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Antioxidant efficacy of a wood degrading fungus *Phellinus gilvus* (Schwein.) Pat., from Indian fungal flora

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Abstract
Phellinus is a basidiomycete genus representing a group of wood rotting fungi. In spite of the wood degrading habit, a number of medicinal uses have been credited to various species of this genus. In the present study, the content of carbohydrates, proteins and phenols was estimated in hydroalcoholic (70% ethanol) extract (HAE) of *P. gilvus*. The same extract was screened for free radical scavenging activity on DPPH (1, 1-Diphenyl-2-picrylhydrazyl), hydroxyl ion, superoxide ion and reducing power. The extract showed good quantity of proteins 42.4 mg/100 mg followed by carbohydrates 34.12 mg/100 mg and phenols 30.2 mg/100 mg. The EC_{50} of test extract was found minimum against DPPH free radical (25±2.30) and maximum against hydroxyl ion (37.67±1.45). The present study demonstrated the antioxidant potential of *P. gilvus* so that it could be used against several free radical related disorders.

Keywords: free radical scavenging, HAE, *P. gilvus*, reducing power, wood rotting

1. Introduction
Synthetic drugs are costly, pose various side effects and not safe for use in several health conditions like gestation period. Therefore the demand of alternative natural drug sources keeps on rising. The use of medicinal fungi including several species of *Phellinus* is well documented in ancient medicinal literature [1-3]. There are 361 taxa of *Phellinus* worldwide [4] and 97 species of this genus have been reported from India [5-7]. Species of *Phellinus* have been reported in association with of a wide range of angiosperms and gymnosperms causing wood rotting [8-10]. Instead of the wood decaying property, several species of *Phellinus* (*P. rimosus*, *P. conchatus*, *P. baumii*, *P. igniarius*, *P. nigricans* and *P. senex*) are known traditionally as folk medicines in different parts of the world and need scientific validation [11–15]. Medicinal potential may be credited to various pharmacological properties contributed by the bioactive constituents reported in these species [16–19]. However, scattered reports are available on the mycochemical composition and pharmacological activities of *Phellinus* species and required much attention. The present study aimed at the estimation of carbohydrates, proteins, phenols and evaluation of antioxidant potential of *P. gilvus* in relation to its free radical scavenging and reducing power.

2. Material and Methods
2.1 Chemicals
All chemicals used in this experimentation were of AR grade and were bought from Himedia, Merck, SD fine Chem. Ltd. and Sigma-Aldrich Chemicals.

2.2 Fungal material
The collection of *P. gilvus* was made from district Dehradun, Uttarakhand, India during October in the year 2011. The morphological and microscopic details of the specimen were observed and used in identification in the laboratory of Department of Botany, Punjabi University, Patiala, Punjab, India. The specimen was compared with the type specimen lying at the internationally recognized herbarium of Department of Botany, Punjabi University, Patiala, Punjab, India and identified as *P. gilvus*. The identified specimen was assigned a unique herbarium number abbreviated as (PUN-5997) and deposited in the herbarium of Punjabi University, Patiala, India.

2.3 Extraction scheme
The dried powder of *P. gilvus* sporocarp (150 g) was put in 1500 mL of 70% ethanol. This mixture was placed for 72 h in orbital shaking incubator at 37^0C and 80 rpm.

The filtrate obtained from this mixture was subjected to evaporation. The residue was dried in oven at 45°C and was preserved at -4^{0}C until use [20].

2.4 Estimation of carbohydrates

Standard anthrone method was used for carbohydrate estimation [21]. Test extract (1 mg/1mL) was taken in 4 mL of anthrone reagent (200 mg/100mL of ice cold 95% H_2SO_4). Heating was done for 8 min followed by rapid cooling. Absorbance was recorded at 630 nm. Total carbohydrates (mg/100mg) were determined using a standard curve of glucose.

2.5 Estimation of total protein content

The method of Folin-Lowry) was used for the estimation of total protein content [22]. The mixture of test extract (1 mg/1mL) and 5 mL (alkaline solution) was kept undisturbed at room temperature for 10 min. This mixture was incubated for 30 min at room temperature after adding 0.5 mL of dilute Folin-Ciocalteu reagent (1:1). Absorbance was taken at 660 nm. Estimation of protein content (mg/100mg) was done using the calibration curve of bovine serum albumin (BSA).

2.6 Estimation of total phenols

A mixture of 100 mg HAE and 15 mL of 50% methanol was extracted for 2 h thrice by maceration. After filtration, the volume was made up to 200 mL with 50% methanol. To 1 mL this solution, 10 mL distilled water was added. It was followed by the addition of 1.5 mL Folin-Ciocalteu reagent. Following incubation at room temperature for 5 min, 4 mL (20%) sodium carbonate was added distilled water was put to make volume up to 25 mL. The above mixture was mixed well and kept untouched at room temperature for 30 min. Absorbance was noticed at 765 nm and total phenol content was denoted as mg GAE/100mg using calibration curve of gallic acid [23].

2.7 DPPH free radical scavenging assay

About 1.5 mL, HAE (0, 10, 50, 100, 500, 1000 µg/mL) was added individually in 2.9 mL (200 µM) DPPH ethanol solution. After vigorous shaking, incubation was done at room temperature for 30 min. Absorbance of the samples was taken at 515. Ethanol solvent was used as blank. The DPPH free radical scavenging (%) was estimated as follows:

Scavenging activity (%) = $[A_0\text{-}A_t/A_0] \times 100$

Where A_0 = Absorbance of control sample. A_t = Absorbance of reaction mixture with HAE [24]. EC_{50} (µg/mL) of HAE against DPPH free radicals was estimated by plotting the graph between scavenging (%) and tested concentrations of HAE.

2.8 Scavenging of hydroxyl ion

Approximately 0.5 mL aliquot of HAE (0, 10, 50, 100, 500, 1000 µg/mL) individually was taken in combination with 100 µL (1 mM) $FeCl_3$, 0.1 mL (1 mM) EDTA, 50 µL (20 mM) H_2O_2, 0.1 mL (1 mM) ascorbic acid and 100 µL (30 mM) deoxyribose in 66 µL (10 mM potassium phosphate buffer; pH 7.4) to obtain 1 mL reaction mixture. Following incubation at 37^{0}C for 1 h, 1 mL (2.8%) trichloroacetic acid and 1 mL (1%) thiobarbituric acid were put. Heating was done for 15 min in boiling water bath. Absorbance was read at 532 nm. The hydroxyl ion scavenging (%) was calculated with the equation:

Percentage inhibition (%) = [A-B/A] × 100

Where A = Absorbance of control. B = Absorbance of reaction mixture containing HAE [25]. EC_{50} (µg/mL) of HAE against hydroxyl ion scavenging was calculated by plotting the graph between hydroxyl ion scavenging (%) against different concentrations of HAE.

2.9 Superoxide ion scavenging

About 1 mL of test extract (0, 10, 50, 100, 500, 1000 µg/mL) was mixed separately with 1.0 mL (0.05 M phosphate buffer; pH 7.4), 0.04 mL of bovine serum albumin (BSA) and 0.04 mL of nitro blue tetrazolium chloride (NBT). Then incubation at 25^{0}C for 10 min was done following the addition of 0.04 mL of 1.5U/mL xanthine oxidase. The reaction mixture was again incubated at 25^{0}C for 20 min. The absorbance was taken at 560 nm. The scavenging activity (%) was calculated as

Scavenging activity (%) = $[A_0\text{-}A_t/A_0] \times 100$

Where A_0 = Absorbance of control. A_t = Absorbance of reaction mixture with HAE [26].

EC_{50} value (µg/mL) of HAE against superoxide radical was determined by the plotting the graph between scavenging activity (%) against the tested concentrations of HAE.

2.10 Reducing power assessment

An aliquot of 0.5 mL HAE (0, 0.0625, 0.125, 0.25, 0.5, 1.0 mg/mL) was mixed individually with 2.5 mL (0.2 M phosphate buffer; pH 6.6) and 2.5 mL of potassium ferricyanide. After incubation at 50^{0}C for 20 min, 2.5 mL of 1% trichloroacetic acid was added and the mixture was centrifuged at 5000 × g for 10 min. To 5 mL of upper layer of the mixture, 5 mL distilled water and 10 mL $FeCl_3$ were added. Absorbance was read at 700 nm [27]. EC_{50} value (mg/mL) of test extract giving absorbance value of 0.5 was determined by the plotting the graph between absorbance and different concentrations of HAE.

2.11 Statistics used

Each assay was conducted in triplicate (n=3) and the results were presented as mean ± standard error mean (SEM) using graph pad prism 5.0 software.

3. Results and Discussion

Phellinus Quél. (Fam ily: *Hymenochaetaceae*) represents a group of wood rotting fungi [28]. Recently species of this genus are gaining attraction for scientific research. As per literature reports, Many species of *Phellinus* possess medicinal value which may be the contribution of bioactive compounds imparting pharmacological activities to these species [29-36]. However, scanty information is available about the mycochemical composition and free radical scavenging activity of this species [37-39]. *Phellinus gilvus*, a medicinal species of this genus. Literature reveals the presence of biochemicals like carbohydrates, proteins and phenols as structural and functional components of fungi. Carbohydrates and proteins are food components and provide energy. Certain proteins also act as enzymes in different metabolic pathways. In the present study, good quantities of carbohydrates, proteins and phenols have been observed (Figure 1; A). As per literature reports, the antioxidant effect of fungal extracts is because of the phenolic compounds which may act as free radical scavengers, hydrogen donors or help in quenching of singlet oxygen and play a role in metal ion chelation. The antioxidant potential of *P. gilvus* HAE was screened by DPPH, hydroxyl ion, superoxide ion free radical scavenging

and reducing power assessment (Table 1). Any molecule that can donate an electron or hydrogen to DPPH can react with it and hence help in declining DPPH absorption. The HAE of *P. gilvus* shows reaction with DPPH and bleach DPPH free radicals from the reaction mixture (Figure 1; B). Highly reactive hydroxyl and superoxide radicals cause oxidative damage at cellular and sub cellular level. Hence, their removal is necessary for normal physiology. Test extract in the present investigation revealed the scavenging effect against hydroxyl (Figure 1; C) and superoxide ions (Figure 1; D). Reducing power of an extract is an indication of antioxidant potential. The results (Figure 1; E) suggest that the extract may consist of polyphenolic compounds showing great reducing power.

Table 1: EC_{50s} values in tested antioxidant assays

Fungus	EC_{50s}			
	DPPH (µg/mL)	OH^- (µg/mL)	O_2^- (µg/mL)	Reducing power (mg/mL)
P. gilvus	25±2.30	37.67±1.45	30.33±0.88	0.4±0.05

Values are expressed as mean ± standard error of mean; n=3.

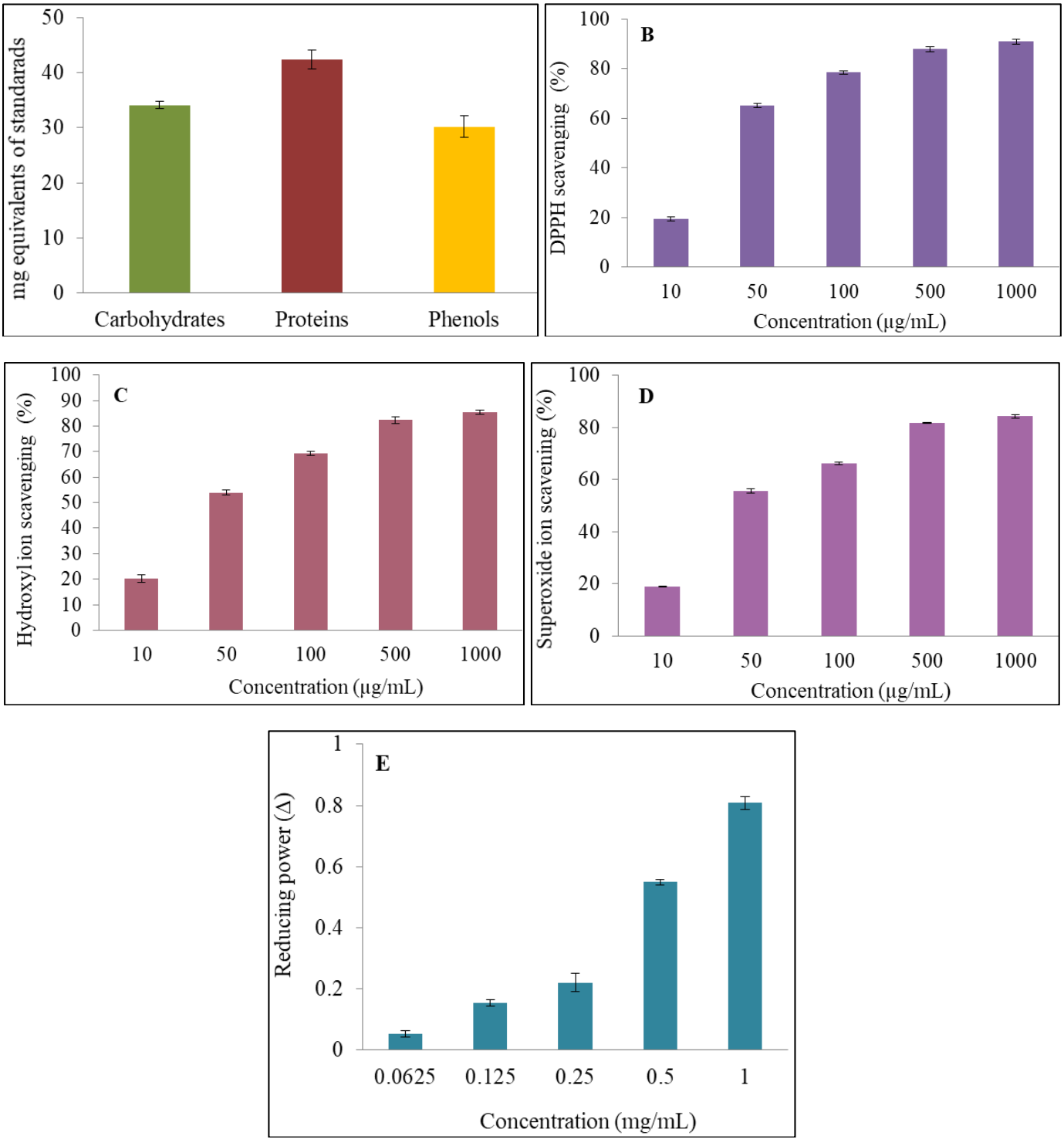

Fig 1: A-Total carbohydrates, proteins and phenol contents; B–DPPH free radical scavenging; C–Hydroxyl ion scavenging; D–Superoxide ion scavenging; E–Reducing power of *P. gilvus* HAE.

4. Conclusion

The preliminary experiments conducted in the present investigation are based on crude HAE of *Phellinus gilvus*. These findings need more sophisticated research to find out the active principle and the underlying mechanism of action. To sum up, the results suggest *P. gilvus* as an excellent source of carbohydrates, proteins, phenols and a promising candidate for antioxidant bioactive products. Hence, in depth extensive *in vitro* and *in vivo* studies are urgent for new and natural drug development.

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