



Strong Radical Scavenging Macrofungi from the Dry Zone Forest Reserves in Sri Lanka

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Abstract: Natural metabolites produced by macrofungi are of great interest as potential antioxidant defensive agents to reduce the oxidative damage caused by free radicals. Primarily, phenolic and flavonoid type metabolites have gained major importance due to the strong capacity of scavenging free radicals. The study was mainly focused to investigate the natural antioxidant properties of macrofungi found in Sri Lankan dry zone forest reserves using DPPH radical scavenging assay and to find out the contribution of phenol and flavonoid substances towards their antioxidant capacity. EC₅₀ values of all extracts were below 1.2 mg/ml. Among the analyzed specimens, *Phellinus repandus* and *Inonotus porrectus* showed the most potent antioxidant activities having EC₅₀ of $7.91 \pm 1.38 \mu\text{g/ml}$ and $19.70 \pm 0.17 \mu\text{g/ml}$, respectively. Ten fungal forms exhibited EC₅₀ < 300 $\mu\text{g/ml}$ and eighteen showed a mean values of EC₅₀ in the range of 300-1200 $\mu\text{g/ml}$. Further, *P. repandus* and *I. porrectus* also exhibited the highest level of total phenols and flavonoids. EC₅₀ values of the species studied were inversely related to the total phenol and flavonoid contents. The analyzed macrofungi specimens exhibited high antioxidant power highlighting their potential as therapeutically useful antioxidant agents. Particularly, *P. repandus* and *I. porrectus* could be an important source of novel antioxidant compounds. In addition, phenol and flavonoid compounds largely contribute to the scavenging activity of studied macrofungi.

Keywords: Macrofungi, Antioxidant Activity, Phenol Content, Flavonoid Content, EC₅₀

1. Introduction

Macrofungi have an established history of use in traditional medical practices worldwide and considered to be a rich therapeutic source for the remedy of various deleterious diseases [1,2]. Macrofungi possess therapeutically useful important pharmacological properties including antioxidant, anticancer and anti-inflammatory activities. Moreover, mushrooms have rich nutritional value with a high protein content, vitamins, fibers, minerals, trace elements and natural antioxidants [3,4].

Over 700 species of higher basidiomycetes have been identified to possess significant pharmacological properties [5,6]. Among the reported pharmacological properties of macrofungi, antioxidant activity studies have been emerging as an intensive research area aiming to develop nutritional

and medicinal provisions for humankind owing to their strong ability to prevent radical mediated toxicity in the human body. All living cells, including human, animal and plant, are continuously exposed to a variety of challenges which exert oxidative stress brought on by a decrease in the antioxidant capacity of the system [7]. Oxidative stress leads to the generation of reactive oxygen species (ROS), including free radicals, which are strongly implicated in the pathophysiology of degenerative diseases associated with ageing, cancer, cardiovascular diseases and brain dysfunction [8,9]. Reactive oxygen species (ROS) include free radicals such as superoxide ($\text{O}_2^{\bullet-}$), hydroxyl (OH^{\bullet}), hydroperoxyl (OOH^{\bullet}), peroxy (ROO^{\bullet}), alkoxy (RO^{\bullet}) radicals and non-free radicals including hydrogen peroxide (H_2O_2) and hypochlorous acid (HOCl), which are continuously produced in the human body during cell metabolism. Others are

reactive nitrogen species (RNS) which include nitric oxide (NO[•]), peroxynitrite (ONOO[•]) and nitrogen dioxide (NO₂) [10,11]. The abundance of free radicals is toxic to every biological molecule in living cells and can cause oxidative damage to functional macromolecules such as DNA, proteins, and lipids if not excluded quickly [12,13]. From all free radical species, OH[•] and O₂^{•-} radicals are the ones that are mainly responsible for the oxidative damage. Naturally, a dynamic balance exists between the amount of free radicals generated in the body and antioxidants to scavenge them to protect the body from harmful effects [14,15]. However, the endogenous mechanisms involved in free radical scavenging in the cell under normal physiological conditions may be inadequate to neutralize free radicals generated [16,17]. Hence, enriching the diet with antioxidants to protect the body against deleterious conditions has become essential. Antioxidants act as a major defense against radical mediated toxicity by protecting the damage caused by free radicals [18,19].

Active secondary metabolites of some of the medicinal mushrooms such as *Ganoderma lucidum*, *Pleurotus florida*, *Thelephora ganbajan*, *Agaricus bisporus*, etc. were found to have promising antioxidant properties leading to the development of drug leads for the remedy of various degenerative diseases caused by radical mediated toxicity [20,21]. Among the secondary metabolites produced by macrofungi, bioactive phenols and flavonoid derivatives are of predominance due to their strong capacity for free radical scavenging [22,23]. Mainly, polyphenols have gained a higher importance in scavenging free radicals which is correlated to their chemical structure which consists of an aromatic ring with hydroxyl substituents. Therefore, there is a recent upsurge in the interest of mushrooms, due to their high content of polyphenol, which positively correlates with the antioxidant activity [24,25,26,27].

Being a tropical country, Sri Lankan biota consists of a variety of wild-growing indigenous fungal species with medicinal and aromatic values. The mycological potential (both gastronomic and economic) of Sri Lanka is attributable to favourable climatic conditions and floral diversity. Approximately 1920 species of fungi have been identified from Sri Lanka, whilst the lack of data on the bioactive properties of wild-growing autochthonous fungal species has increased our interest in the current study [28]. Specifically, most of the mushrooms of Sri Lankan origin are unexplored to a large extent with substantial number of endemic species, many of which are possessing unknown chemical, biological and pharmacological profile. Further, ethno mycological research in Sri Lanka indicates only a rare use of fungi as food or internal medicinal provisions by indigenous practitioners in Sri Lanka [29]. The limited number of scientific research on medicinal importance of Sri Lankan fungal biota have prevented a giant pharmacological potential from being uncovered. Accordingly, it is worthwhile to investigate the bioactive properties of unexplored mushrooms in Sri Lanka to discover novel bioactive substances of fungal origin. The current study is an

extended effort to uncover the antioxidant potential of macrofungi species harvested from the dry zone forest reserves in Sri Lanka and to determine the correlation of antioxidant capacity with total phenol and flavonoid contents of the crude methanolic extracts of macrofungi.

2. Materials and Methodology

2.1. Collection of Macrofungi

The specimens of macrofungi (30) were harvested from the dry zone forest reserves in Dambulla, Minneriya and Sigiriya areas of Sri Lanka during the period of September 2012 to October 2013. 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 Sciences, University of Colombo. The identity of the specimen was achieved by the Department of Plant Science, Faculty of science, University of Colombo, Sri Lanka. Voucher specimens were deposited at the department herbarium of the same institute.

2.2. Solvent Extraction

Mature fruiting bodies of macrofungi were brush cleaned, dried in the oven at 40 °C to a constant mass and pulverized. A fine powder (10 g) of macrofungi sample was extracted by sonication with 150 ml of methanol, methanol: dichloromethane (1:1) mixture and dichloromethane, respectively for 1 hour at 30 °C. Extracts were filtered twice through Whatman No. 1 filter and same extraction procedure was repeated for residue. Filtrates were combined and evaporated to dryness at 40 °C under reduced pressure using rotatory evaporator to obtain the crude extract. Crude extract was dissolved in methanol and used for further experiments.

2.3. Determination of Antioxidant Activity

Antioxidant activity was determined by 1, 1-Diphenyl-2-Picrylhydrazyl (DPPH) scavenging assay according to a previously described method with modifications [30]. 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 DPPH (100 µM) solution and incubated for 30 minutes in dark at 30 °C. Thereafter, absorbance was measured at 517 nm using a UV-Visible spectrophotometer (SHIMADZU AEG-220). Ascorbic acid was used as the positive standard antioxidant. Blank is consisted of DPPH (900 µl) dissolved in the of 100 µl of methanol. The DPPH radical scavenging activity or antioxidant index (%) was calculated by using the following formula:

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

Where A_{control} is the absorbance of blank, and A_{extract} is the absorbance of the test solution. The effective concentration of sample required to scavenge DPPH radical by 50 % (EC₅₀) was obtained by linear regression analysis of dose response

curve plotted with radical scavenging activity (%) and concentration of the mushroom extract.

2.4. Determination of Total Phenol Content

Total phenol content of the methanol extracts was determined by the Folin - Ciocalteu method [31]. Folin-ciocalteu reagent (1N; 250 μ l) was added to 500 μ l of test solution and incubated for 2 minutes. Then, a volume of 1.25 ml of 10 % Na_2CO_3 was added to the mixture and incubated for 45 minutes at room temperature. Absorbance was measured at 760 nm using UV-visible spectrophotometer. A calibration curve was constructed using gallic acid at a concentrations range of 3 – 40 μ g/ ml. The total phenol contents of the samples were expressed as mg of gallic acid equivalents (GAEs) per gram of dry sample.

2.5. Determination of Total Flavonoid Content

Total flavonoid content was determined by the slightly modified method of aluminium chloride colorimetric assay [32]. Sodium nitrite (5 %; 30 μ l), methanol (98 %; 200 μ l) and aluminium chloride (10 %; 30 μ l) was added to 100 μ l of test solution and incubated for 5 minutes at 30 °C. Sodium hydroxide (1 M; 200 μ l) was added at the sixth minute followed by 0.44 ml of methanol (98 %). The absorbance of the resulting solution was measured at 510 nm. Calibration curve was obtained using (-)-Epigallocatechingallate (EGCG) at a concentrations range of 15 – 500 μ g/ ml. The total flavonoid contents were expressed as w/w % EGCG equivalents.

2.6. Statistical Analysis

All experiments were carried out in triplicate. The effective concentration of sample required to scavenge radicals by 50 % (EC_{50}) was obtained by linear regression analysis of the dose response curve. Graphical data were presented as mean \pm standard deviation of the mean (SD).

3. Results and Discussion

3.1. Antioxidant Activity

Macrofungi are found to be a rich source of natural antioxidants with immense radical scavenging activity. Accordingly, antioxidant substances found in mushrooms can act as major protectors against radical mediated toxicity generated in the biological systems. Epidemiological studies also have demonstrated that many of these antioxidant compounds possess anti-inflammatory, antiatherosclerotic,

antitumor, antibacterial, or antiviral activities to a greater or lesser extent signifying the importance of natural antioxidants found in mushrooms [33,34].

In the present study, antioxidant activity of 30 macrofungi species was screened for antioxidant potential using DPPH radical scavenging assay which is commonly used and highly demanding method for assessing antioxidant activity of biogenic fractions of plants and mushrooms. DPPH is a stable free radical which is reduced to DPPH - H in the presence of a free radical scavenger. Consequently, the absorbance is decreased from the DPPH radical to DPPH - H form leading to provide a characteristic absorption at 517 nm in the visible region of electromagnetic spectrum. When antioxidants donate protons to these radicals, characteristic deep purple colour of the DPPH solution become reduced. The degree of discolouration correlates with the scavenging potential of the antioxidant compounds in the extracts in terms of hydrogen donating ability. The decrease in absorption is used as a measure of the extent of DPPH free radical scavenging activity [35,36]. Radical scavenging capacity was found to be inversely related to the EC_{50} values of the investigated species [37,38]. In this investigation, all macrofungal extracts exhibited high antioxidant potential to variable levels (Table 1). Among the analyzed forms, the most potent radical scavenging effect was observed for *Phellinus repandus* and *Inonotus porreus* showing remarkably lower EC_{50} values of 7.90 ± 1.38 μ g/ml and 19.70 ± 0.17 μ g/ml, respectively, compared to the EC_{50} of standard antioxidant, ascorbic acid (EC_{50} ; 5.00 ± 0.13 μ g/ml). The dose response curves of DPPH radical scavenging capacity % and concentration of the crude methanolic extracts of *P. repandus* and *I. porreus* were represented in Figure 1. Figure 1 describes an increase in DPPH free radical scavenging activity with the increase in concentration of the macrofungal extracts of *P. repandus* and *I. porreus* in the initial phase of the dose response curve until it becomes static (Figure 1).

Ganoderma applanatum, *Gyrodontium sacchari*, *Fuscoporia gilva* also showed potent antioxidant activities with EC_{50} values of 50.40 ± 0.43 μ g/ml, 56.70 ± 0.14 and 44.08 ± 1.27 μ g/ml, respectively. Interestingly, other analysed extracts also exhibited high antioxidant potential, with lower EC_{50} values below 1.2 mg/ml, ten forms exhibited $\text{EC}_{50} < 300$ μ g/ml and eighteen forms showed a mean value of EC_{50} in the range of 300-1200 μ g/ml. The antioxidant properties assayed herein were summarized in Table 1 and the results were expressed as EC_{50} values (μ g various extracts per ml).

Table 1. EC_{50} values (mean \pm SD) obtained in the DPPH antioxidant assays of 30 macrofungi species [* Each value is expressed as mean \pm standard deviation (n= 3)].

Name	EC_{50} (μ g/ml)	Specimen no. in the Department's herbarium
<i>Coriolopsis aspera</i>	123.01 ± 0.58	UOC:DAMIA:D01
<i>Schizophyllum commune</i>	425.00 ± 0.65	UOC:DAMIA:D05
<i>Polyporus arcularius</i>	943.00 ± 0.75	UOC:DAMIA:D08
<i>Stereum hirsutum</i>	630.01 ± 0.23	UOC:DAMIA:D09
<i>Xylaria papulis</i>	289.04 ± 0.67	UOC:DAMIA:D11

Name	EC ₅₀ (µg/ml)	Specimen no. in the Department's herbarium
<i>Hexagonia tenuis</i>	531.70 ± 1.01	UOC:DAMIA:D12
<i>Corioloopsis byrsina</i>	557.01 ± 1.14	UOC:DAMIA:D13a
<i>Earliella scabrosa</i>	1088.70 ± 1.38	UOC:DAMIA:D13b
<i>Corioloopsis caperata</i>	365.01 ± 0.45	UOC:DAMIA:D13d
<i>Hexagonia apiaria</i>	91.50 ± 0.65	UOC:WASNP:W09
<i>Microporus vernicipes</i>	552.70 ± 1.38	UOC:DAMIA:D16
<i>Flavodon flavus</i>	77.06 ± 0.005	UOC:DAMIA:D19
<i>Polyporus grammocephalus</i>	951.01 ± 1.12	UOC:DAMIA:D39
<i>Podoscypha petalodes</i>	720.20 ± 0.087	UOC:DAMIA:D44
<i>Trichaptum byssogenum</i>	555.51 ± 0.22	UOC:SIGWI:S10
<i>Pycnoporus coccineus</i>	254.67 ± 0.44	UOC:SIGWI:S19
<i>Trametes elegans</i>	198.75 ± 0.48	UOC:SIGWI:S25
<i>Inonotus porrectus</i>	19.70 ± 0.17	UOC:SIGWI:S61
<i>Daldinia eschscholtzii</i>	735.77 ± 0.76	UOC:SIGWI:S29
<i>Phanerochaete chrysosporium</i>	69.06 ± 0.12	UOC:SIGWI:S44
<i>Termitomyces heimii</i>	1120.10 ± 0.014	UOC:DAMIA:D43
<i>Gyrodonium sacchari</i>	56.70 ± 0.14	UOC:MINNP:MK05
<i>Ganoderma applanatum</i>	50.40 ± 0.43	UOC:KAUNP:MK24
<i>Ganoderma tusage</i>	64.40 ± 0.45	UOC:KAUNP:MK25
<i>Fuscoporia gilva</i>	44.08 ± 1.27	UOC:MINNP:MK71
<i>Xylaria schweinitzii</i>	331.10 ± 1.17	UOC:MINNP:MK33b
<i>Phellinus repandus</i>	7.91 ± 1.38	UOC:DAMIA:D27a
<i>Xylaria feejeensis</i>	83.70 ± 1.11	UOC:MINNP:MK34
<i>Gymnopilus lepidotus</i>	322.10 ± 1.21	UOC:KAUNP:MK64
<i>Hymenochaete rubiginosa</i>	227.07 ± 0.59	UOC:SIGWI:S55

P. repandus and *I. porrectus* are terrestrial basidiomycetes which belong to the family hymenochaetaceae. Majority of the species in this family are of medicinal value, while some are plant pathogens causing a white rot [39]. However, yet there are no reports available on bioactive properties of *P. repandus* and *I. porrectus*.

The DPPH radical scavenging activity evaluated as EC₅₀ values for *Phellinus* spp. namely *Phellinus gilvus*, *Phellinus rimosus* and *Phellinus badius* was 9 mg/ ml, 10 mg/ ml and

13 mg/ ml respectively [40]. *P. repandus* exhibited a strong antioxidant power compared to EC₅₀ values reported for *P. gilvus*, *P. rimosus*, *P. badius*. *Lentinula edodes* which is a widely used edible fungus also belongs to this family. It has been reported that the EC₅₀ of *Lentinula edodes* for methanol extract against DPPH antioxidant assay was 4.4 mg/ml [41]. Interestingly, *I. porrectus* was found to possess a compelling radical scavenging activity compared to the antioxidant capacity of *L. edodes*.

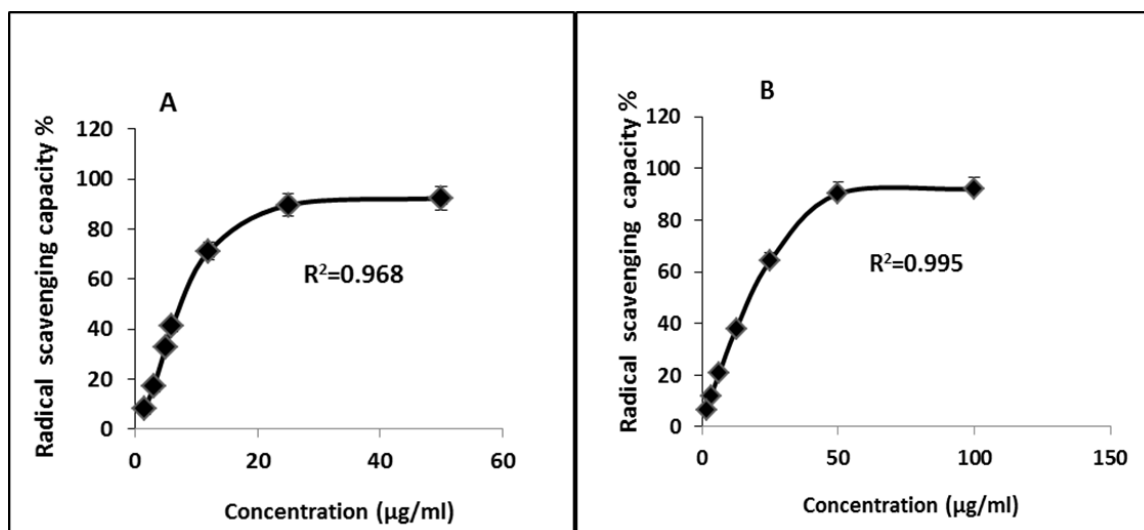


Fig. 1. Dose response curves of DPPH radical scavenging capacity % for methanolic extracts of A) *P. repandus* B) *I. porrectus*.

In addition, *Ganoderma lucidum* is an important medicinal mushroom used in the modern world, acclaimed as “mushroom of immortality” which belongs to Polyporaceae family [42,43]. Hot water extract of *Ganoderma lucidum* was found to have a EC₅₀ of 5.28 mg/ml against DPPH scavenging assay [44]. Methanolic extracts of *Ganoderma lucidum* exhibited free radical scavenging

effect on the 1,1-diphenyl-2-picrylhydrazyl radical with an EC₅₀ value of 1.162 ± 0.016 mg/ml [45]. Currently, the active component which exert antioxidant and anticancer properties of *Ganoderma lucidum* has been identified, isolated and developed as a dietary supplement [46]. Intriguingly, in our investigation, *P. repandus* and *I. porrectus* showed a remarkable radical scavenging effect which is

considerably greater than the “mushroom of immortality”, *G. lucidum*. Above innovation implies the propensity of these species to act as leading sources of therapeutically useful biologically active agents.

3.2. Total Phenol and Flavonoid Contents

Total phenol content and flavonoid content of the each species were determined and the correlation with antioxidant capacity was also investigated. Further, *P. repandus* and *I. porrectus* showed the highest level of total phenols (266.01 ± 5.89 μg Gallic acid/ mg and

75.01 ± 3.16 μg Gallic acid/mg, respectively) as well as highest quantity of total flavonoids (175.10 ± 4.56 μg Epicatechine/mg and 52.20 ± 3.13 μg Epicatechine/mg, respectively) suggesting that phenolic and flavonoid compounds largely contribute to the strong antioxidant activity of these 2 species.

The total phenol and flavonoid contents of all the macrofungal specimens analyzed herein were summarized in Table 2 and the total phenol content were expressed as TPC (μg GAE/mg) and total flavonoid content was indicated as TFC (μg EC/mg).

Table 2. Total Phenol Content (μg GAE/mg) and Total Flavonoid Content (μg EC/mg) of the crude methanolic extracts of 30 macrofungi species [^{*} Each value is expressed as mean \pm standard deviation ($n=3$)].

Name	Mean Value-TPC (μg GAE/mg)	Mean Value-TFC (μg EC/mg)
<i>Coriolopsis aspera</i>	16.5 ± 1.10	5.4 ± 0.99
<i>Schizophyllum commune</i>	4.21 ± 1.21	2.01 ± 1.32
<i>Polyporus arcularius</i>	5.6 ± 0.21	11.45 ± 1.32
<i>Stereum hirsutum</i>	11.6 ± 1.22	3.2 ± 0.43
<i>Xylaria papulis</i>	15.48 ± 0.43	6.6 ± 1.44
<i>Hexagonia tenuis</i>	5.9 ± 1.46	4.02 ± 1.55
<i>Coriolopsis byrsina</i>	7.5 ± 1.78	2.7 ± 0.55
<i>Earliella scabrosa</i>	9.41 ± 1.65	9.41 ± 1.25
<i>Coriolopsis caperata</i>	20.7 ± 0.45	1.44 ± 1.65
<i>Hexagonia apiaria</i>	16.1 ± 0.25	24.4 ± 0.65
<i>Microporus vernicipes</i>	7.4 ± 1.12	1.2 ± 1.10
<i>Flavodon flavus</i>	55.7 ± 0.65	82.4 ± 1.03
<i>Polyporus grammoccephalus</i>	5.01 ± 1.65	2.12 ± 1.23
<i>Podoscypha petalodes</i>	7.01 ± 1.23	4.23 ± 1.43
<i>Trichaptum byssogenum</i>	9.12 ± 1.02	8.01 ± 1.10
<i>Pycnoporus coccineus</i>	12.32 ± 1.24	9.97 ± 1.22
<i>Trametes elegans</i>	15.09 ± 0.89	11.92 ± 1.12
<i>Inonotus porrectus</i>	75.01 ± 3.16	52.20 ± 3.13
<i>Daldinia eschscholtzii</i>	9.93 ± 1.15	8.89 ± 1.54
<i>Phanerochaete chrysosporium</i>	80.98 ± 0.95	45.78 ± 1.22
<i>Termitomyces heimii</i>	5.01 ± 1.41	4.02 ± 1.15
<i>Gyrodontium sacchari</i>	63.03 ± 1.14	61.21 ± 1.21
<i>Ganoderma applanatum</i>	146.21 ± 1.18	135.34 ± 1.24
<i>Ganoderma tusage</i>	105.89 ± 1.22	98.09 ± 1.23
<i>Fuscoporia gilva</i>	64.09 ± 1.14	60.21 ± 1.21
<i>Xylaria schweinitzii</i>	14.12 ± 1.65	8.90 ± 1.54
<i>Phellinus repandus</i>	266.01 ± 5.89	175.10 ± 4.56
<i>Xylaria feejeensis</i>	49.24 ± 1.12	39.12 ± 1.25
<i>Gymnopilus lepidotus</i>	13.90 ± 1.12	7.99 ± 1.45
<i>Hymenochaete rubiginosa</i>	14.76 ± 1.26	8.96 ± 1.21

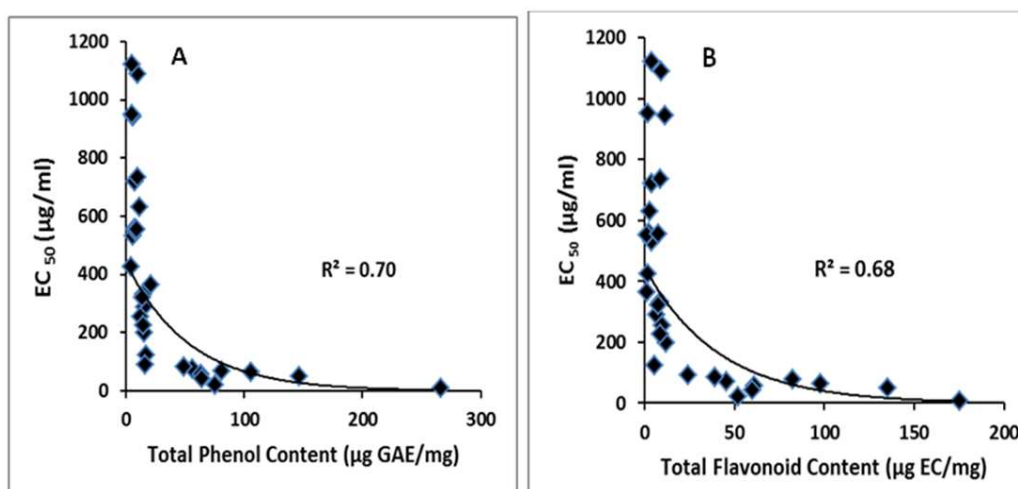


Fig. 2. A) Correlation between EC_{50} values of the macrofungi species and total Phenol content B) Correlation between EC_{50} values and total flavonoid content.

EC₅₀ values of all the species studied were plotted against total phenol and flavonoid contents and correlation of antioxidant activity of macrofungi with total phenol content and flavonoid content of the macrofungi was investigated. Intriguingly, those EC₅₀ values of the species were inversely related to the total phenol and flavonoid contents of the macrofungi extracts implying the contribution of these compounds to their strong antioxidant activities (Figure 2).

To the best of our knowledge, this is the first report on antioxidant activity, total phenol and flavonoid content of *P. repandus* and *I. porrectus*. The rich antioxidant contents of *P. repandus* and *I. porrectus* species make them ideal as nutritional supplements with good medicinal properties. Since the antioxidant activities of *P. repandus* and *I. porrectus* are very high, the isolation and characterization of bioactive compounds from these species are underway to identify potential antioxidant agents for the treatment of degenerative diseases associated with free radicals. Potential antioxidant compounds can be developed as dietary supplements with natural antioxidant properties or as a pharmaceutical composition that can be admixed with one or more pharmaceutically acceptable carriers. Compositions may also include flavours, colourings and coatings. However, added agents must be non-toxic and should not strikingly interfere with the activity of the metabolites of the macrofungi. In addition, it can be added to fruit juice, vegetable juice or all kinds of nutrient drinks containing nutraceuticals of choice such as vitamins, minerals.

4. Conclusion

In conclusion, all analyzed macrofungi species harvested from the dry zone of Sri Lanka possess high antioxidant power. Specifically, *P. repandus* and *I. porrectus* showed promising antioxidant activities and high content of phenols and flavonoid substances implying their importance as potential sources of novel antioxidant compounds. Moreover, phenol and flavonoid contents showed an inverse correlation with EC₅₀ values of each species suggesting that phenolic and flavonoid compounds are largely responsible for the antioxidant activity of analyzed macrofungi. Interestingly, present findings display the importance of isolation and characterization of bioactive compounds from these macrofungi species.

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