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ANTIOXIDANT AND PHYTOCHEMICAL POTENTIAL OF WILD EDIBLE MUSHROOM *TERMITOMYCES RETICULATUS*: INDIVIDUAL CAP AND STIPE COLLECTED FROM SOUTH EASTERN PART OF INDIA

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ABSTRACT

Antioxidant and phytochemical properties of an ethanolic extracts from the wild edible mushroom *Termitomyces reticulatus* and their individual parts (Cap and Stipe) were evaluated. Reducing power, β-carotene bleaching, ABTS and DPPH radicals scavenging activity were used to evaluate their antioxidant activity. Antioxidant components like total phenol, flavonoid, β-carotene and lycopene were also determined and the amount of phenol was correlated with the antioxidant property. All the extracts showed potent antioxidant activities, in which the entire mushroom extract showed more antioxidant property when compared with the other two extracts (Cap and Stipe) and they were in the order, entire mushroom>stipe>cap. Total phenol exhibited a negative correlation with the EC_{50} values of antioxidant assays in all the extracts. The phytochemical profile of the ethanolic extracts, analysed were found to contain alkaloids, saponins, phenols, anthraquinones, steroids, carbohydrates, proteins and cardiac glycosides. The tannins and toxic content cynogenic glycosides were absent. In conclusion, our results supported the consumption of wild edible mushroom that act as a good dietary supplement, taken as whole or entire mushroom.

INTRODUCTION: The search of natural bioactive compounds that can serve as antioxidant and antimicrobial agents has increased tremendously for the last three decades [1]. Because, living cells including those of man, animals and plants are continuously exposed to a variety of challenges, that exert oxidative stress, leads to the generation of reactive oxygen species [2-4]. These generated radicals associated with many diseases in human beings, such as atherosclerosis, cancer and rheumatoid arthritis [5]. Against these radicals, mammalian cells possess intracellular defenses such as super oxide dismutase, catalase and glutathione peroxidase which protects the cells against excessive free radicals [6]. However, these systems are insufficient when excessive free radicals generated under stress or diseased conditions. [7-9]. Epidemiological studies have demonstrated an inverse relationship between the intake of fruits and vegetables that are rich in antioxidants and their effect against free radicals related diseases [10]. In that case natural products like fruits, vegetables, herbs, cereals, sprouts and seeds have been investigated for their antioxidant properties for many years [11-14].

The phytochemicals, especially of phenolics in fruits and vegetables were suggested to be the major bioactive compounds having antioxidant potential that provides health benefits and also found to be associated with the inhibition of atherosclerosis and cancer [15]. Edible mushrooms has become an integral part of the normal human diet and received more attention based on their safety and thus considered as functional food or nutraceutical product [16-17]. In addition to that they are an important source of food and get income in both developing and developed countries [18-19]. Because, many mushroom reported to produce a wide range of secondary metabolites having high therapeutic values such as antioxidant, antitumor, antibacterial, antiviral, cholesterol lowering, hematological agents and immunomodulating properties [20, 21].

The climatic conditions and floral diversity of the Asian regions with a high diversity of wild edible mushrooms are most important, because of their high consumption by the rural population. Herein, we report chemical assays on the antioxidant activity of wild edible mushroom *Termitomyces reticulatus* collected from south eastern part of India. For the first time, the entire mushroom, the cap and the stipe individually were studied in order to compare their antioxidant properties.

MATERIALS AND METHODS:

Mushroom: *Termitomyces reticulatus* collected from south eastern part of India was taken for the study and the mushroom was deposited to the culture collection CAS in Botany, University of Madras.

Preparation of Mushroom Extract: A fine dried powder of the entire fruit bodies and their individual parts were extracted by stirring with the solvent ethanol of 100 mL and incubated in room temperature at 28 ± 30˚C at 150 rpm for 72 hrs and filtered through Whatmann no. 4 filter paper. And the filtered extracts were evaporated using rotary vacuum under reduced pressure. The crude extracts of this mushroom were re-dissolved in appropriate solvents to a determined concentration and stored at 4˚C.

Antioxidant Assays:

Reducing Power Assay: Reducing power assay was determined according to the method, Oyaizu [22]. Various concentrations of the mushroom extracts were mixed with 2.5 mL of 200 mM/L sodium phosphate buffer (pH 6.6)

and 2.5 mL of potassium ferricyanide (10 mg/mL) solution. The mixture was incubated at 50˚C for 20 minutes. Subsequent to incubation 2.5 mL of trichloroacetic acid (100 mg/mL) were added, the mixture was centrifuged at 2000 rpm for 10 minutes. The yellow color layer or upper layer (5 mL) was mixed with 5 mL deionized water and 1mL of freshly prepared ferric chloride (1 mg/mL) solution was added. The absorbance was measured at 700 nm, higher absorbance indicate higher reducing power. α-tocopherol were used as standards.

β- Carotene Linoleic Acid Assay (Lipid Per-Oxidation Inhibition Assay): The antioxidant activity of extracts was evaluated by the β-carotene linoleate model system by Miller [23] as described in Shon, Kim, & Sung [24] with some medication. Firstly, β-Carotene (0.2 mg) was dissolved in 1.0 mL of chloroform. To that mixture 0.02 mL of linoleic acid plus 0.2 mL of Tween 80 was added and the mixture was left standing at room temperature for 15 min. After the evaporation of chloroform, 50 mL of oxygenated distilled water was added and the mixture was shaken to form an emulsion (β-carotene linoleic acid emulsion). Aliquots of 3.0 mL of this emulsion were transferred into test tubes containing 0.2 mL of different concentrations of extracts. The tubes were shaken and incubated at 50°C in a water-bath. As soon as the emulsion was added to each tube, the zero time absorbance (A_0) was measured at 470 nm using a spectrophotometer. A second absorbance (A_1) was measured after 120 min. A blank, with-out β-carotene was prepared for back-ground subtraction. Lipid peroxidation (LPO) inhibition was calculated using the following equation:

$$\textbf{LPO inhibition (\%)} = (A_1/A_0) \times 100$$

The assays were carried out in triplicate and the results were expressed as mean value ± standard deviations. The extract concentration providing 50% antioxidant activity was called as effective concentration inhibiting 50% of the radicals formed.

ABTS Radical Scavenging Activity: The two stock solutions included 7.4 mM ABTS and 2.6 mM potassium persulphate was prepared as described by Arnao, Cano and Asota [25]. The working solution was then prepared by mixing the two stock solutions in equal quantities and allowing them to react for 12 hours and 2 hours at room temperature in dark. The solution was diluted by mixing with 1 mL ABTS solution prepared using 50 mL of methanol, in order to obtain absorbance 1.1 ± 0.02 units at 734 nm. Samples (1.5 mL) were mixed with 2.850 mL of ABTS solution and the mixture was left at room temperature for 12 hours and 2 hours in dark. The absorbance was then measured at 734 nm. The capability to scavenge the ABTS radical was calculated using the following equation:

$$\textbf{ABTS scavenging effect (\%)} = [(A_0\text{-}A_1\ /A_0) \times 100]$$

Where; A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of ABTS scavenging effect against extract concentration, compared with the standard α- tocopherol.

DPPH Radical Scavenging Activity: The free radical scavenging activities of extracts were measured by using 1, 1- diphenyl- 2 - picryl-hydrazyl (DPPH). Briefly, extract concentration

of (0.1–20 mg/mL) in water or ethanol (4 mL) was mixed with 1 mL of methanolic solution containing 1, 1- diphenyl- 2 - picrylhydrazyl (DPPH, Sigma) radicals of 0.2 mM. The mixture was shaken vigorously and left to stand for 30 min in the dark, and the absorbance was then measured at 517 nm against a blank [26]. EC_{50} value (mg/ml) is the effective concentration at which DPPH radicals were scavenged by 50% and the value was obtained by interpolation from linear regression analysis. The capability to scavenge the DPPH radical was calculated using the following equation:

DPPH scavenging effect (%) = $[(A_0-A_1 /A_0) \times 100]$,

Where; A_0 was the absorbance of the control reaction and A_1 the absorbance in the presence of the sample. The extract concentration providing 50% inhibition (EC_{50}) was calculated from the graph of DPPH scavenging effect against extract concentration. BHT was used as standard.

Determination of Antioxidant Component:

Total Phenol: Total phenolic compounds were determined according to Taga, Miller and Pratt [27] using Folin- Ciocalteau method. To 5 mL of 0.3% HCL in methanol/deionized water (60:40, v/v), 100 mg of the ethanolic extract was added. From the resulting mixture (100 µL) was added to 2 mL of 2% aqueous sodium carbonate. The mixture was incubated for 2 mins. To that 100 µL of 50% Folin- Ciocalteau reagent was added and incubated for 30 mins, absorbance was measured at 750 nm against blank. The content of total phenol was calculated on the basis of the calibration curve of gallic acid and the results were expressed as mg of gallic acid equivalents (GAEs) per g of extract.

Flavonoid: Total flavonoid was determined according to Barros *et al.,* [28]. The mushroom extract (250 µl) was mixed with distilled water (1.25 mL) and $NaNO_2$ solution (5%, 75 µL). After 5 minutes the $AlCl_3$ H_2O solution (10%, 150 µL) was added. After 6 min, NaOH (1M, 500 µL) and distilled water (275 µL) were added to the mixture. The solution was mixed well and the intensity of the pink color was measured at 510 nm against blank. The content of flavonoid was calculated on the basis of the calibration curve of quercetin and the results were expressed as mg of quercetin equivalents (CEs) per g of extract.

β- Carotene and Lycopene: For β- Carotene and lycopene determination, the dried ethanolic extract (100 mg) was vigorously shaken with acetone: hexane mixture (4:6, 10 mL) for 1 min and filtered through Whatmann No. 4 filter paper. The absorbance of the filtrate was measured at λ=453, 505 and 663 nm. Contents of β-Carotene and lycopene were calculated according to the following equations:

Lycopene (mg/100mL) = $0.0458\ A_{663} + 0.372\ A_{505} - 0.0806\ A_{453}$;

β- Carotene (mg/100mL) = $0.216\ A_{663} - 0.304\ A_{505} + 0.452\ A_{453}$

The results were expressed as µg of carotenoid/g of extract.

Phytochemical Screening: The ethanolic extract of *Termitomyces reticulatus* was tested for alkaloids, saponins, phenols, flavonoids, carbohydrates, glycosides, sterols, tannins, triterpenoids, anthraquinones, cynogenic glycosides and proteins and amino acids as described by Sofowora [29].

Statistical Analysis: The values were expressed as mean ± standard deviation and one-way analysis of variance (ANOVA) using SPSS/12.0 student software followed by least significant difference (LSD) test. Difference between means at the 5% (*P*-values <0.05) were considered to indicate a statistical significance.

RESULTS AND DISCUSSION: The antioxidant activity of wild edible mushroom *Termitomyces reticulatus* and their individual parts like cap, stipe and the entire mushroom were measured by different biochemical assays such as reducing power, scavenging activity on DPPH, ABTS radicals, and β-carotene- linoleate system.

Reducing Power Assay: The yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each compound. The presence of reducers (antioxidants) causes the reduction of the Fe^{3+}/ferricyanide complex to ferrous form. Therefore, measuring the formation of Perl's Prussian blue at 700 nm can monitor the Fe^{2+} concentration. The reducing power of the *Termitomyces reticulatus* individuals cap, stipe and the entire mushroom extract were parallel to the concentration increased (Fig. 1).

At 3.2 mg/mL the OD values of reducing power of *Termitomyces reticulatus* cap, stipe and the entire mushroom were 0.372, 0.586 and 0.420 respectively. The reducing power of BHA at 3.2 mg/mL and α- tocopherol at 6.4 mg/mL was only 0.112 and 0.092 respectively. The extracts obtained from this mushroom prove to be a better source of antioxidant with low EC_{50} values given in the Table 1, when compared with the standards. The reducing power of the entire mushroom extract was more when compared with the cap and stipe at the maximum concentration of 6.4 mg/mL were in the order; the entire mushroom>stipe>cap. The antioxidant has been reported to be concomitant with the development of reducing power [30]; therefore the antioxidant activity of the extract partially is the results of the reducing power. Reducing power of the various extracts might be due to their hydrogen donating ability [31]. Accordingly *Termitomyces reticulatus* and their parts (Cap and Stipe) may contain the higher amount of reductants which could react with free radicals and terminate radical chain reaction. Similar results were obtained from the wild mushrooms collected from north east Portugal [32].

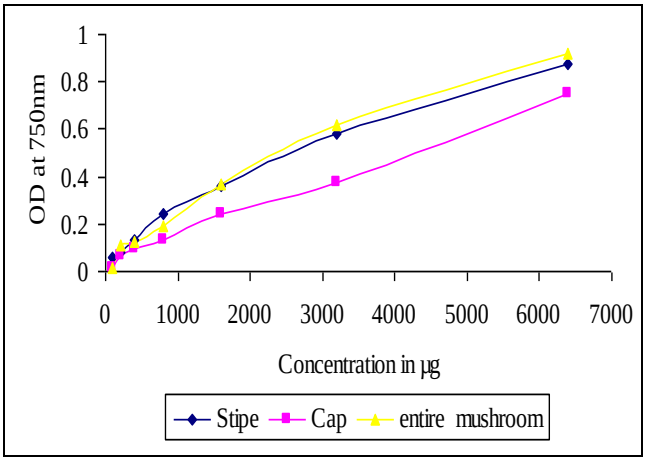

FIG. 1: REDUCING POWER OF ETHNAOLIC EXTRACT FROM *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS

β- Carotene Bleaching Assay: The antioxidant activity of carotenoids is based on the production of carotenoids from linoleic acid. The presence of antioxidant sources stops the chain reaction of β-carotene bleaching by neutralizing the linoleate free radicals. The absorbance decreased rapidly in samples without the antioxidant source whereas; in the presence of antioxidant they retain their color for longer time. The retention of the color was observed in

the tube containing the mushroom extracts. The antioxidant activity was increased with the increased concentration, with low EC_{50} values (Fig. 2, Table 1). At the maximum concentration of 6mg/mL all the three samples inhibited the decolorization of β- carotene and the percentage of activity was 89.14%, 92.1% and 90.05% in stipe, cap and in the entire mushroom respectively. The antioxidant activity by neutralizing the linoleate free radicals and other free radicals formed in the system exhibited by *Termitomyces reticulatus* and their EC_{50} value was around 2.053±0.05 mg/mL in all the extracts. Similar results were reported from the edible mushrooms like *L. gigantus, S. imbricatus* and *Agaricus aruensis* [28].

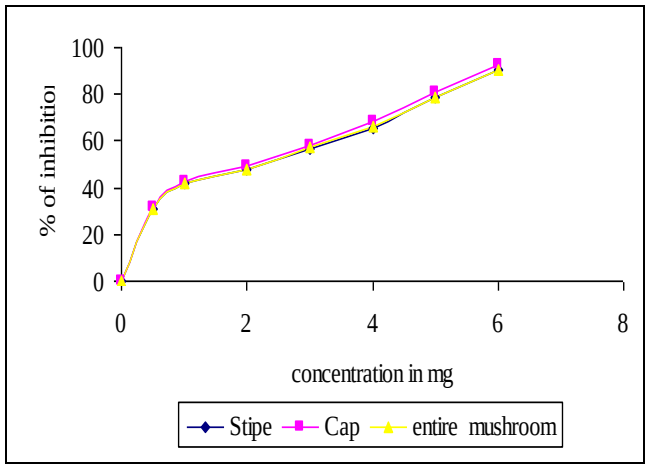

FIG. 2: β- CAROTENE BLEACHING (LPO INHIBITION) EFFECT OF *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS

Radical Scavenging Activity Using ABTS: ABTS a stable free radical with the characteristic absorption at 734nm was used to study the radical scavenging effect of extracts. The results demonstrated that the extracts reacted with ABTS at different concentration ranging from 100, 200, 400 800 1600 and 3200 µg and the readings were observed by measuring the reduction of radical cation generated by $ABTS^{\cdot+}$ at 734 nm. The cap, stipe and the entire mushroom of *Termitomyces reticualtus* showed a maximum decolorization of 99.80%, 98.24% and 98.71% of inhibition at a maximum concentration of 3200 µg/mL. The extent of reduction or decolorization is directly proportional to the increased concentration. The results are illustrated in (Fig. 3, Table 1). ABTS assay is an excellent tool for determining the antioxidant activity of hydrogen-donating antioxidants and of chain breaking antioxidants (scavenger of lipid peroxyl radicals) [33].

Also, ABTS is soluble in both aqueous and organic solvents and is not affected by ionic strength and thus can be used in multiple media to determine both hydrophilic and lipophilic antioxidants components [34]. The EC_{50} value required to reduce the $ABTS^{\cdot+}$ radical was found to be around 200 µg/mL in *Termitomyces reticulatus*. Similar, reports were made in the ethanolic extract of *Pleurotus* sps, and *Agaricus bisporus,* having the scavenging ability of ABTS [35, 36].

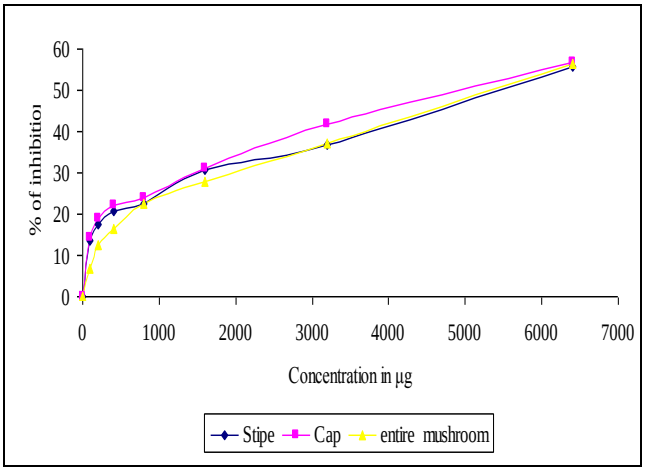

FIG. 3: FREE RADICAL SCAVENGING EFFECT OF *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS AGAINST ABTS RADICALS

Radical Scavenging Activity Using DPPH: DPPH, a stable free radical with the characteristic absorption at 570 nm, was used to study the radical scavenging effects of extract. As antioxidant donate proton to this radical the absorption decreases. The samples were tested against this radical at different concentrations ranging from (100 to 6400 µg) and the readings were observed by decreasing the absorbance taken as a measure indicates the extent of radical scavenging property. The scavenging effects of the samples were evaluated along with the standards such as butylated hydroxyl toluene (BHT) and α- tocopherol. The three samples tested against the DPPH radicals showed a maximum decolorization of 56.42%, 55.53% and 56.12% in cap, stipes and the entire mushroom at the maximum concentration of 6400 µg/mL respectively. The results are given in (Fig. 4 and Table 1).

From the above reading it was observed that increased concentration of the extracts showed increased antioxidant activity. The effective concentration (EC_{50}) was reached upto the higher concentration of 6.4 mg/mL. DPPH a stable free radical of deep purple color which absorption maximum at 570 nm. This purple color generally fades when antioxidant present in the medium. Thus antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by donating electrons resulting in a decreased absorbance. The reactive scavenging activity value of ethanol extract from *Termitomyces reticulatus* and their parts (cap and stipe) revealed antioxidant property in similar fashion. The EC_{50} value against DPPH radicals was found to be 6.4 mg/mL only, this value was found to be less when compared with the other mushrooms such as *Lactarius deliciosus* (8.52 mg/mL) and *Tricholoma protentosum* (22.9 mg/mL) which was reported earlier [32]. This mycelial extract of *Termitomyces albiminosus* is reported from Taiwan for their antioxidant activity [37] against DPPH radical and their EC_{50} value was 5.04 mg/mL, which relates our EC_{50} value of *Termitomyces reticulatus*.

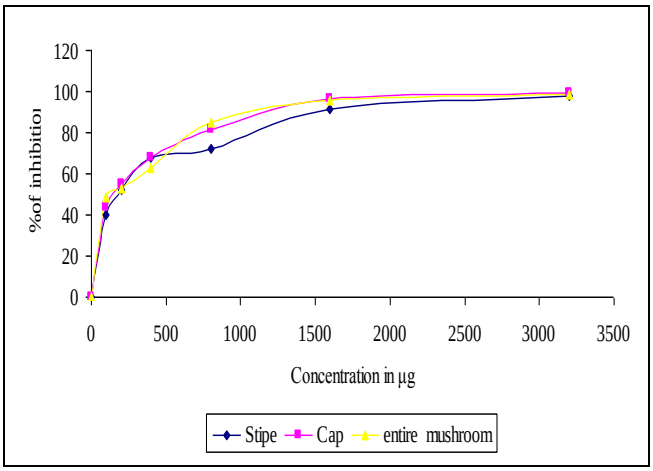

FIG. 4: FREE RADICAL SCAVENGING EFFECT OF *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS AGAINST DPPH RADICALS

TABLE 1: EC_{50} VALUES OF *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS (CAP AND STIPE)

ANTIOXIDANT ASSAYS	EC_{50} VALUE [a] (mg/mL)		
	STIPE	CAP	ENTIRE MUSHROOM
Reducing power	3.66±0.32B	4.393±0.42C	2.587±0.25A
Scavenging ability on ABTS radicals	1.762±0.21A	1.58±0.26A	1.37±0.28A
Scavenging ability on DPPH radicals	5.413±0.38A	5.463±0.47A	4.92±0.34A
LPO inhibition	2.27±0.28A	2.376±0.43A	2.053±0.05A

[a] EC_{50} values: the effective concentration at which the antioxidant activity using the absorbance was 0.5 for

reducing power; ABTS radical was scavenged by 50%; DPPH was scavenged by 50% and β-carotene-linoleic acid assay was inhibited by 50% respectively. EC_{50} values were obtained by interpolation from linear regression analysis. Each EC_{50} values is expressed as mean ± standard deviation (n=3). Means with different capital letters within a row are significantly different (P<0.05)

Determination of Antioxidant Compounds: The major components like phenol, flavonoids, β-carotene and lycopene were quantified in the ethanolic extract of *Termitomyces reticulatus* and their two different parts like cap and stipe. The total phenol was found to be more in entire mushroom 3.2±1.00 mg/g next to that 2.5±1.00 mg/g in stipe and 2.9±1.00 mg/g in cap. The values are equivalent to the standard Gallic acid. Total flavonoids were 4.77±0.690 µg/mg, 2.74±0.28 µg/mg and 3.58±0.43 µg/mg of quercetine equivalent in cap, stipe and the entire mushroom respectively. β- Carotene and lycopene found to be 0.026±0.015 µg/mg, 0.073±0.0007 µg/mg, 0.115±0.007 µg/mg, 0.206±0.04 µg/mg and 0.0543±0.0056 µg/mg, 0.052±0.007 µg/mg of β-carotene/lycopene in the cap, stipes and the entire mushroom respectively. The results of phenol, flavonoid and β-carotene/lycopene are given in the (Table 2).

TABLE 2: BIOACTIVE COMPOUNDS OBTAINED FROM *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS (CAP AND STIPE)

SAMPLE	TOTAL PHENOLS (mg/g)	FLAVONOIDS (µg/mg)	β -CAROTENE (µg/mg)	LYCOPENE (µg/mg)
Stipes	2.5±1.00A	2.74±0.28C	0.115±0.007A	0.206±0.040A
Cap	2.9±1.00A	4.77±0.69A	0.026±0.015C	0.073±0.007B
Entire mushroom	3.2±1.00A	3.58±0.43B	0.054±0.005B	0.052±0.007C

[a] Each value is expressed as mean ± SD (n=3). Mean with different capital letters within a column are significantly different (P<0.05)

Phytochemical Test: Phytochemical profile of the ethanolic extract *Termitomyces reticulatus* was carried out. The extracts contain the following profile like alkaloids, saponins, phenols, anthraquinones, steroids, carbohydrates, tannins and cardiac glycosides. The proteins and toxic content cynogenic glycosides was absent in the mushroom extracts and the results are given in the (Table 3). Total phenol is the major naturally occurring antioxidants found in the ethanolic extracts of *Termitomyces reticulatus* and other mushroom fruit bodies [38]. Phenols such as BHT, Gallates are known to be effective antioxidants [39, 40]. Therefore the contents of total phenol in the three extracts might explain their antioxidant property. The major compounds like phenols, flavonoids, β-carotene and lycopene were quantified whereas, other phytochemicals such as alkaloids, saponins, anthraquinonens, steroids, carbohydrates, tannins and cardiac glycosides were checked only for the presence and concealed that the mushroom extract enriched with essential phytochemicals [41]. Because, these phytochemicals were found to possess a wide range of activities like protection against chronic diseases, such as diabetes, antitumor, antiviral, antioxidative, anticarcinogenic, hepatoprotective properties [42, 43].

TABLE 3: PRELIMINARY PHYTOCHEMICAL SCREENING OF *TERMITOMYCES RETICULATUS* AND THEIR INDIVIDUAL PARTS (CAP AND STIPE)

PHYTOCHEMICAL	INFERENCE
Alkaloids	+
Phenols	+
Flavonoids	+
Carbohydrates	+
Glycosides	+
Saponins	+
Tannins	-
Sterol	+
Triterpenoids	+
Proteins and Amino Acids	+
Anthraquinones	+
Cynogenic glycosides	-

+ = Presence, - = Absent

Correlation between Total Phenol and EC_{50} Values of Antioxidant Assays: Thus, the ethanolic extract of *Termitomyces reticulatus* entire mushroom showed significant antioxidant activity than the cap and stipe with low EC_{50} values. Hence, the ethanolic extract of the entire mushroom and their EC_{50} values was correlated with total phenolic content. Notably negative correlation was established between the phenols and EC_{50} values of antioxidant activities, which are shown in the Fig. 5. These negative linear correlations prove that the sample with highest antioxidant contents show higher antioxidant activity with lowest EC_{50} values.

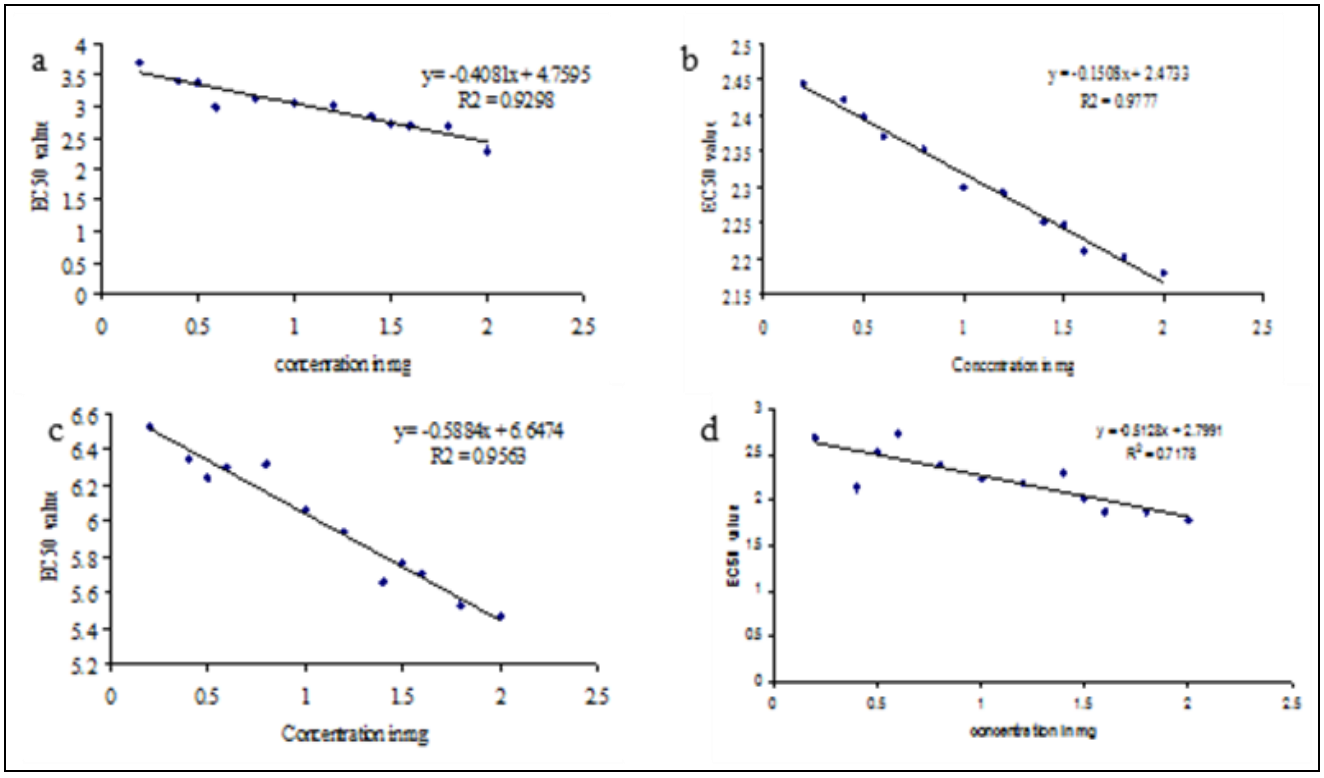

FIG. 5: CORRELATION ESTABLISHED BETWEEN TOTAL PHENOL AND REDUCING POWER, DPPH, ABTS AND B-CAROTENE BLEACHING (LPO INHIBITION) ASSAY (a), CORRELATION BETWEEN REDUCING POWER; (b) B-CAROTENE BLEACHING ASSAY (c) DPPH RADICAL SCAVENGING AND; (d) ABTS RADICAL SCAVENGING ACTIVITY

From the above results and discussion obtained, the use of this entire mushroom is recommended for consumption. The biological properties documented about this mushroom yet a step to make some of them to know, mushroom act as a good dietary supplement as a functional food. Hence it is necessary to identify the biological and pharmacological potential of mushrooms especially of wild edible mushrooms, which are collected indigenously. It is also necessary to do research in identifying and isolating different varieties of mushrooms having nutraceuticals and medicinal properties to commercialize the production in large scale level would create a lot of employment opportunities especially in economically deprived rural area.

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