Quantities of 5.0 ml diluted serum were mixed with 10 µl dimethylsulfoxide (DMSO) or 10 µl DMSO containing various concentrations of the all tested mushroom extracts. CuSO4 solution (2.5 mM), 20 µl, was added to initiate the reaction and the absorbance at 234 nm was recorded then was recorded every 20 min thereafter for 140 min at room temperature (240C±4). The final result was expressed by measure the net area under the curve.

Statistical analysis

All data of antioxidant activity and antimicrobial tests were the average of triplicate analyses. The data were recorded as mean± standard deviation (SD). Significant differences between means were determined by student's-t test, p values ≤ 0.05 were regarded as significant.

Results and discussion

The Effect of extraction methods on the bioactive compounds, vitamins, minerals contents and antioxidant activity of white mushroom (*A. bisporus*)

Table (1) shows bioactive compounds, vitamins and minerals content and antioxidant activity of three white mushroom extracts. The bioactive compounds, vitamins and minerals content of the different mushroom extracts investigated in this study were varied. The methanolic extract showed the highest bioactive compounds including total phenolics content (104.69 mg GAE.g-1), flavonoids (17.54 QE.g-1) carotenoids (2.4 mg.g-1), minerals (Fe, 1.58 mg.g-1, Se, 21.87 mg.g-1 and Zn, 1.12 mg.g-1) and vitamins (A, 0.19 µg.g-1 and E, 0.46 mg.g-1) while the aqueous extract showed the low concentration of bioactive compounds (total phenolics 31.64 mg GAE.g-1, flavonoids, 4.92 QE.g-1 and carotenoids, 0.61 mg.g-1), minerals (Fe, 0.67 mg.g-1, Se, 6.04 mg.g-1 and Zn, 0.62 mg.g-1) and vitamins A, 0.07 µg.g-1 and E, 0.18 mg.g-1). The opposite direction was observed for vitamin C. On the other side, mushroom extracts showed considerable differences in antioxidant activity (AA = 46.43-89.96%). Methanolic extract (MeOH) showed strong activity probably due to its high phenolic content while aqueous extract showed relatively low antioxidant activity and low concentration of total phenolics. When all different mushroom extracts were included in the statistical analysis, there was a relatively positive and significant ($p \leq 0.01$) relationship between total phenolics (r2= 86.45), flavonoids (r2=81.65), carotenoids (r2= 71.56), vitamin C (r2=68.89), vitamin E (r2=62.11) and antioxidant activity. Such data demonstrates that other components beside the determined bioactive compounds of the extracts have a significant effect on the antioxidant activity of the mushroom.

Table (1): Bioactive compounds, vitamins and minerals content and antioxidant activity of white mushroom extracts

Components	Mushroom extract		
	Aqueous	MeOH	EtOH
Bioactive compounds:			
Total phenolics (mg GAE.g^{-1})	31.64±2.43	104.69±7.27	90.22±5.88
Total flavonoids (mg QE.g^{-1})	4.92±0.98	17.54±2.11	11.85±2.31
Total carotenoids (mg.g^{-1})	0.61±0.12	2.45±0.31	2.01±0.23
Vitamins:			
Vitamin A (µg.g^{-1})	0.07±0.02	0.19±0.08	0.11±0.04
Vitamin E (mg.g^{-1-1})	0.18±0.04	0.46±0.11	0.38±0.09
Vitamin C (mg.g^{-1})	5.21±1.02	1.12±0.05	3.32±1.31
Minerals:			
Fe (mg.g^{-1})	0.67±0.11	1.58± 0.34	1.11±0.12
Se (mg.g^{-1})	6.04±1.05	21.87±2.11	16.55±2.32
Zn (mg.g^{-1})	0.62±0.13	1.12±0.21	1.03±0.19
Antioxidant activity (AA, %)	46.43±4.08	89.96±6.34	82.20±4.29

Each value represents the mean value of three replicates ±SD.

In general, plant-based foods generally are considered important sources of antioxidants in the diet. Antioxidants help protect cells from the potentially damaging physiological process known as "oxidative stress" (damage to healthy cells or DNA by unpaired electrons known as free radicals). Oxidative stress is thought to be associated with the development of chronic diseases including cancer, heart disease, conditions of ageing including neurodegenerative diseases such as Parkinson's and Alzheimer's disease. There are a variety of plant antioxidants with different chemical structures. There are the antioxidant nutrients such as vitamins C, E, *β*-carotene and the trace element selenium (found in mushrooms) for which there are Dietary Reference Values (DRVs). However, there are thousands of other bioactive compounds in foods that have antioxidant activity but are not classified as "nutrients." These "non-nutrient antioxidants" include phenolic compounds (found in mushrooms) and have no DRVs (Beelman *et al.*, 2003).

The total antioxidant capacities of some different mushrooms were studied by many authors. For example, a white mutant of *Hypsizigus marmoreus* (Peck) Bigelow (Family, *Tricholomataceae*) is a new edible mushroom currently available in Taiwan (Lee *et al.*, 2008). The ethanolic and hot water extracts were prepared from fruit bodies and mycelia and their antioxidant properties studied. In addition to EC_{50} values [he amount of sample needed to decrease the initial 1,1-diphenyl-2-picrylhydrazyl radicals (DPPH) concentration by 50%] in scavenging abilities on hydroxyl radicals, almost EC50 values were less than 10 mg/mL indicating that these extracts were effective in antioxidant properties assayed. The major antioxidant components found in hot water

extracts were total phenols (10.01–13.14 mg/g) and those in ethanolic extracts were total tocopherols (33.33–10.92 mg/g). Correlations of contents of total antioxidant components and total tocopherols (plus β -carotene) with EC50 values of antioxidant activity, reducing power, scavenging ability on DPPH radicals and chelating ability on ferrous ions were established (r ¼ 0.445–0.940), whereas the correlation of total phenol content was established only with that of scavenging ability on hydroxyl radicals (r ¼ 0.882). Also, selected species of wild edible mushrooms were obtained from the interior areas of East Malaysia to determine the total phenolics and antioxidant properties, including free radical scavenging, reducing power and metal chelating activities (Wong and Chye, 2009). The in vitro antioxidant activities of petroleum ether and methanolic extracts of the edible wild mushrooms were comparable to the cultivated oyster mushroom. The radical scavenging activity was the highest in PE extract of Pleurotus porrigens (angel's wings) (85%), while methanolic extract of Hygrocybe conica (witch's hat mushroom) exhibited the highest (94%) chelating effect at 20 mg/ml. Petroleum ether extracts were more effective than methanolic extract in scavenging ability on DPPH radicals, whereas methanolic extracts were more effective in reducing power and chelating ability on ferrous ions as evidenced by their lower EC50 values. Principal component analysis indicated phenolic group was the primary factor contributing to the metal chelating ability for petroleum ether extract although phenolic was better correlated with reducing power in methanolic extracts.

On the other side, our data indicated that other components beside the determined bioactive compounds of the extracts mainly have a significant effect on the antioxidant activity of the mushroom. Amongst of these compounds, metals such Fe, Se and Zn), are essential trace mineral, functions largely through its association with proteins in the body which defend against oxidative stress, a process that has been implicated in the development of many chronic diseases including heart disease and cancer. A study determining the ergothioneine levels in different mushrooms, found that both white and brown button mushrooms, were rich in ergothioneine and that the levels did not diminish during cooking (Dubost et al., 2006). One antioxidant that has attracted a lot of scientific attention lately is ergothioneine, an intracellular antioxidant first identified in 1909. Ergothioneine levels are high in red blood cells, bone marrow and semen. It is believed that ergothioneine protects the haemoglobin in red blood cells, and protects monocytes against oxidation (Gründemann et al., 2005).

Effect of white mushroom methanol extract (WMME) on Deoxyribonucleic acid (DNA) of EAC cell line

As shown in Table (2) and Figures (1-2) results illustrated a decrement in maximal optical density of intact DNA in Ehrlich Ascites carcinoma (EAC) cell line treated with WMME at concentration 0.50, 1.00, 1.5 and 2.0% with values 88.11, 96.06, 101.98 and 104.68, respectively as compared with control (101.25). So, the concentration of WMME (2%) induced the highest increment in maximal optical density of intact DNA as compared with control. Similarly, DNA fragmentation at 200, 400, 600 and 800 bp in all WMME tested concentrations (0.50, 1.00, 1.5 and 2.0%) illustrated decrements in maximal optical density as compared with control.

Data of the present study with the others indicated that mushrooms are considered a natural source of antioxidants, including phenolics, vitamins and minerals (Beelman et al., 2003 and Sadler, 2003) compounds that can reduce the risk of degenerative diseases caused by oxidative stress, such as cancer, cardiovascular disease, weight control etc. (Cheung, 1996, Muzzarelli, 1999 and Roupas et al., 2010). Anti-tumor effects, primarily in human cell lines, have been reported from polysaccharides extracted from various mushrooms (Roupas et al., 2010). The polysaccharides generally belong to the beta-glucan family of compounds and appear to exert their anti-tumorigenic effects via enhancement of cellular immunity. Anti-

Table (2): The maximal optical density (MOD) of intact DNA and DNA fragmentation in Ehrlich Ascites Carcinoma treated with white mushroom methanol Extract (WMME) at concentration 0.50, 1.0, 1.5 and 2.0% as well as control

WMME Conc. (%)	MOD (%)				
	at 200 bp	at 400 bp	at 600 bp	at 800 bp	Intact DNA
0.5	0.08	0.13	2.12	11.04	88.11
1.0	0.11	0.26	3.41	12.63	96.06
1.5	0.15	0.21	4.16	14.1	101.98
2.0	0.41	0.11	4.39	10.85	104.68
Control	3.01	3.93	9.89	13.51	101.25

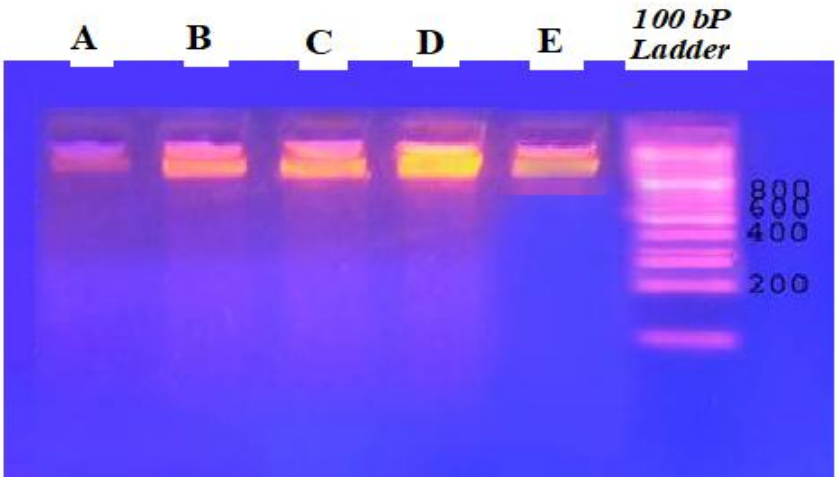

Figure 1. Gel electrophoresis pattern of intact DNA and DNA fragmentation in Ehrlich Carcinoma treated with mushroom (*A. bisporus*) methanolic Extract (MME) in mice cell line (2 x10^6 cells / ml), Lane A, (0.5 %) MME; Lane B, (1 %) MME; Lane C, (1.5 %) MME; Lane D, (2 %) MME; and Lane E, (control).

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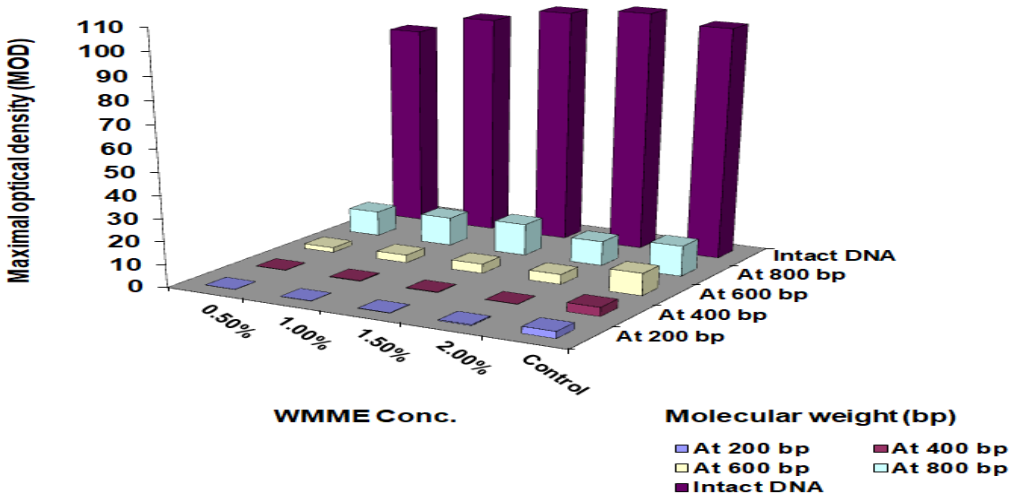

Figure 2. The maximal optical density (MOD) of intact DNA and DNA fragmentation in Ehrlich Ascites Carcinoma (EAC) treated with white mushroom methanol extract (WMME) at concentration (0.50 , 1.0, 1.5 and 2.0% as well as control)

tumor effects of proteoglycan fractions from a variety of mushrooms, including Agaricus bisporus, involve the elevation of natural killer (NK) cell numbers and the stimulation of inducible nitric oxide (NO) synthase gene expression, which is then followed by NO production in macrophages via activation of the transcription factor, NF-kappaB. Activation of NK cells is likely via interferon-gamma and interleukin mediated pathways. While studies in human cell lines provide supporting evidence, well-designed human clinical trials are required before anti-cancer health outcomes in humans can be validated. In recent years, a number of human trials have been undertaken and these are outlined below. Many *in vitro* studies have shown that a heat-labile protein from *A. bisporus* protects Raji cells (a human lymphoma cell line) against H_2O_2 induced oxidative damage to cellular DNA (Shi et al., 2002). Similar protective effects against H_2O_2-induced oxidative damage to cellular DNA have been demonstrated with cold (20 ^{0}C) and hot (100 ^{0}C) water extracts of *A. bisporus* and G. lucidum fruit bodies, respectively. No protective effects were observed with mushroom derived preparations from *F. velutipes*, *Auricularia auricula*, *H. marmoreus*, *L. edodes*, *Pleurotus sajorcaju*, or *Volvariella volvacea* (Rocha *et al.*, 2002). Similar reductions in DNA fragmentation (Comet assay), compared with H_2O_2 as a positive control, have been reported from Chaga mushroom (*I. obliquus*) (Park *et al.*, 2004), while an aqueous extract from *Agrocybe cylindracea* strain B has also been shown to protect against DNA damage in HepG2 cells (Wang *et al.*, 2004). Some edible mushrooms therefore represent a valuable source of biologically active compounds with potential for protecting cellular DNA from oxidative damage, while other mushroom varieties do not. *B*-glucan from *A. brasiliensis* has been reported to be devoid of mutagenic activity and to provide a significant dose-dependent protective effect against DNA damage in the dose range 20–80 lg/ml (Angeli *et al.*, 2006).

Another possible chemoprotective effect of *β*-glucan extracted from *A. Blazei* against DNA damage induced by benzo[a]pyrene, using the comet assay (genotoxicity)

and micronucleus assay with cytokinesis block (mutagenicity) in a human hepatoma cell line (HepG2) has suggested that *B*-glucan did not exert a genotoxic or mutagenic effect, but that it did protect against DNA damage via binding to benzo[*a*]pyrene or by the capture of free radicals produced during its activation (Angeli *et al.*, 2009).

The study of Endo *et al.*, (2010) has ascribed anti-tumor activity of agaritine (from mushrooms) against leukemic cells. Another *in vivo* study demonstrated that crude extracts of *A. blazei* Murrill significantly reduced DNA damage in liver induced by diethylnitrosamine in adult male Wistar rats (Barbisan *et al.*, 2003), while DNA strand breaking by the carbon-centered radical generated from 4-(hydroxymethyl) benzenediazonium salt from *A. bisporus* has been reported in the mouse (Hiramoto *et al.*, 1995). An *in vitro* study using water-based extracts of some mushroom species significantly inhibited growth of both estrogen-receptor positive (ER+) and estrogen-receptor negative (ER-) breast cancer cells, induction of rapid apoptosis on both ER+ and ER- cells, and significantly inhibited MCF-7 tumor colony formation *in vitro*. Also, higher dietary intake of mushrooms decreased breast cancer risk in both pre- and post-menopausal women and an additional decreased risk of breast cancer was observed from a synergistic effect of mushrooms and green tea in a case–controlled study (Zhang *et al.*, 2009). Furthermore, vitamin D_2 could be one of the protective phytonutrients against breast cancer as mushrooms are rich in ergosterol (Furlanetto, 2009).

Effect of white mushroom extracts on arteriosclerosis indices *in vitro*
Inhibition of low density lipoprotein (LDL) oxidation

Dose-dependent inhibition of CuSO4-induced LDL oxidation *in vitro* by white mushroom extracts is shown in Figure (3). From such data it could be noticed that the inhibitive action of the all tested mushroom extracts against CuSO4-induced LDL oxidation, as evidenced by decreased conjugated dienes production in a dose-dependent fashion. Comparison amongst the tested extracts indicated that the white mushroom methyl extract (WMME) and white mushroom ethyl extract (WMEE) acted more dramatically in protecting LDL against oxidation, indicating a possibility those extracts may be more promising in the prevention of atherosclerosis by inhibiting LDL oxidation. Such effects could be attributed to the different bioactive compounds as antioxidants (phenolics, carotenoids, vitamins, minerals and others) contained in such tested extracts.

In similar studies, Aviram et al., (2000) reported that pomegranate juice could effectively protect LDL against oxidation in vitro, which was attributed to the phenolic compounds and ascorbic acid contained in the juice. Also, Li et al., (2006) confirmed the inhibitive action of pomegranate pulp and pulp extracts against LDL oxidation. As compared to the pulp extract, the peel extract acted more efficiency in protecting LDL against oxidation due to its higher content of phenolic compounds. Regarding to the phenolic compounds such as detected in the tested extracts, Majid et al.,(1991) found feeding of phenolic acid (ellagic) significantly ($p \leq 0.05$) increased the levels of reduced glutathione (GSH) and glutathione reductase (GSH-Rd) in liver and lungs of mice as well as increase in inhibition of NADPH-dependent lipid peroxidation. Also, the antioxidant activity of phenolic acids (hydroxycinnamic, chlorogenic, caffeic and ellagic), representative of three chemical groups present in mushroom, upon LDL peroxidation was studied in vitro in a LDL oxidation model by Laranjinha et al., (1994;

Jayakumar et al., 2011 and Lotfy and Rdwan, 2017). All of these phenolic compounds exhibited a complex reaction with peroxyl radicals and inhibition of the LDL oxidation (Aly et al., 2017). It is reported by many studies that the ‘‘oxidative modification of lipoproteins’’ hypothesis proposes that LDL oxidation plays a key role in early atherosclerosis (Chisolm and Steinberg, 2000). The oxidized LDL is atherogenic due to its cytotoxic toward arterial cells and stimulates the monocytes to be adhesive to the endothelium. The uptake of oxidized LDL through scavenger receptors by the monocytes promotes cholesterol accumulation and foam cell formation, which leads to the development of atheromatous plaques (Hong and Cam, 2015 and Lotfy and Rdwan, 2017). Therefore, inhibition of LDL oxidation is proposed to be one of the crucial steps in retarding the foam cell formation and development of aortic lesions. Also, data of the present study proved that the tested mushroom extracts could be used successfully as a promising tool in the prevention of atherosclerosis by inhibiting LDL oxidation.

Peroxyl radical (ROO-)-scavenging activity

Data in Figure (4) shows Dose-dependent ROO- scavenging capacity of white mushroom extracts as determined by improved oxygen radical absorbance capacity (ORAC) assay. The procedure is based on the metal-chelating property of the antioxidants, the so-called preventive capacity against the peroxyl radicals (ROO-) is

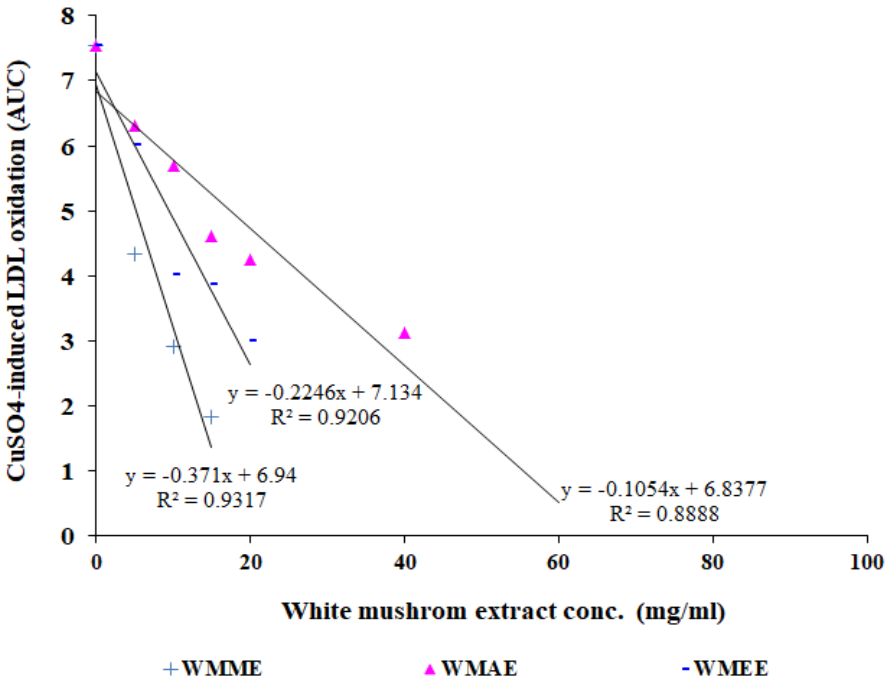

Figure 3. Dose-dependent inhibition of CuSO4-induced LDL oxidation *in vitro* by white mushroom extracts. The conjugated dienes formation was monitored kinetically as the absorbance at 234 nm and the result is expressed as the area under the curve . WMME, white mushroom methyl extract; WMAE, white mushroom aqueous extract and WMEE, white mushroom ethyl extract.

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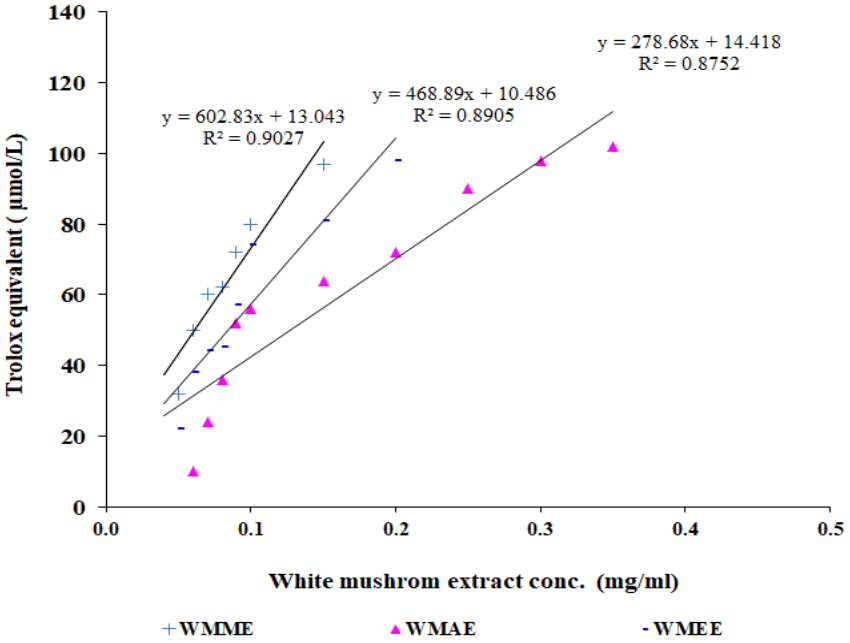

Figure 4. Dose-dependent $ROO^{\cdot}$ scavenging capacity of white mushroom extracts as determined by improved oxygen radical absorbance capacity (ORAC) assay. WMME, white mushroom methyl extract; WMAE, white mushroom aqueous extract and WMEE, white mushroom ethyl extract.

actually related to the metal-chelating capability of the extracts tested. The results revealed that as compared to the mushroom extracts, the WMME and WMEE appeared to be more effective than WMAE in scavenging $ROO^{\cdot}$. $ROO^{\cdot}$ occur during oxidation of lipids in oxidative stress in living cells. Such as reported by Thomas *et al.*, (1990) $ROO^{\cdot}$ may diffuse a considerable distance and can react with sulfhydryl groups, (-SH). Data of the present study indicated that mushroom extracts could be used successfully as a promising tool in the prevention of several diseases including atherosclerosis through scavenging some of the free radicals form during oxidation of lipids in oxidative stress.

In conclusion, studies in human cell lines have provided insights into the possible mechanisms involved for the effects of mushrooms and their components on breast cancer. Also, mushroom extracts are able to suppress the proliferation of breast cancer cell lines, without affecting the proliferation of normal (non-cancer) cell lines. Also, mushroom extracts have the ability in prevention of atherosclerosis by inhibiting LDL oxidation and scavenging $ROO^{\cdot}$. Hence, white mushroom might be useful as antioxidant, anticarcinogenic and anti-atherosclerosis agents, and its extracts especially the methanol one will probably be used successfully for development of dietary foods, food products and pharmaceutical industry.

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مستخلصات فطر عيش الغراب الأبيض (*Agaricus bisporus*) تحمي من أورام/سرطان الثدي وتصلب الشرايين معمليا خارج الجسم

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الملخص : يحتوي فطر عيش الغراب الصالح للإستهلاك الآدمى على العديد من الأنواع الفطرية التي يتم نموها بريا أو فى المزرعة ويتم استهلاكها لقيمتها الغذائية والطبية العالية . وتعد أفراد فطر عيش الغراب الأبيض والتى تشمل النوع *Agaricus bisporus* والتى تنتمى الى العائلة *Agaricaceae* ، هى من أكثر فطريات عيش الغراب انتشارا في العالم بما في ذلك مصر ، وهو ما يمثل ٣٨ ٪ من الإنتاج العالمي من فطر عيش الغراب المزروع. لذلك تهدف الدراسة الحالية إلى إستكشاف التأثيرات الوقائية المحتملة لمستخلصات فطر عيش الغراب الأبيض *Agaricus bisporus* ضد أورام الثدي / السرطان وتصلب الشرايين معمليا خارج الجسم. تم الحصول على عينات فطر عيش الغراب الأبيض من المناطق الداخلية في مصر واستخدمت في تحضير مستخلصات عديدة بإستخدام المذيبات التالية: الماء، الإيثانول، الميثانول. أظهر المستخلص الميثانولي أعلى درجات النشاط المضاد للأكسدة (٨٩,٩٦%) واعلى تزكيز بالنسبة للمركبات النشطة بيولوجيا (باستثناء فيتامين c) بما في ذلك محتوى الفينولات الكلي (١٠٤.٦٩ ملليجرام مكافئ حامض جاليك لكل جرام مستخلص) والفلافونويدات (١٧.٥٤ ملليجرام مكافئ كورستين لكل جرام مستخلص) والكاروتينويدات (٢.٤ ملليجرام لكل جرام مستخلص) والمعادن (الحديد والسيلينيوم بنسب ١.٥٨ ، ٢١.٨٧ ملليجرام لكل جرام مستخلص) ، في حين أظهر المستخلص المائى نشاطا منخفضا فى النشاط المضاد للأكسدة (٤٦.٤٣%) وأقل تزكيز لمحتوى الفينولات الكلي (٣١.٦٤ ملليجرام مكافئ حامض جاليك لكل جرام مستخلص) والفلافونويدات (٤.٩٢ ملليجرام مكافئ كورستين لكل جرام مستخلص والكاروتينويدات (٠.٦١ ملليجرام لكل جرام مستخلص والمعادن (الحديد والسيلينيوم بنسب ٠.٦٧ ، ٦.٠٤ ملليجرام لكل جرام مستخلص). ولقد أشارت نتائج التحليل الإحصائى للمستخلصات المختلفة وجود علاقة نسبية إيجابية ومعنوية ($p \leq 0.01$) بين المحتوى من الفينولات الكلية (r^2= 86.45) والفلافونويدات (r^2= 81.65) والكاروتينات (r^2= 71.56) وفيتامين جـ (r^2= 68.89) وفيتامين هـ ((r^2= 62.1) والنشاط المضاد للأكسدة . أيضا ، أظهرت النتائج أن مستخلص الميثانول لفطر عيش الغراب عاملًا كيميائيًا مفيدًا للوقاية من سرطان أو أورام الثدى وذلك من خلال منع تكسير جزيئات الحامض النووى الديواكسى ريبوز بنسب مختلفة في جميع التركيزات المختبرة ٠.٥٠ ، ١.٠٠ ، ١.٥ ، ٢.٠٪ وذلك بإستخدام خطوط المزارع الخلوية Ehrlich Ascites –EAC Carcinoma.وكذلك.. أكدت النتائج إمكانية مستخلصات فطر عيش الغراب الأبيض الواعدة في الوقاية من تصلب الشرايين عن طريق تثبيط أكسدة البروتين الدهني منخفض الكثافة (LDL) وكنس شقوق البيروكسيل peroxyl radicals التي تتكون خلال عملية أكسدة الدهون في الإجهاد التأكسدي.. وبالتالي ، قد يكون فطر عيش الغراب الأبيض مفيدًا كعامل مضاد للأكسدة ومضاد للسرطان ومضاد لتصلب الشرايين ، كما ان مستخلصاتة خاصة الميثانولية من المحتمل أن تستخدم بنجاح في تطوير الوجبات الغذائية والمنتجات الغذائية وصناعة الأدوية.

الكلمات المفتاحية: فطر عيش الغراب الأبيض ، مستخلص الميثانول، مضاد أكسدة، المركبات النشطة حيويا، المعادن، تجزئة الحامض النووى الديوكس ريبوز، تثبيط ، أكسدة البروتين الدهني منخفض الكثافة.