INNSPUB

International Journal of Biosciences | IJB |
ISSN: 2220-6655 (Print) 2222-5234 (Online)
http://www.innspub.net
Vol. 11, No. 4, p. 312-319, 2017

RESEARCH PAPER OPEN ACCESS

Comparison of *in vitro* antioxidant activity of *Phellinus baumii* and *Trametes versicolor*

Shumaila Shaheen[1], Feroza H. Wattoo*[1], Muhammad Gulfraz[1], Nasir M. Minhas[2], Muhammad F. Nasir[3], Shagufta Perveen[4]

[1]*University Institute of Biochemistry and Biotechnology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.*

[2]*Department of Plant Breeding and Genetics, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.*

[3]*Department of Entomology, Pir Mehr Ali Shah Arid Agriculture University Rawalpindi, Pakistan.*

[4]*National Institute of Genomics & Advance Biotechnology (NIGAB), National Agriculture Research Council, Islamabad, Pakistan.*

Key words: Antioxidants, *Phellinus baumii*, *Trametes versicolor*, Free radicals, Hydrogen peroxide

http://dx.doi.org/10.12692/ijb/11.4.312-319 Article published on October 30, 2017

Abstract

The deleterious effects of oxidative stress caused by accumulation of free radicals in human body has gathered tremendous attention in the recent years. Wild medicinal mushrooms are rich in secondary metabolites that make them effective against a wide range of diseases. The present study was conducted to determine the antioxidant activity of two wild mushroom species; *Phellinus baumii* and *Trametes versicolor* from Pakistan. The 80% methanolic extract of each mushroom species was prepared and tested by using *in vitro* antioxidant assays such as 2, 2-diphenyl-1-picrylhydrazy (DPPH) and hydrogen peroxide (H_2O_2) scavenging assays. The results revealed that *P. baumii* possessed remarkable antioxidant activity against free radicals by showing (83.4±0.1%) and (81.9 %±1.0%) scavenging effects on DPPH and H_2O_2 radicals as compared to *T. versicolor*. In addition, the total phenolic content was measured by Folin-Ciocalteau assay, indicating the presence of high phenolic content in *P. baumii* (27.9±2.68 mg GAE/g dw) as compared to 6.18±2.2mg GAE/g dw for *T. versicolor*. These findings suggested that *P. baumii* could be used as a source of natural antioxidants in pharmaceutical industry.

* **Corresponding Author:** Feroza H. Wattoo ✉ drfhwattoo@uaar.edu.pk

Introduction

Free radicals, which mostly occur in the form of reactive oxygen species (ROS), are produced as byproducts during the cellular metabolism that plays a significant role in cell signaling and homeostasis. Moreover, environmental factors also contribute to enhance their rate of production (Valko *et al.*, 2007). However, when their concentration increases above a desired level, they adversely disturb the natural physiological antioxidant mechanisms in the body. To control increased concentration of free radicals, organisms have evolved several cellular strategies that can detect and detoxify them (Finkel and Holbrook, 2000). Among them is the maintenance of an equilibrium between oxidants and antioxidants for normal body functioning (Ferreira *et al.*, 2009). An imbalance in this equilibrium my lead to over-production of free radicals resulting in oxidative stress that can damage cellular DNA, lipids as well as proteins (Shanlin *et al.*, 1998; Ridnour *et al.*, 2005) resulting in many diseases including neurodegenerative disorders (Perry *et al.*, 2008), aging, cancer, diabetes as well as atherosclerosis (Barja, 2004; Shah and Channon, 2004; Valko *et al.*, 2006).

In more detail, the defense strategy against free radicals include both enzymatic and non-enzymatic antioxidants. All the cells in the eukaryotic organism contain several endogenous enzymes such as catalase, superoxide dismutase, glutathione peroxidases and glutathione reductase among others that can scavenge free radicals (MatÉs *et al.*,1999) The endogenous non-enzymatic antioxidant defenses mechanism include α-tocopherol, glutathione, vitamin C and E, lipoic acid, natural flavonoids and other compounds (McCall and Frei, 1999). The higher level of these free radical scavengers can be achieved through higher consumption of fruits, vegetables and legumes along with avoiding the factors that cause their production such as tobacco, xenobiotics, radiations and environmental pollutants (Lachance *et al.*, 2001).

Interestingly, mushrooms are known to contain phenolic compounds (Kim *et al.*, 2008) that have shown enormous ability of quenching oxygen-derived free radicals by a mechanism in which a hydrogen atom or an electron is donated to free radicals (Yuting *et al.*, 1990). Consequently, these have been frequently used as part of traditional Chinese medicine. A relatively less studied mushroom type, the *Phellinus baumii* (also known as Forest Gold) belongs to the family Hymenochaeteae and is found very effective against heart or liver diseases, gastrointestinal cancer, disorders related to stomach and gynecopathy (Zeng *et al.*, 2008). It has been shown to contain various bioactive substances including triterpenoids, polysaccharides and flavones (*Song et al.*, 2005). Another type, the *Trametes versicolor* (also known as turkey tail) has been similarly used as a medicinal mushroom belonging to the Polyporaceae family. It has been found effective against the infections of the digestive, urinary and upper respiratory tracts and liver diseases including hepatitis B and chronic active hepatitis as well as to improve general immune weakness and tumors (Ying, 1987).

In order to lower the level of oxidants in the body, several synthetic antioxidants such as BHA (2-tert-butyl-4-methoxyphenol) and BHT (2,6-di-tert-butyl-4-methylphenol) are being used but they cause several side effects in long run. Thus, there is a need to discover natural antioxidants. In this study, we assessed the *in vitro* antioxidant activities of *P. baumii* and *T. versicolor* to explore more about their beneficial effects on human health.

Materials and methods

P. baumii was collected from *Acacia nilotica* trees growing on the Margalla Hills in Islamabad while *T. versicolor* was collected from Murree, Pakistan. All mushrooms were washed thoroughly with distilled water, cut into small pieces, finely milled to a fine powder (200 mesh), lyophilized, and stored at -80°C. DPPH, Folin-Ciocalteau reagent, hydrogen peroxide, gallic acid and vitamin C were purchased from Sigma Chemical Co. (St. Louis, MO, USA). All other chemicals used in the study were of analytical grade.

Preparation of Mushroom Extracts

Extraction was done by shaking 2g of each mushroom powder with 100 mL of 80% methanol for 24 h at 25°C under dark conditions, centrifugation was done

at 5000*g* for 10min and extract was filtered by using Whatman filter paper 1. The extract was saved while the residue was extracted twice with 80% methanol. To get dried extract, rotary evaporator at 45°C was used to evaporate the methanol while lyophilizer was employed for the removal of the remaining water content. The dried extract thus obtained was weighed and stored by preventing light exposure at -20°C (Liu *et al.*, 2013).

In vitro antioxidant activity assays

DPPH (2, 2-diphenyl-1-picrylhydrazy) radical scavenging Assay

The DPPH radical scavenging activity of the selected mushrooms were evaluated by using the method described by (Shimada *et al.*, 1992). Briefly, different concentrations of the extract (0.08mg/mL-1.3mg/mL) was added to 450µL of Tris-HCl. Then 1 mL solution of DPPH (0.2mM DPPH in methanol) was added and the reaction mixture was kept at room temperature for 30min in a dark room. The absorbance of the mixture was measured at 517nm. The percentage scavenging activity was measured by using following formula:

Scavenging activity (%) = $[1- (A_1-A_0)/A_0] \times 100$

Whereas A_0 = absorbance of control (80% methanol),

A_1 = absorbance of samples.

Hydrogen Peroxide (H_2O_2) Scavenging Assay

The assay was performed by using the method described by (Ruch *et al.*, 1989). A mixture was prepared by using different concentration of mushroom samples (0.1-2mg/mL), 0.6mL of H_2O_2 solution (40mM) and 2.4mL of phosphate buffer (0.1M) was incubated at room temperature for 10min. The absorbance of the reaction mixture was measured spectrophotometrically at 230nm. Vit C was taken as positive control. The following formula determined the scavenging activity:

Scavenging activity (%) = $[1- (A_1-A_0)/A_0] \times 100$

Whereas, A_0 = absorbance of control (water instead of sample),

A_1 = absorbance of sample

Determination of Total Phenolic Content

For the determination of total phenolic content in the methanolic extracts of mushrooms, Folin-Ciocalteu method was used as described by (Liu *et al.*, 2009). Briefly, each sample extract (1mg/mL) was mixed with 10% Folin-Ciocalteu reagent in a dark room and allowed to react at 30°C for 5min. Then 800µL of 700mM sodium carbonate solution was added and left for 2h after which absorbance (760nm) of the reaction mixture was measured. Gallic acid was used to calculate standard curve and the results were expressed as mg of gallic acid equivalents (GAE) per gram of dry weight (mg GAE/g dw).

Statistical Analysis

The results were expressed as mean of three measurements ± standard deviation, computed by using Excel program from MS office 2016. One-way analysis of variance (ANOVA) was used for the comparison of means of two mushroom species followed by t-test by using GraphPad Prism Software (La Jolla, CA, www.graphpad.com). The statistical significant level was set at $p < 0.05$.

Results

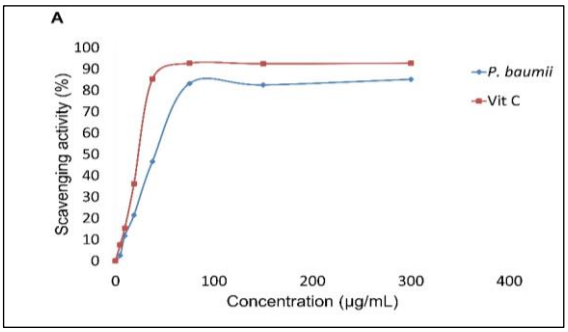
DPPH Radical Scavenging Assay

As shown in Fig. 1A, at a concentration of 50µg/mL, the DPPH scavenging activity for the Vc was 92.7±0.21%. Whereas in case of mushrooms, *P. baumii* showed 83.2±0.11% scavenging activity against DPPH radicals while *T. versicolor* scavenged 13.7±0.16% DPPH radicals. The IC_{50} values for Vc, *P. baumii* and *T. versicolor* were 0.14±0.18mg/mL, 0.27±0.002 mg/mL and 4.37±0.025mg/mL respectively (Table 1).

Table 1. IC_{50} values of mushrooms by using DPPH scavenging assay expressed as mg/mL.

No. of obs.	Samples	IC_{50} (mg/mL)
1	Ascorbic acid (Vc)	0.14 ± 0.001
2	*P. baumii*	0.27 ± 0.002
3	*T. versicolor*	4.37 ± 0.025

Data are expressed as mean ± SD (n=3).



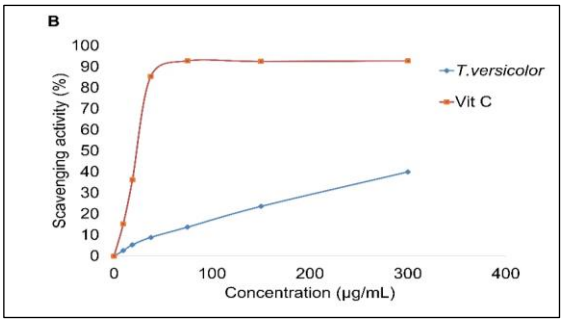


Fig. 1. Scavenging activity of methanolic extract of *P. baumii* (A) *T. versicolor* (B). Data are presented as means ± SD of triplicates. The linear equations of Vc and the extracts were shown as follows. Vc: y = 0.364x-1.5598 (R^2 = 0.9992*); *P. baumii*: y = 0.0017x-3.2943 (R^2 = 0.9952*); *T. versicolor*: y = 0.0983x + 6.951 (R^2 = 0.9981 *). The symbol * followed with the determination coefficient shows the significant level at 0.05.

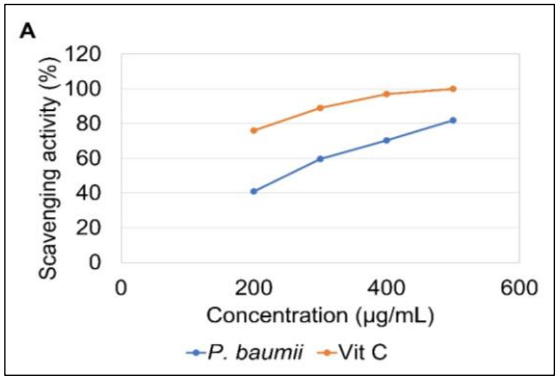
Assay of H_2O_2 Scavenging Activity

As depicted in Fig. 2, mushrooms methanolic extracts and V_C all exerted concentration dependent H_2O_2 scavenging activities. At the concentration of 500µg/mL, V_C showed 100% scavenging activity against H_2O_2 while methanolic extracts of *P. baumii* and *T. versicolor* showed 81.9±1.0% and 65±0.72% scavenging activity respectively. The IC_{50} value for standard V_C, *P. baumii* and *T. versicolor* were 0.04±0.03mg/mL, 0.06±0.01mg/mL and 0.42±1.6mg/mL respectively (Table 2). Since the phenolic compounds are good electron donors so they denote electrons to free radicals H_2O_2 to convert it to H_2O (Ruch *et al.*, 1984).

Table 2. IC_{50} values of mushrooms by using H_2O_2 scavenging assay expressed as mg/mL.

No. of obs.	Samples	IC_{50} (mg/mL)
1	Ascorbic acid (Vc)	0.04 ± 0.03
2	*P. baumii*	0.06 ± 0.01
3	*T. versicolor*	0.42 ± 1.6

Data are expressed as mean ± SD (n=3).



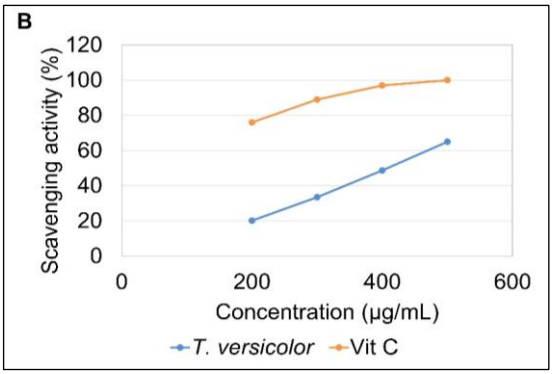


Fig. 2. The scavenging activity on H_2O_2 by methanolic extracts of *P. baumii* (A) and *T. versicolor* (B). Data are represented as mean ± SD of triplicates.

Determination of Total Phenolic Content

In the present study, the mean total phenolic content of the extracts was measured by using equation Y = 0.0577x+0.0203 for the quantitative determination of total phenolic content expressed as gallic acid equivalent per gram dry weight of the sample (Fig. 3). As depicted in Table 3, TPC in *P. baumii* methanolic extract was found to have a value (27.9±2.68mg GAE/g dw) while *T. versicolor* showed (6.18±2.2mg GAE/g dw) of total phenolic content that was 4.5 folds lesser than *P. baumii*. It was also less than previously reported TPC (46.01±0.98mg GAE/g dw) in the ethanolic extracts of *T. versicolor* from India (Sheikh *et al.*, 2014).

Table 3. Total phenol content of mushrooms.

No. of obs.	Mushrooms Total phenols (mg GAE/g dw)	
1	*P. baumii*	27.9 ± 2.68*
2	*T. versicolor*	6.18 ± 2.2*

Total phenolic content (mg GAE/g dw), *Indicates significant difference ($p < 0.05$). Data is presented as mean ± SD (n=3).

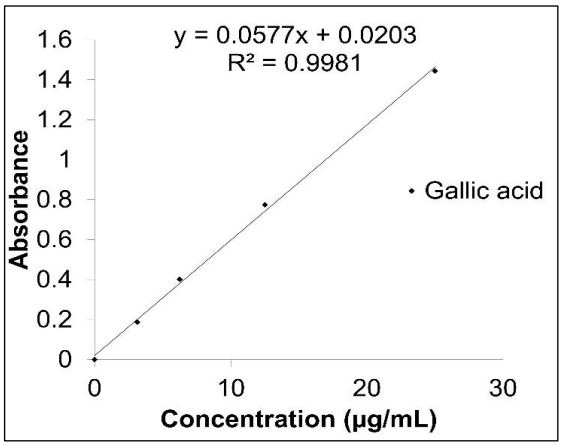


Fig. 3. Gallic acid standard curve.

Discussion

Mushrooms are an important functional food that not only fulfills dietary requirements but also have beneficial effects in terms of reducing the risks of many diseases. It is well known that polyphenols are the key constituents that contribute to the antioxidant properties of vegetables, fruits and mushrooms (Ferreira *et al.*, 2007). The DPPH radical scavenging assay is an extensively used method to evaluate the antioxidant activity of natural products. In this assay, the presence of antioxidants in the sample is determined through the color change of stable DPPH radical from purple to yellow due to reduction of DPPH to DPPH-H. Spectrophotometric assays were done by using five different concentrations of 80% v/v methanolic extracts of mushrooms and it was found that the scavenging activity was directly correlated to the concentration of extracts. Results demonstrated that *P. baumii* showed considerable antioxidant activity in all *in vitro* assays as compared to *T. versicolor*. In this study, 50µg/mL of *P. baumii* extract scavenged 83.2±0.11% DPPH radicals which is almost 10 folds more active than earlier study on *P. baumii* from South Korea where 500µg/mL of methanolic extract showed 85% scavenging activity on DPPH radicals (Shon *et al.*, 2003). The other mushroom, *T. versicolor* showed higher inhibitory activity than methanolic extracts from China (24.6% scavenging activity at a level of 0.64mg/mL) (Mau *et al.*, 2002), whereas lower than the methanolic extracts of *T. versicolor* from Japan where 0.5µg/mL of extract showed 40.0% scavenging activity against DPPH radicals (Kamiyama *et al.*, 2013) and also lower than ethanolic extracts from India (71.88 ± 0.84% scavenging activity at the level of 100µg /mL) (Sheikh *et al.*, 2014).

In addition to DPPH, H_2O_2 is another type of free radical, extensively used in *in vitro* antioxidant studies. Although not very reactive, but it forms hydroxyl radicals in the cell on reaction with superoxide or ferrous anion radicals as it can easily penetrate in cellular membranes (Chun-hui *et al.*, 2007). Because of this, H_2O_2 is the most precarious free radical that may harm various important cellular processes.

In our study, both mushrooms showed considerable scavenging activity against H_2O_2 radicals with *P. baumii* being more active with an IC_{50} value of 0.06±0.01mg/mL as compared to 0.42±1.6mg/mL for *T. versicolor*.

Phenolics are aromatic compounds having one or more hydroxyl groups that allows them to act as reducing agent by donating hydrogen atom or electron to the free radical (Sheikh *et al.*, 2014; Li *et al.*, 2009). A higher phenolic content was observed for *P. baumii* (27.9±2.68 mg GAE/g dw) as compared to *T. versicolor* (6.18±2.2mg GAE/g dw) that could be directly correlated to its higher antioxidant activity. It was also much higher than previously reported (338±8µg/mL of caffeic acid equivalents) in *P. baumii* from South Korea (Shon *et al.*, 2003). The results revealed that the *P. baumii* exhibited significantly higher scavenging activity against free radicals as compared to *T. versicolor* that could be related to the presence of high amounts of phenolic compounds in it. The concentration of phenolic compounds in mushrooms also vary depending on the nature of substrate that effects its functional, organoleptic and chemical properties (Michael *et al.*, 2011). The higher antioxidant activity shown by *P. baumii* may be due to the fact that it was growing on the *Acacia nilotica* tree that is rich in phenolics and other secondary metabolites (Sultana *et al.*, 2007) while *T. versicolor* was growing on dead wood log. Previous studies have also shown that Phellinus species produce a yellowish polyphenol called hispidin which made this mushroom a very good source of natural antioxidant (Wang *et al.*, 2007). So, more rich the mushroom is in the concentration of phenolic compounds higher will be its antioxidant activity.

Conclusion

This study clearly demonstrated the effectiveness of methanolic extracts of *P. baumii* against free radicals as compared to *T. versicolor* which was possibly due to the accumulation of high amounts of phenolic compounds in it. Further investigations on the isolation of potential compounds responsible for antioxidant activity may contribute to beneficial medicinal effects of this mushroom and it could be used as a new source of natural antioxidants.

Acknowledgements

This research was supported by Pir Mehr Ali Shah-Arid Agriculture University Rawalpindi, Pakistan and Higher Education Commission of Pakistan (HEC). The authors acknowledge Dr. Kishwar Sultana (Ex-Plant Pathologist at PMAS-Arid Agriculture University, Rawalpindi) for the identification of mushrooms.

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