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Chemical, enzymatic and cellular antioxidant activity studies of *Agaricus blazei* Murrill

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ABSTRACT

Mushrooms possess nutritional and medicinal properties that have long been used for human health preservation and that have been considered by researchers as possible sources of free radical scavengers. In this work, the antioxidant properties of water extracts from *Agaricus blazei* Murill, produced by maceration and decoction, are demonstrated *in vitro*. Resistance to oxidation is demonstrated through three mechanisms: i) inhibition of enzymatic oxidative process, with 100% inhibition of HRP (horseradish peroxidase) and MPO (myeloperoxidase); ii) inhibition of cellular oxidative stress, with 80% inhibition of the oxidative burst of polymorphonuclear neutrophils (PMNs); and iii) direct action over reactive species, with 62% and 87% suppression of HOCl and superoxide anion radical ($O_2^{\bullet -}$), respectively. From the data, it was concluded that the aqueous extract of *A. blazei* has significant antioxidant activity, indicating its possible application for nutraceutical and medicinal purposes.

Key words: *Agaricus blazei* Murrill, antioxidant, oxidant species, chemiluminescence, polymorphonuclear neutrophils.

INTRODUCTION

Oxidations are essential reactions in the biological processes of many organisms. However, reactive oxygen species (ROS), which are continuously produced *in vivo*, are known to promote cell death and tissue damage (Calabrese et al. 2007, Halliwell and Gutteridge 1999). Aging and diseases, including atherosclerosis, diabetes, cancer and cirrhosis, have been linked to oxidative damage (Vellosa et al. 2007a, Morton et al 2000, Eastwood 1999, Vinson et al. 1995, Halliwell et al. 1995a).

There is an increasing emphasis on research aimed to identify and utilise antioxidants from natural sources (Ramarathnam et al. 1995). Antioxidants are of great interest because of their possible role in protecting the organism

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against damage by ROS (Halliwell et al. 1995b) such as superoxide anion radical ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^{\bullet}$). These species are by-products of normal metabolism that can attack biological molecules such as lipids, proteins, DNA and RNA, leading to cell or tissue injury associated with degenerative diseases (Jung et al. 1999).

Agaricus blazei Murrill (Agaricaceae), an edible mushroom, is native to the small Brazilian town of Piedade (Mizuno 1995, Heinemann et al. 1993). Today, *A. blazei* is consumed globally as food or in tea on account of its putative medicinal properties (Mshandete and Cuff 2007, Kaneno et al. 2004). Previous studies with isolated fractions of the *A. blazei* fruit bodies indicated that some samples exhibited antimutagenic, anticarcinogenic and immunostimulative activities (Mizuno et al. 1998, Kawagishi et al. 1990, Itoh et al. 1994, Osaki et al. 1994, Mizuno et al. 1990).

Natural products with antioxidant activities are used to aid the endogenous immune system. As a result, increasing interest has been expressed in the antioxidative roles of nutraceutical products (Pietta et al. 1998). Polyphenols and flavonoids are plant constituents with a probable effect on the organism's ability to scavenge free radicals (Vellosa et al. 2006). Studies by Barros et al. (2007), Ferreira et al. (2007) and others (Turkoglu et al. 2007, Oliveira et al. 2007, Cheung et al. 2003, Cheung and Cheung 2005, Lo and Cheung 2005, Yang et al. 2002) indicate a correlation between the mushrooms' antioxidant activity and their phenolic content.

The aim of this study is to evaluate the antioxidant properties of aqueous extracts of *A. blazei*, particularly the reduction in activity of oxidoredutase enzymes such as HRP, the scavenging effects on radicals such as superoxide radical and HOCl and the inhibition of the oxidative burst of activity of polymorphonuclear neutrophils (PMNs).

MATERIALS AND METHODS

The enzyme horseradish peroxidise (HRP) type VI, phorbol-12-myristate-13-acetate (PMA), 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS), 5-thio-2-nitrobenzoic acid (TNB), phenazine methosulphate (PMS), nicotinamide adenine dinucleotide reduced (NADH), nitrobluetetrazolium (NBT), luminol and 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB) were purchased from Sigma-Aldrich Chemical Company, (St Louis, MO, USA). Hydrogen peroxide (30% solution) was purchased from Peroxides, Brazil (São Paulo, SP, Brazil). All of the reagents used for buffer preparation were analytical grade.

Apparatus

All chemiluminescence measurements were carried out using a luminescence photometer Luminometer 1251 (BioOrbit model, Finland) and were monitored on a computer workstation running the program Multiuse v 2.0. All assays were conducted on an HP 8453 Diode Array Spectrophotometer.

Mushroom Material

Agaricus blazei Murrill (*A. blazei*) fruiting bodies were collected at Valemar Ranch (São José do Rio Preto, SP, Brazil) and identified by Dr. Arailde Fontes Urben (EMBRAPA researcher).

Two types of *A. blazei* aqueous extracts were prepared. First, a DECOCTION extract was obtained by decocting 1 g of powdered mushroom in 100 mL of distilled water until the volume was reduced by half. Next, a MACERATION extract was obtained by macerating 1 g of powdered mushroom in 50 mL of distilled water. Material was refrigerated at 4°C for 60 minutes and then filtered and fractionated. The extract was used within 4 hours.

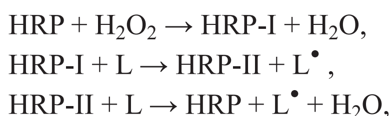
HRP/H_2O_2/Guaiacol Kinetics Activity Measurements

Both macerate and decoction extracts were subjected to the peroxidase colorimetric assay.

All horseradish peroxidase activity assays were conducted by measuring the oxidation of guaiacol as substrate. H_2O_2 and HRP solutions were prepared in MilliQ water and their concentrations were determined spectrophotometrically using the molar absorption coefficients of $\varepsilon_{230\,nm} = 80\ M^{-1}cm^{-1}$ (Brestel 1985) and $\varepsilon_{403\,nm} = 1.02 \times 10^5\ M^{-1}cm^{-1}$ (Ohlsson and Paul 1976), respectively. Typical reaction mixtures contained 10^{-9} M HRP enzyme, 50 mM phosphate buffer, pH 7.0 and 25 mM guaiacol, at 25°C. The reaction was started by the addition of H_2O_2 and product formation was followed spectrophotometrically at 470 nm (Makinen and Tenovuo 1982, Gazarian and Lagrimini 1996). The initial reaction rate (v_o) was determined by the angular coefficient of the plot of absorbance at 470 nm versus time (seconds), extrapolated to time zero. All reactions were conducted in triplicate (Desser et al. 1972).

CHEMILUMINESCENCE ASSAY OF HRP ACTIVITY

Inhibition of HRP activity by *A. blazei* was measured by chemiluminescence. Luminol solutions were prepared in MilliQ water and their concentrations were determined spectrophotometrically using the molar absorption coefficient $\varepsilon_{347\,nm} = 7{,}636\ M^{-1}cm^{-1}$ (Allen and Loose 1976). Typical reaction mixtures contained 3×10^{-8} M HRP enzyme, 50 mM phosphate buffer, pH 7.0 and 5×10^{-6} M luminol, at 37 °C (Brestel 1985). All reactions were conducted in triplicate. The net reaction is illustrated in the following scheme:



$HRP + H_2O_2 \rightarrow HRP\text{-}I + H_2O$,
$HRP\text{-}I + L \rightarrow HRP\text{-}II + L^{\bullet}$,
$HRP\text{-}II + L \rightarrow HRP + L^{\bullet} + H_2O$,

where HRP, HRP-I and HRP-II are the ferric peroxidase and intermediate compounds of horseradish peroxidase; and L and $L^{\bullet}$ are luminol and its radical product of one electron oxidation, respectively. The radical product of luminol oxidation is then converted into 3-aminophthalate, resulting in light emission (Dodeigne et al. 2000). The integrated light emission was taken as the analytical readout.

MPO/H_2O_2/TNB KINETIC ACTIVITY MEASUREMENTS

All myeloperoxidase (MPO) activity assays were conducted by measuring the oxidation of 5-thio-2-nitrobenzoic acid (TNB). TNB was prepared according to Ching et al. (1994). The concentration of TNB was determined by measuring the absorbance at 412 nm using $\varepsilon = 13{,}600\ M^{-1}\ cm^{-1}$.

Typical reaction mixtures contained 0.04 U/mg MPO enzyme, 50 mM phosphate buffer, pH 7.0, 0.07 mM TNB, 0.1 mM taurine, 50 µL of *A. blazei*, and 0.1 mM H_2O_2, at 25°C. The reaction was started by the addition of H_2O_2 and the TNB consumption was monitored spectrophotometrically at 412 nm. The initial reaction rate (v_o) was determined by the angular coefficient of the plot of the absorbance at 412 nm versus time (in seconds), extrapolated to time zero. All reactions were conducted in triplicate.

The antioxidant activity of the extract from macerated A. blazei was determined by comparing the results of three reactions:

Reaction **A**: MPO + Taurine + Catalase + TNB;
Reaction **B**: MPO+Taurine+H_2O_2+Catalase+TNB;
Reaction **C**: MPO + Taurine + H_2O_2 + *A. blazei* + Catalase + TNB

The antioxidant activity was calculated as the percent inhibition of TNB oxidation to DTNB by the following equation:

$$\%\ inhibition = \frac{\Delta(A-B) - \Delta\ (A-C)}{\Delta(A-B)}\, x100$$

where A, B and C correspond to the absorbance change observed for reactions A, B and C, respectively.

CHEMILUMINESCENCE ASSAY USING PMNS

Primary polymorphonuclear neutrophils (PMNs) were obtained according to the following protocol. A 5% oyster glycogen solution (dissolved in

0.85% NaCl) was injected into the peritoneum of anesthetized rats. This work was submitted to the Ethics Committee of FCFAR / UNESP and authorized by the protocol CEP nº 65/2009. The animals were kept with food and liquid *ad libitum* and sacrificed 12 h after injection. Calcium-free Dulbecco's phosphate-buffered saline (PBS-D, 20 mL), containing 10 IU heparin per mL, was injected into the peritoneal cavity (Babior and Cohen 1981, Paino et al. 2005). The discharge was collected and centrifuged for 3 min at 2,000 g. The cell pellet was layered on Ficoll-Hypaque 1077 and centrifuged for 25 min at 2,000 g. PMN cells were collected, washed and kept in ice-cold PBS-D until required.

The effect of mushroom macerate on PMN activity was assayed by chemiluminescence (Parij et al. 1998). PMNs (1 x 10^6 cells/mL), suspended in PBS-D buffer, pH 7.2, were stimulated by the addition of phorbol-12-myristate-13-acetate (PMA, 1x10^{-5} mM) in the presence of luminol (10^{-5} M). The chemiluminescence emission was measured in millivolts (mV) at 37°C in 1-s intervals for 1 hour. The ideal dose was determined by the addition of different volumes of *A. blazei* extracts. PMN activity was expressed as a percent reduction in the maximum chemiluminescence response of PMNs to PMA in a positive control reaction. Negative controls were performed by omitting PMA. The integrated light emission was taken as the analytical readout.

Assay of Superoxide Anion Radical ($O_2^{\bullet -}$) Suppression

Superoxide radicals, produced by reduced nicotinamide adenine dinucleotide (NADH) and phenazine methosulphate (PMS), reduce nitrotetrazolium blue chloride (NBT) to produce a formazan compound. The intensity of the colour is inversely proportional to the antioxidant concentration (Vellosa et al. 2007B, Kakkar et al. 1984). The assay was carried out in sodium pyrophosphate buffer (25 mM, pH 8.3) and the mixture contained 25 μL of 0.372 mM PMS, 75 μL of 0.6 mM NBT, 50 μL of 1.56 mM NADH, macerate mushroom extract (several volumes) and buffer to a final volume of 1 mL. Reactions were started by the addition of NADH. After a 90 seconds incubation at 25°C, 100 μL of glacial acetic acid and 900 μL of sodium pyrophosphate buffer were added. After vigorous homogenisation, the colour intensity of the mixture was measured at 560 nm. All reactions were conducted in triplicate.

Statistical Analysis

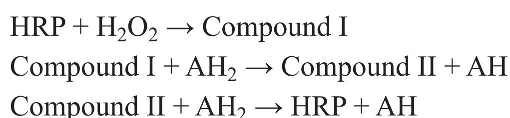
Data are reported as the mean ± SD. The Student t-test was used to determine the difference between test and control preparations, with the level of significance set at $p < 0.05$.

RESULTS AND DISCUSSION

The goal of this study was to evaluate the aqueous extract from *A. blazei* as a source of pharmacological agents against oxidative stress. The aqueous extracts used for most experiments were obtained by maceration at 25°C, as it was observed that extracts obtained by decoction had low activity (Figure 1). A possible explanation for this observation is that the high temperature (~100°C) of the extraction induces polymerization of lower molar mass phenols, leading to a lower antioxidant capacity.

Kinetics Enzymatic HRP/Guaiacol/H_2O_2 Assay

Job and Dunford (1976) have studied the oxidation of several phenols and aromatic amines through horseradish peroxidase (HRP). The one-electron oxidation of organic compounds (AH) by horseradish peroxidase may be represented as follows:



$HRP + H_2O_2 \rightarrow$ Compound I
Compound I + $AH_2 \rightarrow$ Compound II + AH
Compound II + $AH_2 \rightarrow$ HRP + AH

The HRP activity was studied by spectrophotometrically monitoring guaiacol oxidation, which generates a chromophore with an absorbance at 470 nm within 1 minute. Figure 1 presents the

inhibitory effects of aqueous extracts of *A. blazei*, prepared by either maceration or decoction, on the HRP/guaiacol/H_2O_2 assay. HRP activity was completely inhibited in the presence of the macerate. The mechanism by which the extracts exert their antioxidant properties cannot be determined by this method alone; however, it is likely that the macerate extract provides phenolic compounds able to act on the enzyme system or to reduce the oxidised product.

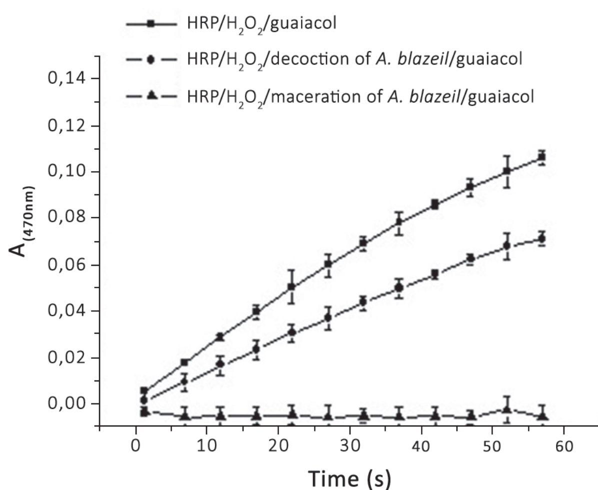


Figure 1 - HRP/guaiacol/H_2O_2 assay in aqueous extract, macerated and decoction of *A. blazei*. The incubation mixture contained HRP/ $3x10^{-5}$ mM; H_2O_2 /$3.0x10^{-1}$ mM; Guaiacol /$5.0x10^{-4}$ mM, (Mean ± SD; n=3).

Chemiluminescence Assay Using HRP and *Agaricus Blazei* Murill

The luminol-dependent chemiluminescent assay lacks specificity regarding the ROS generated upon neutrophil activation (Dodeigne et al. 2000); therefore, experiments were performed to determine the effect of *A. blazei* extracts on the activity of the HRP/H_2O_2 enzymatic system. Peroxidases catalyse the oxidation of luminol by hydrogen peroxide.

Figure 2 presents the inhibitory effects of aqueous macerated extracts from *A. blazei* on the HRP-catalysed, luminol-dependent chemiluminescence assay. Note that in the control reaction of luminol, HRP and H_2O_2, the light intensity is 2,000 mV; the addition of 100 µL of aqueous macerated extract reduces the light intensity to 18.5 mV and, hence, reduces oxidation by HRP.

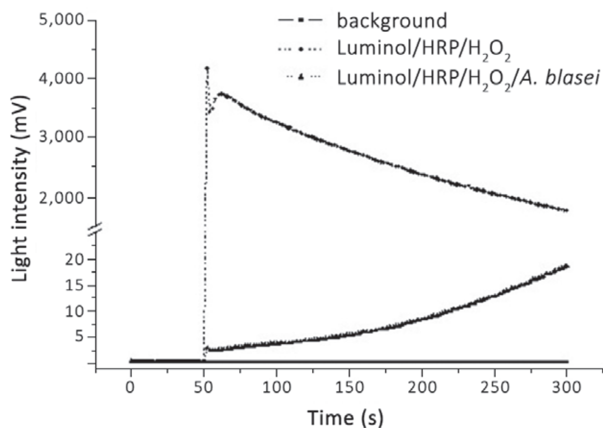


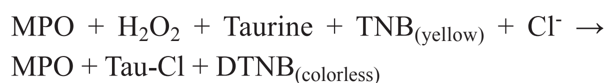
Figure 2 - Effect of the mushroom macerate extract over chemiluminescence intensity during luminol oxidation by hydrogen peroxide catalyzed by HRP. The incubation mixture contained 50 mM of Phosphate buffer, pH 7.0, $3x10^{-8}$ mM HRP, and $5x10^{-6}$ mM luminol. Chemiluminescence intensity was registered 5 min, by 37°C, after the start of the reaction.

Antioxidant Activity of *Agaricus blazei* Murill by the MPO/H_2O_2/TNB System

The neutrophils' oxidative burst is the result of the assembly of the multi-enzyme NADPH-oxidase system that promotes the one-electron reduction of oxygen to superoxide anion radical (Babior 2000). Next, this species is reduced to hydrogen peroxide by superoxide dismutase. Finally, hydrogen peroxide is used by myeloperoxidase (MPO) to oxidise chloride to hypochlorous acid (HOCl) (Hampton et al. 1998, Podrez et al. 2000, Lapenna and Cuccurullo 1996). This highly oxidising molecule has been proposed to be the primary agent responsible for the antimicrobial action of PMNs. Figure 3 represents the antioxidant activity of aqueous extracts of *A. blazei* in the following reactions:

Column **A**: MPO + Taurine + Catalase + TNB; Column **B**: MPO + Taurine + H_2O_2 + Catalase + TNB; Column **C**: MPO + Taurine + H_2O_2 + *A. blazei* + Catalase + TNB

This assay is based on the ability of a substance (scavenger) to inhibit the oxidation of 5-thio-2-nitrobenzoic acid (TNB) to 5,5'-dithio-2-nitrobenzoic acid (DTNB) in the presence of the oxidants HOCl and chloramine-taurine (Tau-Cl, a species also formed *in vivo*), (Ching et al. 1994) generated *in vitro* by MPO and H_2O_2. The antioxidant activity of the macerated extract of *A. blazei* is expressed by the following reaction:



$$MPO + H_2O_2 + Taurine + TNB_{(yellow)} + Cl^- \rightarrow MPO + Tau\text{-}Cl + DTNB_{(colorless)}$$

The antioxidant activity, calculated as the percent inhibition of TNB oxidation, was 62%.

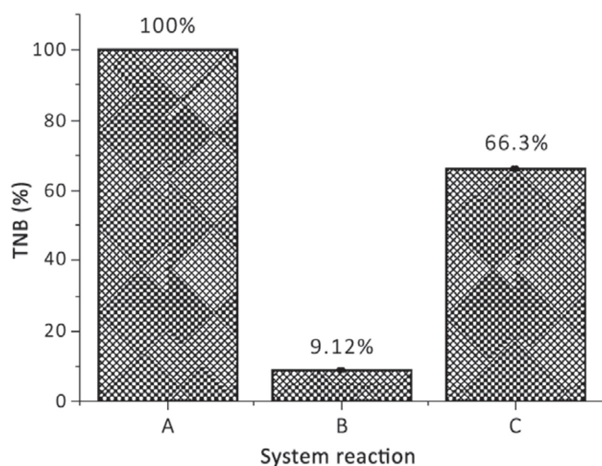


Figure 3 - Antioxidant activity of aqueous macerate extract of *A. blazei* on the system MPO/Taurine/H_2O_2/Cl^-. (Mean ± SD; n=3). (Mean ± SD; n=3). The incubation mixture contained: MPO/0.04 U/mg, H_2O_2/$2x10^{-5}$ mM, TNB/0.074 mM, Tau/0.1 mM, PBS-D buffer by Cl^-, pH 7.4. Columns: **A**, MPO/Taurine/Catalase/TNB, by absorbance of 1.130; **B**, MPO/Taurine/H_2O_2/Catalase/TNB, by absorbance of 0.130; and **C**, MPO/Taurine/H_2O_2/Catalase/TNB/*A. blazei*, by absorbance of 0.749.

Luminol-Dependent Chemiluminescence Assay Using Polymorphonuclear Neutrophils (PMNS): Oxidative Burst Inhibition by *Agaricus Blazei* Murill

Luminol-dependent chemiluminescence (LDCL), commonly believed to result from the production of $O_2^{\bullet -}$, HOCl and H_2O_2, is observed during the oxidative burst of PMNs (Dahlgren and Karlsson 1987, Allen 2000).

The oxidative burst of PMNs was monitored using the soluble stimulant phormol-12-myristate-13-acetate (PMA). Studies by Dahlgren and Karlsson (1987) showed that LDCL produced by PMNs depends largely on the generation of HOCl by the MPO/H_2O_2/Cl^- system. Thus, the oxidation of luminol can occur either by the peroxidase reaction or by the direct reaction of luminol with HOCl.

PMN assays conducted in the presence of the *A. blazei* aqueous extracts demonstrate reduced LDCL on PMA-stimulated PMNs (Figure 4). This effect may be linked to the action of compounds in the *A. blazei* extract on the oxidative burst enzymes or to a direct reaction with HOCl and possibly other reactive oxygen species such as superoxide anion.

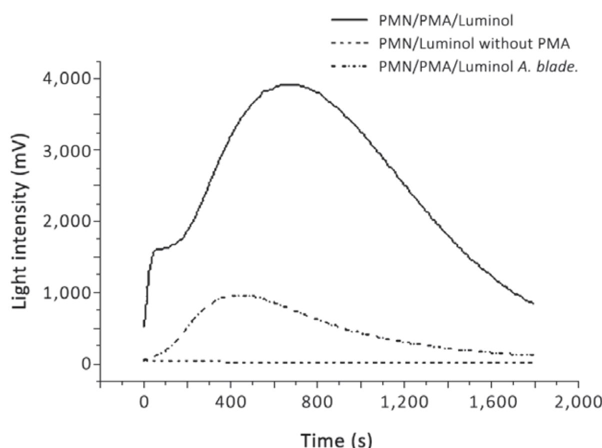


Figure 4 - Effect of the mushroom macerate extract over luminol-dependent chemiluminescence assay using PMN. The incubation mixture contained PMN/$1x10^6$ cells/mL stimulators by PMA/$1x10^{-5}$ mM the absence end presence of 50µL of *A. blazei*.

Suppression of superoxide anion radical ($O_2^{\bullet -}$)

We have also evaluated the potential of macerated extracts of *A. blazei* to suppress superoxide anion formation using a non-enzymatic superoxide generation method (Kakkar et al. 1984). The radical is generated by reacting phenazine methosulphate with NADH, resulting in NBT reduction. We used this reaction to evaluate if the extract (Figure 5) was able to suppress superoxide anion formation

in vitro. Note that in this reaction, NBT must be present in excess to evaluate the real potency of samples in suppressing superoxide anion (Vellosa et al. 2007B). The ability of *A. blazei* extracts to scavenge superoxide anion radical ($O_2^{\bullet -}$) (% inhibition = 51.8 ± 2.1%) may be expected to contribute to its possible anti-radical action.

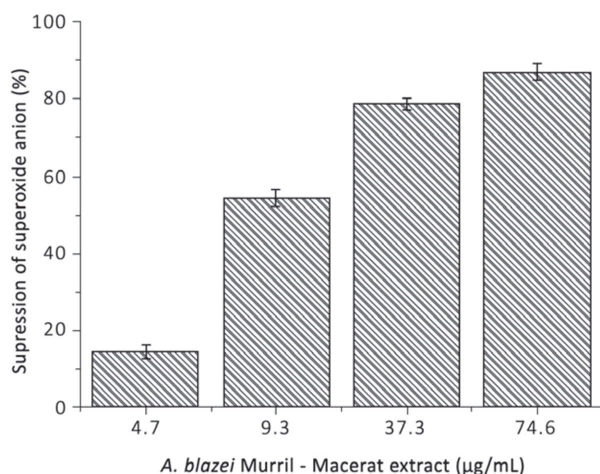


Figure 5 - Suppression of superoxide anion radical ($O_2^{\bullet -}$) by different volumes (µL) of *A. blazei* macerate extract. The incubation mixture contained: Phenazine Methosulphate – $168x10^{-6}$ mM; NBT – $30x10^{-6}$ mM; NADH – $78x10^{-6}$ mM; Sodium pyrophosfate buffer – 25 mM. (Mean ± SD; n=3).

CONCLUSION

The results indicate that *A. blazei* aqueous macerated extracts are capable of diminishing ROS levels by interfering with enzymatic ROS generators or by suppressing different reactive species. Evidence in support of these conclusions came from the inhibited formation of superoxide anion, the lessened extent of HRP-dependent luminol-dependent chemiluminescence, the inhibition of the oxidative-burst in neutrophils and the inhibition of HRP and MPO enzymatic oxidative kinetics. From these observations, it may be suggested that the *A. blazei* mushroom can be used as a possible pharmacological agent against oxidative stress and as a nutritional and pharmaceutical source of new therapeutic compounds, due to the mushroom's excellent antioxidant properties.

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RESUMO

Cogumelos apresentam propriedades nutricionais e medicinais bastante empregadas na manutenção da saúde humana e têm sido considerado por pesquisadores como possíveis fontes de seqüestradores de radicais livres. Neste trabalho, as propriedades antioxidantes de extratos aquosos de *Agaricus blazei* Murill, produzidos por maceração e decocção, são demonstrados *in vitro*. A resistência à oxidação é demonstrada através de três mecanismos: i) inibição de processos oxidativos enzimáticos, with 100% de inibição da HRP (horseradish peroxidase) e MPO (myeloperoxidase); ii) inibição do estresse oxidativo celular, com 80% de inibição do *oxidative burst* de neutrófilos polimorfonucleares (PMN) e iii) ação direta sobre espécies reativas, com 62% e 87% de supressão do HOCl e ânion superóxido ($O_2^{\bullet -}$), respectivamente. A partir destes dados, conclui-se que o extrato aquoso deste cogumelo tem significativa capacidade antioxidante, indicando sua possível aplicação para propósitos nutracêuticos e medicinais.

Palavras-chave: *Agaricus blazei* Murril, antioxidantes, espécies oxidantes, quimiluminescência, neutrófilos polimorfonucleares.

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