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Assessment of the antioxidant efficacy of *Agaricus bisporus* against bleomycin-induced pulmonary oxidative stress in albino rats

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ABSTRACT

Oxidative damage is an important factor in many disorders. *Agaricus bisporus* is the world's most popular edible mushroom species. This study investigated how *Agaricus bisporus* can protect against bleomycin-induced pulmonary oxidative damage in rats. The study involved 42 male rats divided into 7 groups, 6 rats per each, for treatment over 3 weeks. The control-negative group (I) administered one ml saline, orally, once daily, while the intoxicated group (II) administered bleomycin sulfate at a dosage of 0.54 mg /rat subcutaneously, twice weekly. Test groups (III, IV) were given *Agaricus bisporus* extract (ABE) at low and high doses of 250 or 500 mg/kg, respectively, orally, daily with bleomycin. The standard group (V) received vitamin C at a dose of 200 mg/kg, orally, daily with bleomycin, and the last two groups (VI, VII) received only *Agaricus bisporus* extract at the same dose. By the end of the experiment, blood samples and lung tissues were taken to evaluate antioxidant enzyme activity and gene expression. Results showed that bleomycin reduced antioxidant enzymes (SOD, GPx, and Catalase) activities and increased lipid peroxidation (MDA), accompanied by the downregulation of the lung SOD, GPx and CAT enzymes' genes. While *Agaricus bisporus* extract significantly countered these effects. Pathological findings supported the biochemical and gene expression findings. The study concluded that *Agaricus bisporus* had notable antioxidant properties and potentially treats oxidative stress-related diseases by enhancing antioxidant enzyme gene expression.

1. INTRODUCTION

Oxidative stress refers to disequilibrium between reactive oxygen species and detoxification processes within the body (Romá-Mateo C. et al., 2015). When the cells' natural redox state is interrupted, damaging peroxides and free radicals are produced, resulting in damage of DNA, proteins, and lipids (Bhattacharya S. et al., 2015). Reactive oxygen species (ROS) induce indirect base damage by generating hydrogen peroxide, superoxide, and hydroxyl radicals (Bhattacharya et al., 2015). Numerous diseases are influenced by oxidative stress such as Atherosclerosis. (Bonomini et al., 2008), depression (Jiménez-Fernández et al., 2015), Alzheimer's disease (Romá-Mateo et al. 2015), and cancer in humans (Hayes et al., 2020).

Bleomycin (BLM) is a cytostatic glycopeptide antibiotic produced by *Streptomyces verticillus*. BLM has been clinically proven to be primarily utilized as a chemotherapy. It is employed in treating carcinomas, tumors of the testicles, and Hodgkin's lymphoma. However, it eventually causes dose-related interstitial lung damage and inflammation. (Della Latta. et al.,2015).

Antioxidant research is ongoing to seek out new, safe, and natural sources of antioxidants that can prevent oxidative stress disorders. Mushrooms contain compounds with various healing properties, including anticancer, immune-stimulating, anti-atherosclerotic, antibacterial, antifungal and antioxidants. (Krishnamurthy. et al., 2012) . Mushrooms have the potential to treat a variety of ailments, including cardiovascular disease, diabetes, malignancies, infection, immune-modulating disorders, and metabolic diseases. (Lindequist et al. 2005)

Agaricus bisporus is the world's most extensively consumed edible mushroom species. It has antimicrobial, anti-inflammatory, cancer-fighting, and immunomodulatory effects. (Yahaya et al., 2014; Meng et al., 2016)). For many years *Agaricus bisporus* has been studied for its potential preventive activity in hypertension, hypercholesterolemia, and malignancies (Elmastas et al., 2007; Tsai et al., 2008.(

As a result, the current study was designed to investigate *Agaricus bisporus* extracts protective capabilities against bleomycin-induced oxidative stress in the lung. The antioxidant enzymes genes expression (CAT, GPX, and SOD), oxidative state (biochemical analysis of plasma antioxidant enzymes (MDA, CAT, GPX, and SOD), and histological changes in the pulmonary tissue were all assessed..

2. MATERIAL AND METHODS

2.1. Identification of the plant

Agaricus bisporus is a type of fungus belonging to the Basidiomycota phylum, Agaricomycetes class, *Agaricales* order, *Agaricaceae* family, and *Agaricus* genus. (Marks, et al., 1991). *Agaricus bisporus* is a young, light-yellow or light-brown mushrooms known as button mushrooms. (Bhushan et al., 2018). The plant was recognized by Dr. Gamal Ashour figure (1), Plant Pathology Professor, Faculty of Agriculture, Benha University. The plant was obtained from El-Abed Mall, Toukh, Qalubiya, Egypt.

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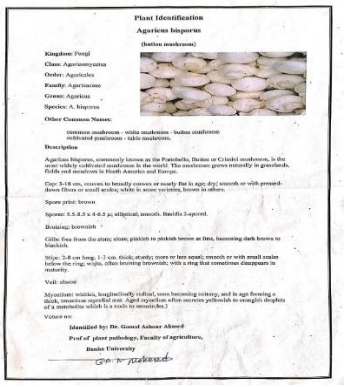
Plant Identification

Agaricus bisporus

(button mushroom)

Kingdom: Fungi
Class: Agaricomycetes
Order: Agaricales
Family: Agaricaceae
Genus: Agaricus
Species: A. bisporus

Other Common Names:

common mushroom - white mushroom - button mushroom
cultivated psalliota - table mushroom.

Description

Agaricus bisporus, commonly known as the Portobello, Button or Cricket mushroom, is the most widely cultivated mushroom in the world. The mushroom grows naturally in grasslands, fields and meadows in South America and Europe.

Cap: 3-18 cm, convex to broadly convex or nearly flat in age; dry; smooth or with pressed-down fibres or small scales; white in some varieties, brown in others.

Spore print: brown

Spore: 5.5-8.5 x 4-6.5 µ; elliptical; smooth. Basidia 2-spored.

Bruising: brownish

Gills: free from the stem; stem; pinkish to pinkish brown as first, becoming dark brown to blackish.

Stipe: 3-8 cm long, 1-3 cm. thick; sturdy; more or less equal; smooth or with small scales below the ring; white, often bruising brownish; with a ring that sometimes disappears in maturity.

Veil: absent

Mycelium: whitish, longitudinally rolled, soon becoming cottony, and in age forming a thick, tenacious mycelial mat. Aged mycelium often secretes yellowish to orangish droplets of a metabolite which is a toxic to nematodes.)

Voucher no:

Identified by: Dr. Gamal Ashour Ahmed
Prof of plant pathology, Faculty of agriculture,
Benha University

Fig. 1 Identification of the plant *Agaricus bisporus*

2.2.1. Preparation of different doses (HD &LD) to be suitable for rats

The hydro-ethanolic extract was prepared at concentrations of 50 mg/ml and 100 mg/ml, representing low (250 mg/kg) and high doses (500 mg/kg) of *Agaricus bisporus* extract respectively, (Yamac et al., 2010, Ruthes. et al., 2015). If a 200 g rat receives 1 ml of the extract, the yield percentage was calculated using the formula: yield% = (weight of extract) / (weight of plant material) ×100.

2.3. Reagents, Chemicals and Kits

Bleomycin sulphate injection (15 units) was obtained as a vial named (bleocip)® from Bristol-Myers Squibb Company in Cairo, Egypt. used to cause oxidative injury in the rat's lung at a dose of 0. 54 mg/rat subcutaneously (Juul Jorgensen et al., 1972). Vitamin C was purchased as a capsule (C retard® 500 caps) Hikma plc company, Egypt, with a dose of 200 mg/kg orally (Kalender et al.,2010).

2.4. Experimental animals

The current research involved 42 male Wistar albino rats weighing 180−220 g, obtained from the animal house, Faculty of Veterinary Medicine, Benha University, Egypt. The animals were acclimated over two weeks. at about 25 °C and given a standard diet and water freely. The study protocol and the use of rats were approved by the Ethics Committee, Benha University's Faculty of Veterinary Medicine (BUFVTM15-09-24).

2.4.1. Animal grouping

The study involved 42 acclimated male rats divided into 7 groups, 6 rats /group with different treatments.

Group I: control negative, received 1 ml of saline orally once daily for three weeks.

Group II: The control positive group received Bleomycin sulfate at 0. 54 mg per rat, twice weekly, subcutaneously for three weeks.

Group III: Intoxicated low dose, received a small dose of Agaricus bisporus extract (250 mg/kg) once daily per os, along with Bleomycin sulfate at 0. 54 mg per rat, twice a week, subcutaneously for three weeks.

Group IV: Intoxicated high dose, received a high dose of *Agaricus bisporus* extract (500 mg/kg) once daily per Os., along with Bleomycin at 0. 54 mg per rat, twice a week, subcutaneously for three weeks.

Group V: Standard group, which received Vitamin C at 200 mg/kg once daily per Os, along with Bleomycin for three weeks.

Group VI: ABE low dose received a small dose of *Agaricus bisporus* extract (250 mg/kg) once daily per Os for three weeks.

Group VII: ABE high dose received a high dose of *Agaricus bisporus e*xtract (500 mg/kg) once daily per Os for three weeks.

2.4.2. Sampling

Blood samples were collected after three weeks of treatment and placed in test tubes with lithium heparin. They were centrifuged to separate plasma, which was stored at -40°C for measuring oxidative stress indicators (catalase, SOD, GPx, and MDA). Animals were euthanatized by cervical dislocation and tissue samples from the lung were kept at -80°C for gene expression analysis. Specimens for histopathology were fixed in 10% formalin.

2.5. Assessments

2.5.1. Oxidative stress assessment

The oxidative stress biomarkers, including SOD, GPx, Catalase, and MDA activities were assessed using commercial kits from Bio diagnostic, Doki, Giza, (Catalog No. TA2513 for GPx, Catalog No. TA2529 for MDA, Catalog No. TA2521 for SOD, Catalog No. TA2517 for catalase)., following the manufacturer's instructions at the Central Laboratory of Veterinary Medicine College, Benha University, Egypt.

2.5.2. Gene Expression Assessment

Total RNA was isolated employing TRIzol Invitrogen following the manufacturer's directions. Each cDNA reaction involved one microliter of RNA. Pratrimer sequences to be used in the experiment were as follows:

Gene	Forward primer	Reverse primer
CAT	5′-GTCCGATTCTCCACAGTCGC-3′	5′-CGCTGAACAAGAAAGTAACCTG-3′
SOD	5′-ATGGGGACAATACACAAGGC-3′	5′-TCATCTTGTTTCTCGTGGAC-3′
GPx	5′-CACAGTCCACCGTGTATGCC-3′	5′-AAGTTGGGCTCGAACCCACC-3′
ß-Actin	5′-TCACTATCGGCAATGTGCGG-3′	5′-GCTCAGGAGGAGCAATGATG-3′

The fold change in mRNA levels among the treated and untreated groups was corrected for ß-ACTIN levels which was selected for normalization due to its well-established stable expression in oxidative stress and antioxidant-related studies (Bustin et al., 2009). Its expression stability was validated which confirmed minimal variability (M-value < 0.5) across all experimental conditions (Vandesompele et al., 2002). Additionally, the coefficient of variation (CV) for ß-Actin expression was <5%, further supporting its suitability as a reference gene (Bustin et al., 2009). 25 µl reaction mixture contained 0. 1 µl of each primer, 12.5 µl of Power SYBR Green PCR Master Mix, 11 µl of nuclease-free water, and 1. 25 µl of cDNA sample. The data was processed to calculate relative gene expression, employing the equation: fold change = $2^{-\Delta(\Delta Ct)}$ (Pfaffl. et al., 2002), where $\Delta Ct = Ct(target) - Ct(ß\text{-}ACTIN)$ and $\Delta(\Delta Ct) = \Delta Ct(treated) - \Delta Ct(untreated)$. Each reaction had been carried out in duplicate on the CFX96 (BioRad, USA) .

2.5.3. Histopathological assessment

Lung tissues were fixed with formalin 10%, then washed, dehydrated, and enclosed in paraffin. Tissue paraffin sections 5 µm in thickness were processed, stained with H&E according to (Suvarna. et al. 2018), and analyzed microscopically using light microscope.

2.6. Statistical analysis

All data are shown as the mean ± SE. To assess significant differences between groups, one-way ANOVA was performed, followed by Tukey's post-hoc testing using GraphPad Prism v. 6. P-values ≤ 0.05 were considered statistically significant.

3. RESULTS

3.1. Oxidative stress measurements

As shown in Table (1), rats treated with bleomycin at a dose of 0.54 mg/rat had higher MDA levels compared to the control group. In contrast, the groups co-treated with a low dose of ABE showed a reduced MDA concentration, while those receiving a high dose displayed even lower MDA values. co-treated group with Vitamin C showed the lowest MDA levels, although it was still greater than the control group's MDA level. The groups treated with ABE at doses of 250-500 mg/kg showed MDA levels similar to the control group. Additionally, Rats treated with bleomycin showed lower catalase, SOD, and GPx enzyme activity relative to the control group. However, coadministration of 250 mg/kg ABE with bleomycin normalized enzyme activity. Moreover, coadministration of 500 mg/Kg ABE with bleomycin normalized the enzyme activities more than ILD, and the group treated with Vit C showed higher activities of the enzymes but still lower than the control group. Moreover, treated groups with ABE at a low and a high dose of (250-500) mg /kg show the normal activity of the enzymes SOD, GPx, and CAT.

Table 1 The effect of *Agaricus bisporus* extract and bleomycin on oxidative stress biomarkers (catalase, SOD, GPx, and MDA) in the plasma.

	SOD (U/ml)	GPX (U/l)	CAT (U/l)	MDA (nmol/ml)
G 1: Control Negative	215.73±5.85^{a}	33.92±1.74^{a}	83.75±5.23^{a}	54.27±4.48^{e}
G2: BLM Intoxicated	76.50±4.29^{e}	14.52±1.39^{e}	25.57±4.17^{e}	250.19±17.23^{a}
G3: Intoxicated Low Dose	104.97±13.28^{d}	18.48±0.89^{d}	37.45±2.66^{d}	193.94±8.36^{b}
G4: Intoxicated High Dose	139.66±8.21^{c}	23.59±0.80^{c}	52.78±2.40^{c}	160.59±8.54^{c}
G5: Standard	170.70±5.93^{b}	27.64±1.22^{b}	64.91±3.65^{b}	107.62±6.25^{d}
G6: ABE low dose	221.70±12.41^{a}	33.33±1.66^{a}	81.98±4.98^{a}	53.66±3.04^{e}
G7: ABE high dose	216.55±9.44^{a}	34.56±1.10^{a}	85.69±3.31^{a}	59.02±3.34^{e}

SE (standard error), SOD (superoxide dismutase), GPx (glutathione peroxidase), CAT (catalase), and MDA (malondialdehyde). Comparisons were made using one-way ANOVA and a post-hoc Tukey's test with a significance level of 0.05. The data is presented as (Mean ± S.E), with various superscript letters signifying significant differences at $P < 0.05$

3.2. Gene Expression

As shown in Table (2), relative catalase, SOD, and GPx gene expression in the lung decreased in rats injected with bleomycin in comparison to the untreated rats. However, co-treatment with 250 mg/kg ABE increased catalase, GPx, and SOD gene expression slightly compared to bleomycin alone, but not as much as the control. Additionally, co-treatment with 500/kg ABE enhanced the rise in catalase and SOD gene expression compared to the control. On the contrary, concurrent treatment with vitamin C showed increased catalase, SOD, and GPx gene expression in comparison with treatment with bleomycin only and the control group, which resembled the impact of ABE 500mg/kg.

Table 2 Lung gene expression of antioxidant genes (CAT, SOD and GPx) among treated and control groups.

	CAT	SOD	GPx
G 1: Control Negative	1.02±0.05^{a}	1.03±0.04^{a}	1.04±0.09^{a}
G2: BLM Intoxicated	0.30±0.02^{e}	0.47±0.04^{e}	0.39±0.02^{e}
G3: Intoxicated Low Dose	0.51±0.01^{d}	0.59±0.03^{d}	0.54±0.02^{d}
G4: Intoxicated High Dose	0.66±0.02^{c}	0.72±0.05^{c}	0.71±0.02^{c}
G5: Standard	0.80±0.01^{b}	0.86±0.02^{b}	0.85±0.02^{b}
G6: ABE low dose	1.05±0.09^{a}	1.05±0.01^{a}	1.06±0.04^{a}
G7: ABE High Dose	1.07±0.06^{a}	1.08±0.05^{a}	1.15±0.04^{a}

Standard error (SE), superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx). Comparisons were made using one-way ANOVA and a post-hoc Tukey's test with a significance level of 0.05. The data is presented as (Mean ± S. E), with various superscript letters signifying significant differences at $P < 0.05$

3.3. Pathological findings

Microscopic examination of lung tissue sections in the control group revealed normal histologic structure of lung tissue (Fig 2A). Meanwhile, the bleomycin-treated group exhibited intense peri-bronchiolar mononuclear inflammatory cell infiltration with desquamation of the bronchiolar epithelial Extensive interstitial pneumonia masks the alveoli with mononuclear inflammatory cells infiltrating the alveolar wall and the perivascular spaces (Fig 2B). Moderate interstitial pneumonia was recorded in the intoxicated low-dose group as the examined sections revealed variable mononuclear inflammatory cell infiltrations around the bronchioles with infiltrations in the interstitial tissue (Fig 2C). Marked improvement was noticed in the intoxicated high-dose group as almost all examined sections were normal with fewer sections revealing mild perivascular inflammatory cell aggregation (Fig 2D). Moderate improvement was noticed in the Vitamin C treated group as the examined lung sections showed fewer perivascular lymphocytic cuffing with fewer interstitial mononuclear inflammatory cell infiltrations (Fig. 2E).

4. DISCUSSION

Oxidative stress is a complex process caused by internal and external factors, significantly influencing aging, raising the risk of chronic diseases, and leading to serious outcomes. It is linked to various diseases like cardiovascular issues, mutations, cancer, and neurodegenerative disorders. (Sachindra et al., 2010). Bioactive compounds having potent antioxidant qualities, such as phenolics and glycosides, are found in naturally occurring sources originating from plants, fungi, and algae. (Chen et al., 2012). As a result, studying bioactive-rich edible mushroom samples is critical for discovering new and safe antioxidants that can prevent and/or mitigate the detrimental consequences of oxidative stress.

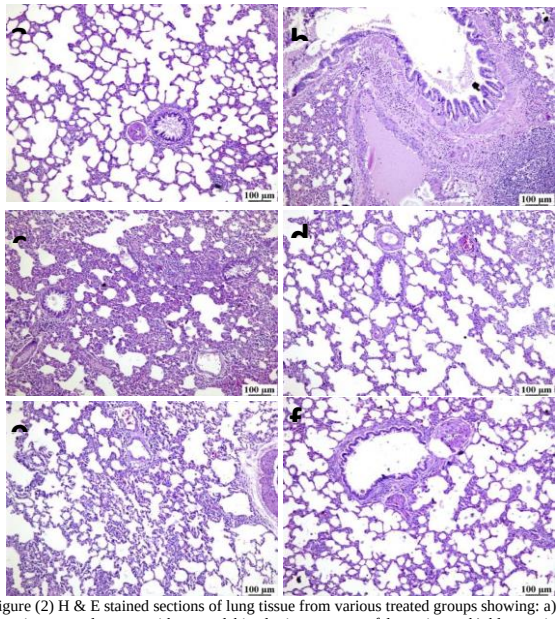

Figure (2) H & E stained sections of lung tissue from various treated groups showing: a) negative control group with normal histologic structure of lung tissue, b) bleomycin treated group showed intense peri-bronchiolar mononuclear inflammatory cell infiltration and Extensive interstitial pneumonia, c) intoxicated low dose group showed variable mononuclear inflammatory cell infiltrations around the bronchioles, d) intoxicated high dose group showed normal lung structure with fewer sections revealing mild perivascular inflammatory cell aggregation, e) Vit C treated group showed fewer perivascular lymphocytic cuffing with fewer interstitial mononuclear inflammatory cell infiltrations, f) ABE low dose treated group with normal alveoli structure.

In the current investigation, the levels of antioxidant enzymes (SOD, CAT, and GPX) in plasma were considerably lower, and the oxidative stress indicator (MDA) was significantly greater in rats given a dosage of bleomycin (0.54 mg/rat) subcutaneously twice a week for three weeks. Combining ABE (250 and 500 mg/kg) with bleomycin resulted in a dose-related rise in plasma CAT, SOD, and GPx enzymes ($P < 0.05$) and reduced plasma MDA level. Similarly, vitamin C (200 mg/kg) coupled with bleomycin produced the same effect .

These findings were consistent with the findings reported by Liu, et al., (2018), who demonstrated that ABE polysaccharides significantly reduced MDA levels in livers damaged by CCl4 in a mouse model and improved antioxidant activity of liver GPx. and SOD. The data presented also supports the work of Khazri et al. (2016), indicating that Bleomycin raised lung lipoperoxidation and diminished activities of antioxidant enzymes like catalase, SOD, and GPx. Similarly, Bahri et al. (2020) noted that BLM raised lipid peroxidation and lowered SOD and CAT activities. However, these findings contrast with Chang, et al., (2011), who found no significant reversal of GPx and catalase changes after treatment with *Agaricus blazei Murrill.*

Relative catalase, SOD, and GPx gene expression in the lung was decreased in animals receiving BLM relative to control rats. However, co-treatment with (250-500) mg/kg ABE enhanced the catalase and SOD gene expression when compared with BLM alone., but less than the control. and similar to the vitamine C treated group Additionally, co-treatment with 250-500 mg / kg ABE alone showed a similar effect to the control group.

The present study demonstrated an increased MDA, reduced catalase and SOD enzyme activity in conjunction with lower gene expression of catalase, SOD, and GPx enzymes in animals exposed to BLM compared to control, demonstrating oxidative damage. In conformity with our results, a study of mice under restraint stress showed decreased catalase and SOD enzyme activity and increased lipid peroxidation in their brains, which were notably avoided on *Agaricus bisporus* supplemented diets (García-Sanmartín. et al., 2022). Consistently, a recent study on the impact of ABE on antioxidant activity and oxidative stress in treated rats, the SOD, CAT, and GPx activity was enhanced in the group treated with ABE (Iqbal, et al.,2024). Another study reported a decreased activity of SOD, CAT, and GPX enzymes after treating mice with 4U/ kg/2ml BLM for 14 days (Shariati et al., 2019).

Pathological observations in this study supported biochemical findings and antioxidant gene expression results. which demonstrated that *Agaricus bisporus* extract led to a significant reduction in the pulmonary index, inflammatory cell infiltration and bronchiolar epithelial desquamation in animals supplemented with "ABE" low and high doses along with BLM, which was associated with increased levels of plasma GPx, CAT, and SOD enzymes and decreased level of MDA in conjunction with increased gene expression of catalase, SOD, and GPx enzymes. These results indicated that ABE played a positive protective role in BLM-induced pulmonary fibrosis. these findings align with Kan et al., (2015) who found that treatment with *Ganoderma lucidum* mushroom polysaccharides significantly reduced pulmonary fibrosis and inflammation in rats with BLM-induced pulmonary fibrosis. Similarly, Su et al. (2019) who noted that the lung of BLM-treated mice displayed significant inflammatory alterations. While mushroom *phellinus linteus* extract supplementation for 4 weeks improved BLM-induced lung histopathological damage in a dose-dependent manner. additionally, our findings are consistent with Shariati et al. (2019) who found that the treated mice group with BLM is damaged with the obvious lesions, cell infiltration, and decomposed tissue. In MT staining, the collagen can be seen as blue strings, and Bahri et al. (2023) noted pulmonary fibrosis in the lungs of rats treated with BLM..

5. CONCLUSIONS

In conclusion, *Agaricus bisporus* serves as an effective antioxidant against Bleomycin-induced oxidative injury in rats, suggesting its potential as a natural nutraceutical for the protection against oxidative damage-related diseases.

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