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Research

Antioxidant potential and content of the polyphenolic secondary metabolites of white rot macrofungi; *Flavodon flavus* (Klotzsch.) and *Xylaria feejeensis* (Berk.)

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ABSTRACT:
White rots are a variety of wood-decay fungi that digests moist wood, causing it to rot. White rot fungi produce structurally diverse polyphenolic secondary metabolites which are known to responsible for the renowned antioxidative system of this fungal category. The current study was aimed at investigating the antioxidant properties of terrestrial white rots *F. flavus and X. feejeensis* harvested from the dry zone forest reserves in Dambulla and Mahiyanganaya areas of Sri Lanka for the first time. Furthermore, the contribution of phenolic and flavonoid substances towards the antioxidant properties of studied white rots were determined. Studied white rot species exhibited a strong antioxidant capacity implying that studied forms possess an effectual antioxidative system. Furthermore, analyzed species also contained high contents of phenolic and flavonoid substances suggesting that phenolic and flavonoid substances secreted by *F. flavus* and *X. feejeensis* contribute to their prominent antioxidant activity.

KEY WORDS: White rot fungi, Antioxidant activity, EC50, Phenol content, Flavonoid content

INTRODUCTION

Recent upsurge in the applications of bioactive compounds produced by white rot fungi is inspiring a worldwide exploration for novel bioactive compounds of fungal origin. White rot fungi are wood-decay macrofungi which decompose cellulose, hemicellulose and lignin while the wood turns pale in colour with a fibrous to stringy consistency. White rot macrofungi were found to accumulate a spectrum of polyphenolic secondary metabolites which possess great antioxidant potential (Blanchette *et al.*, 1988 and Raghukumar *et al.*, 2001). Among the polyphenolic metabolites secreted by fungi, phenols and flavonoid derivatives are predominant since they are capable of exerting strong antioxidant potential (Keles *et al.*, 2011 and Barros *et al.*, 2008).

The physiological life cycle events of the white rot macrofungi are strongly associated with the elevated levels of free radicals, which in turn induce the decay process of secondary cell wall in the wood. Therefore, white rot fungi are found to possess highly efficient antioxidative system consisting of enzymatic elements such as catalases, peroxidases and polyphenolic secondary metabolites (Jaszek *et al.*, 2013).

Numerous bioactive properties of fungal secondary metabolites which are extensively described in epidemiological studies are associated with their antioxidant properties.

Antioxidants found in macrofungi can act as a major protector against radical mediated toxicity generated in human body. Human cells are continuously uncovered to oxidative stress conditions brought on by a decrease in the antioxidant capacity of the system (Diaz *et al*., 2012 and Menikpurage *et al.,* 2012). Oxidative stress leads to the generation of reactive oxygen species (ROS), including free radicals, which are strikingly concerned in the pathophysiology of degenerative diseases associated with ageing, cancer, cardiovascular diseases and atherosclerosis (Adebayo *et al*., 2012 and Turkoglu *et al*., 2007). Antioxidant properties of macrofungi are directly associated with relieving oxidative stress exerted in the human body due to the scavenging ability of generated free radicals (Chang *et al*., 2007 and Ravipati *et al*., 2012). Bioactive secondary metabolites of certain white rot fungi such as *Ganoderma lucidum, Lentinula edodes, Agaricus bisporus* were found to have promising antioxidant properties leading to development of drug leads in the treatment of cancer and other degenerative diseases described above (Ramırez-Anguiano *et al*., 2007 and Rawat *et al.,* 2013). However, most of the white rots have not been explored yet for their pharmacological importance. Being a tropical country, Sri Lankan fungal biota also consists of a variety of macrofungi species with medicinal and aromatic properties (Fernando *et al.,* 2015 and Karunarathna *et al*., 2012). The current study was aimed at determining the antioxidant capacities of the white rots *F. flavus and X. feejeensis* collected from the dry zone woodlands in Dambulla and Mahiyanganaya areas of Sri Lanka. Furthermore, total phenolic and flavonoid contents of the white rots were also investigated. *F. flavus* is often attached to a decaying wood with slightly raised folds in a gill like manner (Figure 1A). *X. feejeensis* appears in black colour and often knobby with a finely roughened or cracked surface. Moreover, *X. feejeensis* shows irregularly club-shaped or fingerlike appearance (Figure 1B). Crude extract of *F. flavus* was pale red in colour while that of *X. feejeensis* had a stringy appearance with black in colour.

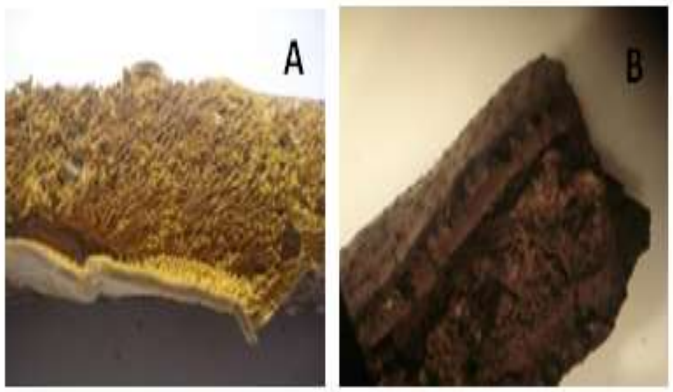

Figure 1. Morphological appearance of fruiting bodies of A) *F. flavus* and B) *X. feejeensis* found in forest reserves of Dambulla and Mahiyanganaya, Sri Lanka.

METHODOLOGY

Collection of sample

The fruiting bodies of *F. flavus and X. feejeensis* were collected from the forest reserves in Dambulla and Mahiyanganaya areas in Sri Lanka during the period of September 2012 to October 2013. Dambulla is situated at 80°40' East longitude and 7°51' North latitude in Matale district of Sri Lanka. Mahiyanganaya is located in Badulla district of Sri Lanka with latitude of 7°33' and longitude of 80°99'. They were collected into paper bags and packed loosely with proper ventilation. The collected material was transported within 24 hours to the laboratory at Department of Plant Science, University of Colombo**.** The identity of *F. flavus* (Genbank Accession No.: KJ919974) and *X. feejeensis* (Genbank Accession No.: KR348864) were authenticated by the Department of Plant Science, Faculty of Science, University of Colombo. Voucher specimens of *F. flavus* (UOC:DAMIA:D19) and *X. feejeensis* (UOC:MINNP:MK34) were deposited at the same institute.

Sequential solvent extraction

Mature fruiting bodies of collected specimens were cleaned and dried in the oven at 40 °C to a constant mass. Thereafter, dried material was pulverized. The dried powder of each specimen (10 g) was sonicated sequentially with 150 ml of 100 % methanol, 99 % dichloromethane and methanol: dichloromethane (1:1) mixture at 30 °C for 1

hour. Each extract was filtered twice through Whatman No. 1 paper. The filtrates were pooled together and evaporated to dryness at 40 °C under reduced pressure using a rotatory evaporator (BUCHI Rota vapor R-200) to obtain the crude extract.

Determination of antioxidant activity

All experiments were carried out in triplicate. Antioxidant capacity of white rot fungi was determined by 1, 1-Diphenyl-2-Picrylhydrazyl scavenging assay according to the method of Brand-Williams *et al.,* (1995) with slight modifications of the test volumes and concentrations. Different concentrations (0.01-10 mg/ml) of crude extract were prepared in methanol. The effective concentration range was determined by performing a pretest. A volume of 100 µl of the test solution was added to 900 µl of 1, 1- diphenyl-2-picrylhydrazyl (100 µM) solution. The mixture was incubated for 30 minutes in the dark at 30 °C. Thereafter, absorbance was measured at 517 nm using a UV-Visible spectrophotometer (SHIMADZU AEG-220). The 1, 1- diphenyl-2-picrylhydrazyl radical scavenging activities were expressed as the percentage inhibition of free radical production or antioxidant index (AI %) using the following formula:

$$\text{Radical scavenging activity (\%)} = [(A_{control} - A_{extract})/ A_{control}]*100$$

Where $A_{control}$ is the absorbance of blank, and $A_{extract}$ is the absorbance of the fungal extract.

Determination of total phenol content (TPC)

Preparation of Folin-ciocalteu reagent

Folin-ciocalteu reagent was prepared according to the method of Bray and Thrope, 1954. Sodium tungstate (10.0 g) and sodium molybdate (2.5 g) were dissolved in 70 ml of distilled water. About 5 ml of 85% phosphoric acid and 10 ml of concentrated hydrochloric acid were added to the reaction mixture followed by refluxation for 10 hours. Lithium sulfate (15 g), 5 ml of distilled water, and 1 drop of bromine were added to the mixture and refluxed for 15 minutes. Reaction mixture was allowed to cool till obtain the room temperature and filled up to 100 ml with distilled water.

Total phenol content (TPC)

All experiments were carried out in triplicate. Total phenol contents of the fungal extracts were determined according to the method of Folin *et al.*, (1927) with slight modifications of the test volumes and concentrations. Six different concentrations of methanolic extract of the crude, ranging from 0.01 mg/ml to 2 mg/ml were prepared. Folin-ciocalteu reagent (1N; 250 µl) was added to 500 µl of fungal extract and allowed to stand for 2 minutes. A volume of 1.25 ml of 10 % Na_2CO_3 was thereafter added to the mixture followed by incubation at 30 °C for 45 minutes. Absorbance was measured at 760 nm. A calibration curve was constructed using gallic acid at a concentrations range of 3.75 – 40 µg/ ml. The total phenol contents of the fungal extracts were expressed as mg of gallic acid equivalents (GAEs) per gram of dry sample.

Determination of total flavonoid content (TFC)

All experiments were carried out in triplicate. Total flavonoid contents were determined by the method of Chang *et al.*, (2002). Crude extract was dissolved in methanol and six different concentrations ranging from 0.02 mg/ml to 1 mg/ml were prepared. Sodium nitrite (5%; 30 µl), methanol (98 %; 200 µl) and aluminium chloride (10%; 30 µl) were added to 100 µl of fungal extract. Reaction mixture was incubated for 5 minutes at 30 °C. Sodium hydroxide (1 M; 200 µl) was added to the mixture at the sixth minute after incubation followed by addition of 0.44 ml of methanol (98 %). The absorbance of the resulting solution was measured at 510 nm. Calibration curve was obtained using (-)-Epigallocatechingallate (EGCG). The total flavonoid contents were expressed as w/w % EGCG equivalents.

Statistical analysis

The effective concentrations of sample required to scavenge 1, 1- diphenyl-2-picrylhydrazyl radicals by 50 % (EC_{50}) were obtained by regression analysis of the dose response curves of percentage antioxidant

index and concentration of the extracts using Microsoft Excel.

RESULTS AND DISCUSSION

In the present study, crude extracts of the *F. flavus* and *X. feejeensis* exhibited strong antioxidant capacity against 1, 1- diphenyl-2-picrylhydrazyl radical. Moreover, crude organic extracts of white rots were screened for the presence of phenols and flavonoid substances. Polyphenolic derivatives are of great interest due to their strong capacity of scavenging free radicals which is correlated to their chemical structure consisting of an aromatic ring with hydroxyl substituents (Tiwari *et al*., 2001).

Currently, bioactive polyphenols and flavonoid compounds isolated from certain white rot macro fungi species are used as a source of functional food or nutraceuticals. For instance, important bioactive compounds isolated from *Ganoderma lucidum*, a popular wild edible white rot fungus which exert bioactive properties have been developed as a dietary supplement (Nahata, 2013). *G. lucidum* is an important medicinal mushroom used today and is commended as "mushroom of immortality". Methanol and chloroform extracts of *G. lucidum* are known to exhibit free radical scavenging effect on the 1,1-diphenyl-2-picrylhydrazyl radical with an EC_{50} value of 1.162 ± 0.016 mg/mL and 0.684 ± 0.31 mg/ml (Baskar *et al*., 2008 and Samarakoon *et al*., 2013).

Radical scavenging capacity is inversely related to the EC_{50} values of the fungal species. In the present study, *F. flavus* showed a potent antioxidant activity with an EC_{50} of 77.00 ± 0.18 µg/mL and *X. feejeensis* exhibited promising antioxidant capacity with an EC_{50} value of 98.4 ± 0.28 µg/mL supporting the fact that, radical scavenging capacity is inversely related to the EC_{50} values of the species. In this investigation, an increase in 1, 1- diphenyl-2-picrylhydrazyl free radical scavenging activity with the increase in concentration of the extract was observed for both species during the early stages (Figure 2, 3).

Both species have demonstrated remarkable radical scavenging effect exceeding the antioxidant capacity of "mushroom of immortality", *G. lucidum*. Further, this outcome proves that white rot fungi possess a highly efficient antioxidative system.

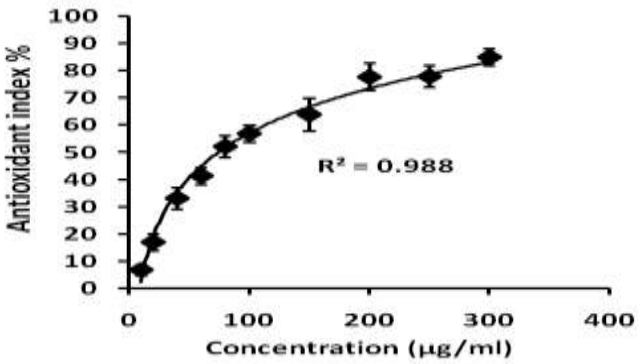

Figure 2. 1,1- diphenyl-2-picrylhydrazyl Radical Scavenging Capacity of *Flavodon flavus*. The percentage antioxidant index was plotted against concentration of the extract. Each value represents mean ± S.D.

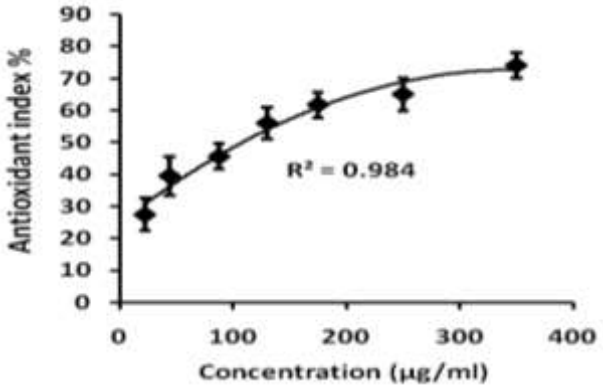

Figure 3. 1, 1- diphenyl-2-picrylhydrazyl Radical Scavenging Capacity of *Xylaria feejeensis*. The percentage antioxidant index was plotted against concentration of the sample. Each value represents mean ± S.D.

Ascorbic acid was found to possess an EC_{50} of 5.87 ± 0.17 µg/ml as the standard (Figure 4).

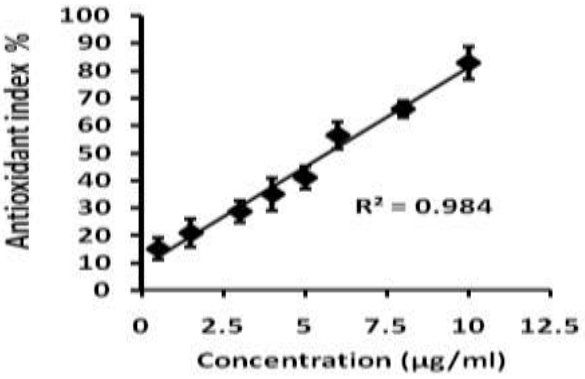

Figure 4. 1, 1- diphenyl-2-picrylhydrazyl Radical Scavenging Capacity of Ascorbic Acid. The percentage antioxidant index was plotted against concentration. Each value represents mean ± S.D.

Further, *F. flavus* and *X. feejeensis* showed higher contents of total phenols of 55.7 ± 10.89 µg Gallic acid/mg and 31.33 ± 8.87 µg Gallic acid/ mg, respectively. *F. flavus* and *X. feejeensis* also exhibited a high level of total flavonoids, 82.4 ± 4.0 µg Epicatechine/mg and 23.35 ± 7.0 µg Epicatechine/mg, respectively. However, the highest amount of total phenolics and flavonoid substances were found in *F. flavus* compared to *X. feejeensis* (Figure 5).

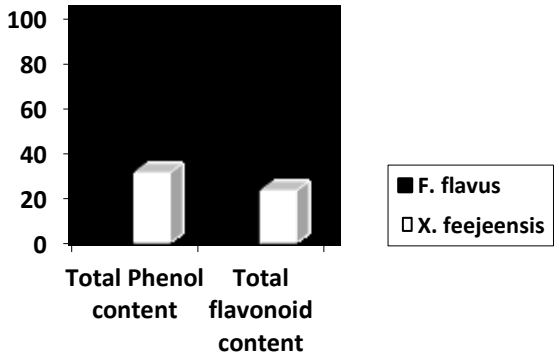

Figure 5. Total phenolic and flavonoid compounds of the crude extracts of *F. flavus* and *X. feejeensis*. Total phenol content and total flavonoid content were expressed as mg GAE/mg dry extract and µg Epicatechine/mg dry extract.

With more understanding of the compounds involved in these activities, *F. flavus* and *X. feejeensis* could become a ideal source of nutritional supplements having antioxidant properties and drug leads in the treatment of serious illnesses such as cancer. Moreover, these findings suggest that phenolic and flavonoid compounds play a dominant role in the development of greater propensity for scavenging free radicals by *F. flavus* and *X. feejeensis*. As the results of this study showed that antioxidant activities of *F. flavus* and *X. feejeensis* are very high, the isolation and characterization of the bioactive compounds from these species are underway to identify potential antioxidant agents for medicinal use. To the best of our knowledge, this is the first report of antioxidant activity, total phenol content and flavonoid contents of the white rot fungi *F. flavus* and *X. feejeensis.*

Conclusion

Studied white rot fungal species demonstrated a strong antioxidant potential leading to generate an important source of natural antioxidant compounds. Hence, these findings imply that studied white rot fungi possess a powerful antioxidative systems. Furthermore, present study suggests that phenolic and flavonoid substances secreted by *F. flavus* and *X. feejeensis* contribute to the observed antioxidant activity of studied white rots.

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