Chapter – 2: Evaluation of *Termitomyces* species for their suitability for human consumption in terms of nutritional value and also to screen for valuable bioactive principles
2.1 Introduction

There is a huge macrofungal diversity of different types, from truffles to milk-caps, chanterelles to termite mushrooms, with more than 1100 species recorded till today. They provide notable contributions to diet for indigenous people during the months of the year when the supply of food is often perilously low. They are a valued and valuable addition to diets of rural people. In central southern Africa, China, India. Wild edible fungi (WEF) are a significant source of nutrition for indigenous people who live in rural and tribal belts. Wild edible fungi are being collected for food and for money in more than 80 countries (Mattila et al., 2000; Rai, 1994).

Macrofungi are reported to be a good source of protein and minerals and bioactive molecules and some investigators have even contended that the amino acid compositions of mushrooms are comparable to animal proteins and contain all the essential amino acids required for man (Ogundana and Fagade, 1982; Zakhary et al., 1983; Senatore, 1988; Adewusi et al., 1993; Aletor, 1995). Today they are potential protein sources especially in developing countries where animal protein is scarce and expensive (Fink and Hoppenhaus, 1958; Suzuki and Oshima, 1976; Gruen and Wong, 1982). Fresh mushrooms contain very little fat and low in energy (calories). They do, however, contain significant quantities of B vitamins, including thiamin (B1), riboflavin (B2), pyridoxine (B6), niacin, vitamin C and also a source of vitamin D. The low total fat content, and the high proportion of polyunsaturated fatty acids (72 to 85%) relative to total fatty acids, is considered a significant contributor to the health benefit of wild macrofungi. Fresh mushrooms contain relatively large amounts of carbohydrate and fibre ranging from 51% to 88% and 4% to 20% in dry weight (Crisan and Sands, 1978; Ayodele and Okhuoya, 2007).

Termitomyces species contain highest protein content with values ranging from 33-45g per 100 g dry weight basidiocarps (Parent and Thoen, 1977). The data provided by the Chinese Academy of Sciences (1978) revealed that 100g of fresh Termitomyces contains; 92.61g of water and 7.30g of dry matter. The dry matter contains 36.94% crude protein, 21% pure protein, 3.40% of rude fat, crude fiber 13.91%, 38.42% soluble non-nitrogen quality materials, 4.57% soluble sugar, 9.59% of the hydrolysis of sugar, 7.17% ash.
The review of literature revealed that some species of *Termitomyces* contain all the essential amino acids and Glycine, glutamate and alanine are the most abundant amino acids in all the *Termitomyces* species (Alofe, 1991; Botha and Eicker, 1992). Wild edible fungi are also a good source of minerals, including iron, potassium, phosphorus, magnesium, copper, manganese and selenium.

Almost all organisms are well protected against free radical damage by enzymes such as superoxide dismutase and catalase, or compounds such as ascorbic acid, tocopherols and glutathione (Mau *et al.*, 2004). However, the antioxidants present in human diet are of great interest as possible protective agents to help the human bodies to reduce oxidative damage.

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Since ancient times, spices in different types of food to improve flavors are well known for their antioxidant capacities (Lee *et al.*, 1999). In recent decades, the essential oils and various extracts of plants have been of great interest as they have been the sources of natural products. In order to prolong the storage stability of foods, synthetic antioxidants are used for industrial processing. But according to toxicologists and nutritionists, the side effects of some synthetic antioxidants used in food processing such as butylated hydroxytoluene (BHT) have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms (Baardseth, 1989; Ames *et al.*, 1993). The many pharmacological effects of phenolic compounds and flavonoids are linked to their ability to act as strong antioxidants and free radical scavengers to chelate metals and to interact with enzymes, adenosine receptors and bio membranes (Middleton and Kandaswami, 1993). Phenolic compounds, especially phenolic acids and flavonoids are ubiquitously present in vegetables, fruits, seeds, tea, wines and juices; thus they are an integral part of the human diet. These protective effects have partly been ascribed to the antioxidant properties especially of flavonoids (Hollman and Katan 1999; Kaur and Kapoor, 2001; Cakir *et al.*, 2003; Inga *et al.*, 2007). Among flavanone aglycons, naringenin, hesperetin, eriodictyol and iso sakuranetin are the most common, but they are present in much smaller quantities than are glycosides. Citrus flavonoids, especially hesperidin, have shown a wide range of therapeutical properties such as anti-inflammatory, antihypertensive, diuretic, analgesic and hypolipidemic.
activities (Monforte et al., 1995; Inga et al., 2007). Several studies revealed that, they do contain appreciable level of phenolic compounds and hence macrofungi become a valuable source of natural antioxidants (Oyetayo and Akindahunsi, 2004; Puttaraju et al., 2006; Oyetayo, 2008). Since research has tended to focus on the dietary value of edible mushrooms, there is relatively little information pertaining to their antioxidant properties of *Termitomyces* species and their possible use to treat oxidative stress.

A nutraceuticals can be a substance that may be considered as food or part of a food that provides medical or health benefits like the prevention and treatment of disease. Some examples of nutritive nutraceuticals or “functional food ingredients” are dietary fiber, poly unsaturated fatty acids (PUFA), proteins, peptides, amino acids, keto acids, minerals, antioxidative vitamins and other antioxidants (Andlauer and Furst, 2002; Kruger and Mann, 2003). Different mushrooms studied by the scientific community discovered new therapeutic alternatives, and the results proved that they do contain bioactive properties (Lindequist et al., 2005). Macrofungi are rich sources of nutraceuticals (Caglarirmak, 2007; Elmastas et al., 2007; Ribeiro et al., 2007) responsible for their antioxidant (Mau et al., 2002; Lo and Cheung, 2005; Barros et al., 2007), antitumor (Wasser and Weis, 1999), and antimicrobial properties (Smania et al., 1995; Hirasawa et al., 1999; Hatvani, 2001; Barros et al., 2007; Turkoglu et al., 2007).

In India, particularly the alternative systems of medicine, utilize the curative properties of mushrooms. The secondary metabolites of these mushrooms are chemically diverse and possess a wide spectrum of biological activities, which are explored in traditional medicines and in new targets of molecular biology. Pharmaceuticals worth $700 million US dollars are produced annually in Japan alone from *Lentinus, Trametes, Schizophyllum and Ganoderma*. Extracts of various edible fungi, viz. *Lentinus edodes, Flammulina velutipes, Pleurotus ostreatus, Agaricus bisporus, Pholiota nameko, Tricholoma matsutake and Auricularia auricula-judae* possess antitumour effects also. In USA and Japan Maitake (*Grifola frondosa*) and Shiitake (*Lentinus edodes*) have been reported to be inhibitory to the AIDS virus. Various biologically active substances generated in mushrooms have been recorded in the literature; they include polysaccharides, terpenoids, polysaccharide-peptide complexes and proteins (Wang et al., 1998; Wasser et al., 1999). These bioactive
components have become popular sources of natural antioxidative, antitumor, antiviral, antimicrobial and immunomodulatory agents.

Tumor diseases are one of the main causes of death worldwide. Experience from Asian and Eastern Europe countries, it shows that mushrooms could play an important role in prevention and treatment of cancer. *Piptoporus betulinus* was used traditionally in Bohemia for the treatment of rectal cancer and stomach diseases (Semerdzieva and Veselsky, 1986). It is also known as fungus of the ‘iceman’ from the Copper Age found in 1991, who carried *P. betulinus* fruiting bodies attached to his clothing on his journey in the Alpines.

In Eastern Europe, the fruiting bodies of *I. obliquus* have been used as a folk medicine for cancer and stomach diseases since the 16th or 17th century (Molitoris, 1994). Antitumor effects of several extracts and isolated compounds could be demonstrated in tumor cell systems and in animal assays (Kahlos et al., 1994; Burczyk et al., 1996). Several triterpenes and ergosterol peroxide contribute to the activity. The melanin complex of *I. obliquus* has high antioxidant and genoprotective effects on peroxidase-catalyzed oxidation of aminodiphenyls (Babitskaya et al., 2002). So called ‘immunomodulators’ (biological response modifier, immunopotentiators and immunostimulants) are the most important medicinal mushroom drugs used especially in Japan, China, Korea and other East Asian countries today.

Bioactive macrofungi proteins mainly identified as agglutinins and lectins, fungal immunomodulatory proteins (FIP), ribosome inactivating proteins (RIP), ribonucleases, and laccases are the most important agents providing diverse antitumor and immunomodulation activities. Over 40 proteins, found not only in the fruiting body but also in the mycelium, have been investigated; they have been found to exhibit a range of chemical characteristics and various biological effects on immune cells (Guillot et al., 1997; Wang et al., 1998). They can activate lymphocytes and stimulate cell proliferation as well as cytokine secretion in vitro (Sze, et al., 2004), stimulate macrophage (Sheu et al., 2004), inhibit the growth of implanted tumor cells (Yang et al., 2005) or cause cytotoxic or immunosuppressive affects in vivo (Mejia et al., 2005). These capabilities have led us to consider it as a potential remedy for
treating diseases in various states (Mejia et al., 2005; Jeurink et al., 2008). Accordingly, mushroom proteins may affect the host’s immune system, and are therefore thought to have potential in treating various disease states. Numerous mushroom protein complexes have therefore been approved for clinical application in immunomodulation and cancer therapy (Monro, 2003).

*Termitomyces* species occurring in the region of Kodagu district were not documented for their potential food value, pharmaceutical and nutraceutical applications. Hence work was taken up to analyse the nutritional parameters of *Termitomyces* species occurring in Kodagu district and their evaluation for bioactive peptides if any.

### 3.4 Materials and Methods

#### 2.2.1 Proximate analysis of *Termitomyces* species

Based on the availability of *Termitomyces* species, five species of *Termitomyces* namely *T. heimii*, *T. mammiformis*, *T. indicus*, *T. globules* and *T. clypeatus* were collected from different areas of Kodagu district as mentioned in chapter-1 and subjected for proximate analysis and antioxidant activity and bioactive proteins.

1. **Sample preparation**

   To carry out different analyses, the basidiocarps were first washed thoroughly in clean water to free them from mud, ferns and other extraneous materials, and were cut into small pieces and thoroughly mixed and dried at 50°C in an air dry oven for 3 hours. Dried samples were ground to a fine powder so as to pass through 40 mesh sieve and preserved under cold condition for further analysis. All the analyses were carried out in triplicates to ensure replicability of the results.

2. **Moisture content**

   The fresh weight of each mushroom sample was recorded using electronic balance. The samples were then oven dried separately at 80°C for 48 h. The loss in weight recorded after drying was regarded as the moisture content of fruiting bodies (Manzi et al., 1999).

   \[
   \text{Moisture (\%) = } \frac{\text{Initial weight} - \text{Final weight}}{\text{Weight of samples taken}} \times 100
   \]
2.2.1.3 Protein content

The crude protein content of the mushroom samples was estimated by the Kjeldhal method (Anon., 1990). Because of the significant content of non-protein nitrogen in mushrooms, the protein was determined by using the adjusted conversion factor (4.38) for mushroom protein, because it was known that mushrooms contain a significant amount of non-protein nitrogen, generally in the form of chitin in their cell walls (Oei, 1991; Shashirekha et al., 2002).

2.2.1.4 Ash content

About 5-10 g of each sample was weighed accurately into a Porcelain crucible previously heated to about 550°C and cooled. The sample was placed in a muffle furnace at 600°C for 3-4 hours and cooled inside the desiccators. The ash content was then calculated using following equation:

\[
\text{Ash content (\%) = } \frac{\text{weight of the ash}}{\text{Weight of samples taken}} \times 100
\]

2.2.1.5 Fat content

About 10 g of dry each sample was weighed and extracted with Petroleum Ether in Soxhlet apparatus for about 16 hours. The ether extract was filtered into a weighed conical flask. The flask containing the ether extract washed 4-5 times with small quantities of ether and the washings were also transferred. The ether was then removed by evaporation, the flask with the residue was dried in an oven at 80-100°C, cooled in a desiccator and weighed (Raghuramulu et al., 2003).

\[
\text{Fat content (\%) = } \frac{\text{Weight of ether extract}}{\text{Weight of samples taken}} \times 100
\]

2.2.1.6 Crude fiber content

The crude fiber content was estimated by using the AOAC (1990) protocols. About 5g of mushroom sample was extracted using petroleum Ether. The fat free material was transferred in a beaker and 200 ml of dilute sulfuric acid was added and boiled. Whole boiling acid in a flask is connected to reflux condenser and heated for 30 minutes. The flask was removed and filtered and washed thoroughly with boiling water followed by washing in boiling 0.313 N (1.25%) sodium hydroxide and again
refluxed for 30min. The contents were filtered and washed with boiling water and finally washed with ethanol. The residue was dried and incinerated in muffle furnaces at 660˚C and the crucible along with ash was weighed and percentage of fiber was calculated.

\[
\text{Crude fiber content (\%) = \frac{(\text{Wt. of crucible before ashing} - \text{Wt. of crucible after ashing})}{\text{Weight of samples taken}}} \times 100
\]

2.2.1.7 Carbohydrate content

AOAC protocol (1990) used for estimation of total carbohydrate content of samples by difference method (100-total moisture + total ash + total protein + total fat + total fibers) the percentage of carbohydrate was calculated.

2.2.1.8 Vitamin D estimation

Vitamin D in *Termitomyces* species extracts were analyzed by HPLC (model LC-10A, Shimadzu) on a Silica column using a diode array UV-detector (operating at max 254 nm). A solvent system consisting of hexane: amyl alcohol (isocratic, 99.2:0.8 v/v/v) was used as mobile phase at a flow rate of 1 mL / min. To the conical flask containing 500 µL of the mushroom extract 500 µL of toluene, 5 mL of alcohol, 1 mL of sodium ascorbate and 600 µL of KOH (1.5 %) were added and refluxed at 60 ˚C for 30 min in a water bath. After incubation, 3 mL of water, 2 mL of alcohol and 10 mL of diethyl ether was added; shaken well and transferred to narrow tubes and vortexed. To the lower aqueous phase added 2 ml of ethyl alcohol and 10 mL of hexane; vortexed. To 6 mL of upper hexane layer 6 mL of 3% KOH and 10 mL of water was added. Centrifuged and collected the upper layer after all KOH is removed from the aqueous phase. After 5-6 times water wash, 500µL of hexane layer was collected, dried under nitrogen gas and dissolved in 100 µL of mobile phase and subjected to HPLC for quantification of vitamin D (Roberts et al., 2008; Phillips et al., 2008; Byrdwell, 2009). The experiments were conducted in the Department of Biochemistry and Nutrition, CFTRI, Mysore.
2.2.2 Analysis of minerals

2.2.2.1 Preparation of Ash

About 5g of dried sample placed in a muffle furnace at 1000˚C. The residue was cooled in a desiccator and used for further mineral estimation.

2.2.2.2 AAS (Atomic Absorption Spectrophotometry)

Calcium, Magnesium, Manganese, Phosphorus, Iron, Copper and Zinc were determined using an atomic absorption spectrophotometer (Varian Techtron Model AAS 1000). The ash of each sample, which were digested in an acid solution of HNO3, were passed through the AAS system using different lamps, and calibrated with related minerals in different concentrations for different micronutrients (Anon, 1990). To check for possible contamination by reagents or glassware, blanks containing 4mL of ultra pure concentrated HNO₃ and 4ml of H₂O₂ were run together with analytical samples and every batch of the analytical samples were run together with the standard matrix.

2.2.2.3 Flame photometry

Sodium and Potassium were determined by a flame photometry method using standard sodium and potassium solutions (Latiff et al., 1996). All samples were analyzed in triplicates and results were recorded as mean ± S.D.

2.2.3 Antioxidant activities assay and determination of antioxidant components

Five species of Termitomyces fruiting bodies collected from natural growth in different geographic locations of the Kodagu district were procured. The complete names of these species, their geographical locations, and natural habitats were presented in (Table- 2).

2.2.3.1 Preparation of aqueous and solvent extraction

All the collected and dried varieties of Termitomyces species were cleaned to remove any residual compost/soil and subsequently air-dried in an oven at 50 °C for about 3 hrs. All of the dried mushrooms were ground to fine powder (ca. 1 mm size) and stored in airtight plastic bags in desiccators at room temperature for further
analysis. One gram of each of dried mushroom sample was mixed with 10 mL of boiled water or 10 mL of methanol. Samples were stirred for 15 min. for effective extraction and centrifuged at 2000g for 15 min. The supernatants were referred to as water extract (WE) and methanolic extract (ME), respectively, and stored at 4 °C until the completion of the analysis.

2.2.3.2 Total phenol content

The total phenolic contents of water extract of all of the *Termitomyces* samples were determined using colorimeter by Folin-phenol method (Lo and Cheung, 2005). A sample aliquot of 100 µL was added to 900 µL of water, 1 mL of Folin-Ciocalteu reagent, and 2 mL of 10% sodium carbonate solution was mixed in a cyclo-mixer, and incubated for 1 hr at room temperature. The absorbance was measured at 765 nm with a UV-visible Spectrophotometer. The standard curve was drawn using 10-100 µg of gallic acid. The total phenolic content was expressed as gallic acid equivalent (GAE) in milligrams per gram of sample.

2.2.3.3 Total flavonoids

Total flavonoid was determined according to Barros et al. (2007). *Temitomyces* extract (250 µl) was mixed with distilled water (1.25 mL) and NaNO₂ solution (5%, 75 µL). After 5 min. AlCl₃ H₂O 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 (QEs) per g of extract.

2.2.3.4 β- Carotene estimation

*Termitomyces* extracts (1 mL) were vortexed with 3 mL dichloromethane: methanol (1:2, v: v) followed by addition of dichloromethane (1 mL) and hexane (1.5 mL) to each sample. Samples were centrifuged at 1000 g for 15 min at 4° C. The upper hexane/dichloromethane phase was collected and the extraction was repeated twice with the lower phase. Extracts were pooled and evaporated to dryness under a stream of nitrogen. The residue was dissolved in a mixture of dichloromethane:
methanol (2:1 V/V) and subjected to HPLC for quantification of beta-carotenes in the extracts (Khalil and Varananis, 1996). The work was carried out at the Department of Biochemistry and Nutrition, CFTRI, Mysore.

### 2.2.3.5 HPLC analysis of phenolics in water and solvent extracts of *Termitomyces* species

Phenolic acids of aqueous and methanol extract were analyzed according to the method of Wulf and Nagel (1976), on a reverse phase Shimpak C18 column (4.6×250 mm), using a diode array detector (operating at 280 nm). A solvent system consisting of water/acetic acid/methanol (80:5:15) (v/v/v) was used as mobile phase at a flow rate of 1 mL/min. Phenolic acid standards such as gallic acid, tannic acid, caffeic acid, p-coumaric acid, ferulic acid, gentisic acid, protocatechuic acid, syringic acid, vanillic acid, and cinnamic acid were used for the identification of phenolic acids present in WE and ME. Quantitation of phenolic acids was achieved by the absorbance recorded in the chromatograms related to external standards at 320 nm (Valentao *et al.*, 2005). The experiments were conducted at the Department of Biochemistry and Nutrition, CFTRI, Mysore.

### 2.2.3.6 Free radical scavenging (FRS) DPPH assay

The effect of water extract of *Termitomyces* species on DPPH radical was estimated according to the method of Lai *et al.* (2001). Amounts of 10-250 µL of water extracts (reached the volume to 500 µL with double distill water), were mixed with 100 mM Tris-HCl buffer (800 µL, pH 7.4). One milliliter of 500 µM DPPH in ethanol was added to the sample to a final concentration of 250 µM. The mixture was shaken vigorously and left to stand for 20 min at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation:

\[
\text{Scavenging effect (\%)} = \frac{1 - \text{Absorbance of sample at 517 nm}}{\text{Absorbance of control at 517 nm}} \times 100
\]
2.2.3.7 Reducing power assay (RPA)

The reducing power of water extract of all seven *Termitomyces* species was determined according to the method of Yen and Chen (1995). Amounts of 50-250 µL of *Termitomyces* extracts (reached the volume to 500 µL with Double distill water) were mixed with an equal volume of 0.2 M phosphate buffer, pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10% trichloro acetic acid was added to the mixture and centrifuged at 3000g for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% FeCl₃ in a ratio of 1:1:2 (v/v/v) and the absorbance were measured at 700 nm. Increase in the absorbance of the reaction mixture indicated increased reducing power. A higher absorbance indicates a higher reducing power. Gallic acid in the range of 2-10 µg was used as control.

2.2.4 Bioactive proteins extraction, purification and characterization

2.2.4.1 Protein extraction

The protein extraction was performed in accordance with the procedure of Di Toppi *et al.* (1996) with some modifications. The dried fruiting bodies of *T. mammiformis* (400 g) were homogenized with cold phosphate buffered saline (PBS), consist of 0.14 mM NaCl in 5 mM pH 7 Na phosphate buffer in the presence of 0.05 M 2-mercaptoethanol (Lin, 1984), homogenate was stirred overnight at ± 4 °C, filtered and the supernatant was centrifuged at 10,000 g for 25 min. Soluble proteins in the supernatant were then precipitated by adding saturated ammonium sulfate to 95% of saturation. The precipitates were stirred overnight, and then collected by centrifugation at 20,000 × g for 30 min. The precipitate was collected and then dialyzed against 2 L 10 mM Tris/HCl pH 8.0 at 4°C for 36 hrs with three changes of dialysis solution. The extra salt removed by passing the crude protein from 3K Amicon filters.1% Salt traces to be removed with help of Desalting column (All purification steps were performed out at 4°C).

2.2.4.2 Anion exchange chromatography using DEAE column and Mono Q column

The desalted crude protein was subjected to a DEAE-cellulose column (2.2X 10 cm) which was previously equilibrated with 10 mM Tris/HCl pH 8.0. The column
was washed with 200 ml equilibration buffer and then eluted with 200 mL 0-0.5 M NaCl in 10 mM Tris/HCl pH 8. The eluted fractions from above column was loaded and purified on Mono Q HR 5/5 column that had also been pre-equilibrated using 10 mM Tris buffer, pH 8.2. The purified fractions subjected to protein estimation and further assay which was carried at the c-CAMP, NCBS and IISc, Bangalore.

2.2.4.3 Electrophoresis and periodic acid-Schiff’s (PAS) staining

The purified proteins were routinely analyzed by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a Bio-Rad mini protein gel apparatus to detect their identification and purity using the method described by Laemmli (1970) and then the gels were stained with Coomassie Brilliant Blue R250. The molecular weights of the subunits were measured by using the low-range rainbow molecular weight markers consisting of the following proteins: ovalbumin (45,000), carbonic anhydrase (30,000), trypsin inhibitor (20,100), lysozyme (14,300), and aprotinin (6,000). Apparent molecular weights were determined by comparison with protein standards of known molecular weight. The polyacrylamide gels were further stained with periodic acid-Schiff’s (PAS) reagent (Merck, Darmstadt, Germany) to determine the carbohydrate content according to the method of the Zacharius et al. (1969).

2.2.4.4 Estimation of protein content of purified fractions by Bradford’s method

The protein content in the sample was determined by Bradford’s method (Bradford, 1976) wherein Comassie Brilliant Blue (CBB) was used which was dissolved in 70% ethanol with ortho-phosphoric acid having $\lambda_{\text{max}}$ of 670nm on which protein-dye complex was formed.

2.2.4.5 MALDI TOF MS protein characterization (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry)

MALDI-TOF MS has been extensively used in the last decade for the exact molecular mass determination of peptide and proteins (Chait and Kent, 1992), polymers, oligonucleotides (Dai and Whittal, 1996) and other non-volatile, low molecular mass compounds (Fukai et al., 2000). MALDI has been introduced as a rapid and simple method of obtaining molecular weight information at picomole to
femtomole sensitivity. In protein chemistry, this analytical tool typically complements or even replaces conventional protein biochemical techniques, such as sodium dodecyl sulphate polyacrylamide gel electrophoresis (Chait et al., 1993; Wada et al., 1994).

Equal volume of purified protein fractions and matrix (1µL each) were mixed on microcentrifuge tube, mixed properly and the uniform mixtures were deposited directly on the MALDI plate (Mtp 384 Ground Steel, Target Plate, Bruker). Spotting was also being accomplished using the sandwich method (Mirgorodskaya, 2000; Baldwin, 2004). After drying the plate is inserted in MALDI instrument (MALDI MODEL/MAKE: Ultraflex TOF/TOF, Bruker Daltonics Germany) and analysis is performed (Method used for acquiring: 25KvA Reflector mode), with a N2, laser 337-nm laser, 50Hz. (Kyselova et al., 2007). The experiment conducted in IISc. Department of Molecular Biology and Biophysics, Bangalore, India.

2.2.4.5.1 Matrix preparation

Saturated solution of matrix was prepared with 50% ACN (Merck, HPLC grade) /H2O (milli Q/ DD H2O) with 0.1% TFA (Trifluoroacetic acid).

2.2.4.5.2 Trypsin In-solution digestion of active protein fractions

a) Trypsin Stock (concentration of 0.1µg/µL): Adding 200µl of trypsin resuspension buffer to 20µg of trypsin. Store the solution at -20˚ C for up to two months.

b) Digestion Buffer: Weighed 10mg of the Ammonium bicarbonate and dissolved in 2.5ml ultrapure water for a final concentration of 50mM. The solution was stored at 4˚C up to two months.

c) Reduction Buffer: Weighed 8mg of DTT and dissolved with 500µL of ultrapure water for a final concentration of 100mM and was stored the reducing buffer at -20˚ C.

d) Alkylation Buffer: Weighed 9 mg of iodoacetamide and added it to a foil wrapped tube to avoid exposure to light and added 500µL of ultrapure water for a final concentration of 100mM.
e) **Protein concentrations:** The procedure described was useful for protein concentration in the range of 0.1-1.0mg/mL.

f) **Procedure for in-solution digestion:** To protein (12µL), added 15 µL of digestion buffer (Ammonium Bicarbonate) and 1.5 µL of reducing buffer (100mM DTT) in 0.5ml tube. Added 10 µL of the protein solution to the tube and adjusted the final volume to 27 µL with ultrapure water. Incubated the sample at 95°C for five min. and allowed the sample to cool at room temperature. Added 3 µL of alkylation buffer to the tube and incubated in the dark at room temperature for 30min. Added 2 µL of Trypsin (0.1µg/µL) to above tube and incubated at 37°C for overnight. Digests were spotted (four replicates) on a MALDI target using α-cyano 4-hydroxy cinnamic acid (2 mg/ml in 50% acetonitrile, 0.1% TFA containing 10 mM ammonium phosphate as matrix. Dilutions of the digests were made at 1/8 and were spotted in the same manner.

2.2.4.5.3 **MALDI TOF data analysis**

Mascot is a software developed by Matrix Science firm for prediction of mass fingerprint of proteins/peptides followed by sequence analyses (-N and -C terminal). Based on the fragmentation of mass/charge (m/z) ratio (ms) each peak list of measured peptide masses was then used to search in the National Center for Biotechnology Information (NCBI) sequence database for protein identifications using Mascot (http://www.matrixscience.com). In this study, searches were performed allowing for variable modifications that include methionine oxidation and fixed modification carbamidomethyl, as well as allowing for up to one missed cleavage site on the peptide. The same samples of proteins were further characterized by tandem mass spectrometry using the same instrument (ms²). Data was analyzed using Mascot analyser.

2.2.5 **Antioxidant assay of purified fractions**

2.2.5.1 **Free Radical Scavenging (FRS)/ DPPH Assay**

DPPH radical was estimated according to the method of Lih-Shiuh *et al.* (2001). Amounts of 10-50 µL of purified protein fractions (reached the volume to 500 µL with double distilled water), were mixed with 100 mM Tris-HCl buffer (800 µL,
One (1) ml of 500 µM DPPH in ethanol was added to the sample and made the final concentration to 250 µM. The mixture was shaken vigorously and left to stand for 20 min. at room temperature in the dark. The absorbance of the resulting solution was measured spectrophotometrically at 517 nm. The capability to scavenge the DPPH radical was calculated using the following equation.

\[
\text{Scavenging effect (%) = } \frac{1 - \text{absorbance of sample at 517 nm}}{\text{absorbance of control at 517 nm}} \times 100
\]

### 2.2.5.2 Reducing power assay (RPA)

The reducing power of the purified fractions extracted from *T. mammiformis* was determined according to the method of Yen and Chen (1995). 10-50 µL of fractions were taken and made up to 500µl with double distilled water and were mixed with an equal volume of 0.2 M phosphate buffer having pH 6.6, and 1% potassium ferricyanide. The mixture was incubated at 50 °C for 20 min. An equal volume of 10% trichloro acetic acid (TCA) was added to the mixture and centrifuged at 3000g for 10 min. The upper layer of the solution was mixed with distilled water and 0.1% FeCl3 in a ratio of 1:1:2 (v/v/v) and the absorbance were measured at 700 nm. (Increased absorbance of the reaction mixture indicates increased reducing power). Higher absorbance indicates a higher reducing power. Gallic acid in the range of 2-10 µg was used as control.

### 2.2.6 Cytotoxicity study on HT-29

#### 2.2.6.1 Cell lines and culture medium

HT-29 (Colon cancer) was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO2 at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock culture was grown in 25 cm² culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., Kolkata, India).
2.2.6.2 Preparation of test solution

For cytotoxicity studies, each test fraction was dissolved in buffer and made up to with DMEM supplemented with 2% inactivated FBS to obtain a working solution from 50 to 1.56% (v/v) concentrations and sterilized by filtration.

2.2.6.3 Determination of cell viability by MTT assay

The ability of the cells to survive a toxic insult has been the basis of most cytotoxicity assays. This assay is based on the assumption that dead cells or their products do not reduce tetrazolium. The assay depends both on the number of cells present and on the mitochondrial activity per cell. The principle involved is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a blue coloured product (formazan) by mitochondrial enzyme succinate dehydrogenase. The number of cells was found to be proportional to the extent of formazan production by the cells used (Francis and Rita, 1986).

The monolayer cell culture was trypsinized and the cell count was adjusted to $1.0 \times 10^5$ cells/ml using DMEM medium containing 10% FBS. To each well of the 96 well microtitre plate, 0.1 ml of the diluted cell suspension (approximately 10,000 cells) was added. After 24 h, when a partial monolayer was formed, the supernatant was flicked off, washed the monolayer once with medium and 100 µl of different test concentrations of protein fractions were added on to the partial monolayer in microtitre plates. The plates were then incubated at 37º C for 3 days in 5% CO$_2$ atmosphere, and microscopic examination was carried out and observations were noted every 24 h interval. After 72 hrs, the drug solutions in the wells were discarded and 50 µl of MTT in PBS was added to each well. The plates were gently shaken and incubated for 3 h at 37º C in 5% CO$_2$ atmosphere. The supernatant was removed and 100 µl of propanol was added and the plates were gently shaken to solubilize the formed formazan. The absorbance was measured using a microplate reader at a wavelength of 540 nm. The percentage growth inhibition was calculated using the following formula and concentration of test drug needed to inhibit cell growth by 50% ($CTC_{50}$) values is generated from the dose-response curves for each cell line. The study was conducted in Radiant Research Laboratory Bangalore.
Growth Inhibition (%) 

\[ \text{Growth Inhibition} \% = 100 - \frac{\text{Mean OD of individual test group}}{\text{Mean OD of control group}} \times 100 \]

2.2.7 Determination of apoptotic activity of samples in HT-29 cells by DNA ladder assay

2.2.7.1 Cell lines and culture medium

HT-29 (Human, colorectal cancer) cell culture was procured from National Centre for Cell Sciences (NCCS), Pune, India. Stock cells of HT-29 were cultured in DMEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100 µg/ml) and amphotericin B (5 µg/ml) in an humidified atmosphere of 5% CO₂ at 37°C until confluent. The cells were dissociated with TPVG solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in PBS). The stock cultures were grown in 25 cm² culture flasks and experiments were carried out in 6 well plates (Tarsons India Pvt. Ltd., India).

2.2.7.2 DNA fragmentation studies

HT-29 cells (3 x 10⁶ /ml) were seeded into 6 well plates and incubated at 37°C with 5% CO₂ atmosphere for 24 h. The cells were washed with medium and were treated with selected doses of protein fractions and incubated at 37°C, 5% CO₂ for 24 hrs. At the incubation time ended, the chromosomal DNA of cancer cells was prepared with Roche apoptotic DNA ladder kit. Briefly, cells were harvested and lysed with lysis buffer for 10 min. Then the samples were mixed with isopropanol before passing through the filter and washed. The DNA was eluted from the filter and treated with RNAse at 37°C for 30 min before loading onto 2% agarose gel electrophoresis and run 50 V/cm for 3 hrs. The gel was visualized under UV transilluminator and photographed (Montririttigri, 2008).

2.2.8 Statistical analysis

Statistical analysis was performed using SPSS version 10.1 for Windows (USA). All the experiments were performed in triplicates and the data represents the mean ± standard error per replicate. The experiments were also carried out in three independent point of time.
CHAPTER 2  
Nutritional Value and Bioactive Principles

2.3 Results

2.3.1 Nutritional value of *Termitomyces* species

The results of the nutritional composition of the edible *Termitomyces* species are shown in Table 4. The moisture content of the *Termitomyces* species was found to be ranged from 86.06% in *T. heimii* to 90% in *T. indicus*. Protein content was very high range from 30.99 g/100g in *T. mammiformis* to 32.62 g/100g in *T. clypeatus*. Fat content was ranged from 0.44 g/100g in *T. heimii* to 0.56 g/100g in *T. indicus*. In general, wild edible *Termitomyces* species were richer sources of protein and had a lower amount of fat than commercial mushrooms, making them ideal materials for food. This study was in agreement with other studies of different mushroom species (Longvah and Deosthale, 1998; Barros *et al.*, 2007). The carbohydrate content, calculated by difference, was also in abundant macronutrient and ranged from 31.57 g/100g in *T. globulus* to 35.11 g/100g in *T. indicus*. Polysaccharides such as chitin and starch content were the most available mushroom carbohydrates (Manzi *et al.*, 2001). The fiber and ash content varied from 3.50 g/100g in *T. indicus* to 5.0 g/100g in *T. heimii* and 7.90 g/100g in *T. mammiformis* to 8.9 g/100g in *T. heimii* respectively.

Vitamin D of the selected *Termitomyces* species was found to be less when compared with other wild edible mushrooms and they were estimated in water and methanol extract by HPLC method (Table 5). The maximum concentration of vitamin D reported in metanolic extract of *T. mammiformis* was 0.079µg/g (3 IU) followed by *T. globulus* and *T. clypeatus*. The presence of macronutrients and micronutrients in *T. heimii*, *T. indicus*, *T. mammiformis*, *T. globulus* and *T. clypeatus* were also present in sufficient quantities.
Table 5: Proximate analysis of Termitomyces species (g/100g).

<table>
<thead>
<tr>
<th>species</th>
<th>*Moisture (%)</th>
<th>**Protein</th>
<th>**Fat</th>
<th>**Fiber</th>
<th>**Carbohydrate</th>
<th>**Ash</th>
</tr>
</thead>
<tbody>
<tr>
<td>T. heimii</td>
<td>86.06±0.92</td>
<td>31.51±0.32</td>
<td>0.44±0.01</td>
<td>5.00±0.05</td>
<td>31.91±1.31</td>
<td>8.90±0.02</td>
</tr>
<tr>
<td>T. indicus</td>
<td>90.30±0.61</td>
<td>32.55±0.41</td>
<td>0.56±0.04</td>
<td>3.50±0.03</td>
<td>35.11±0.40</td>
<td>8.61±0.05</td>
</tr>
<tr>
<td>T. mammiformis</td>
<td>88.93±1.00</td>
<td>30.99±0.56</td>
<td>0.49±0.01</td>
<td>3.67±0.14</td>
<td>32.03±1.45</td>
<td>7.90±0.05</td>
</tr>
<tr>
<td>T. globulus</td>
<td>86.17±0.7</td>
<td>31.86±0.28</td>
<td>0.50±0.02</td>
<td>4.62±0.02</td>
<td>31.57±0.60</td>
<td>8.41±0.17</td>
</tr>
<tr>
<td>T. clypeatus</td>
<td>88.50±0.5</td>
<td>32.62±0.52</td>
<td>0.51±0.01</td>
<td>4.07±0.08</td>
<td>34.40±0.53</td>
<td>8.72±0.18</td>
</tr>
</tbody>
</table>

P=.000  P=.004  P=.000  P=.000  P=.003  P=.000

*expressed on %  ** expressed on g/100

Table 6: Vitamin D content of Termitomyces species in water and solvent.

<table>
<thead>
<tr>
<th>species</th>
<th>Concentration (µg/g)</th>
<th>Concentration in IU</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WE</td>
<td>ME</td>
</tr>
<tr>
<td>T. mammiformis</td>
<td>0.056</td>
<td>0.079</td>
</tr>
<tr>
<td>T. globulus</td>
<td>0.055</td>
<td>0.085</td>
</tr>
<tr>
<td>T. clypeatus</td>
<td>0.027</td>
<td>0.038</td>
</tr>
</tbody>
</table>

P=.000  P=.004  P=.000  P=.000  P=.003  P=.000

*One µg equal to 40 IU (International Unites)

Table 7: The composition of minerals (macronutrient and micronutrient) presents in Termitomyces species.

<table>
<thead>
<tr>
<th>Macrowtrients (mg/100 g) dry weight</th>
<th>Micronutrients (mg/100g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P</td>
<td>K</td>
</tr>
<tr>
<td>------------------------------------</td>
<td>--------------------------</td>
</tr>
<tr>
<td>T. heimii 30.20±0.28</td>
<td>36.30±0.42</td>
</tr>
<tr>
<td>T. indicus 20.40±0.57</td>
<td>34.85±0.21</td>
</tr>
<tr>
<td>T. mammiformis 26.35±0.49</td>
<td>31.00±0.28</td>
</tr>
<tr>
<td>T. globulus 36.15±0.21</td>
<td>32.75±0.21</td>
</tr>
<tr>
<td>T. clypeatus 28.90±0.14</td>
<td>34.85±0.35</td>
</tr>
</tbody>
</table>

P=.000  P=.000  P=.00  P=.010  P=.823 (NS)  P=.000  P=.004  P=.000
Results of the proximate analysis of mineral values of the edible species of *Termitomyces* clearly indicated the potentials for these species used as sources of food. The phosphorous content of *Termitomyces* species varied between 20.40 mg/100 g in *T. indicus* to 36.15 mg/100g in *T. heimii*. Potassium content was ranged between 31 mg/100g in *T. mammiformis* to 36.30mg/100g in *T. heimii*. Magnesium content varied between 5mg/100g in *T. indicus* to 6.85 mg/100g in *T. heimii*. Sodium was ranged between 2.13 mg/100g in *T. globulus* to 2.39 mg/100g in *T. indicus*. In terms of micronutrients iron content varied between 0.31 mg/100g in *T. heimii* to 0.69 mg/100g in *T. globulus*. Copper was available in low quantity and ranged between 0.02 mg/100g to 0.05 mg/100g. *T. heimii* contains highest zinc quantity.

### 2.3.2 Determination of total antioxidant components and antioxidant activity

#### 2.3.2.1 Total phenolic content

The total phenolics were the major naturally occurring antioxidant components found in the water extracts of several mushroom species (Mau et al., 2002; Tsai et al., 2006). The phenolics concentrations of all the *Termitomyces* varieties in WE were estimated and expressed as milligrams of GAE per gram of dry mushroom. The total phenol content was found to be higher in *T. mammiformis* (28mg/g) followed by *T. indicus* (25.5mg/g), *T. heimii* (23.75mg/g), *T. globulus* (18.5mg/g) and *T. clypeatus* (17.5mg/g) respectively. Among *Termitomyces* species *T. mammiformis* ranked first in terms of phenolics content (Table - 7).

#### 2.3.2.2 Total β carotene

β carotene content of *Termitomyces* species (Table- 7) varied from trace amount in *T. heimii* and *T. indicus* to 2474pmoles/g in *T. mammiformis*. This shows a significant variation of a biomolecule within the species itself (Figure- 38).

#### 2.3.2.3 Total flavonoid

Flavonoid concentration of all the *Termitomyces* species in water extracts were recorded and expressed as microgram of QEs per gram of dry sample (Table- 7). High levels of flavonoids were observed in *T. mammiformis* (4.4 mg/g) followed by *T. heimii* and *T. microcarpus*. 

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### Table- 8: Total phenols, \( \beta \)-carotene and flavonoid contents of *Termitomyces* species.

<table>
<thead>
<tr>
<th>No.</th>
<th>Parameters</th>
<th><em>T. heimii</em></th>
<th><em>T. indicus</em></th>
<th><em>T. mammiformis</em></th>
<th><em>T. globulus</em></th>
<th><em>T. clypeatus</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Total phenols (mg/g)</td>
<td>23.75±0.07</td>
<td>25.5±0.03</td>
<td>28.0±0.06</td>
<td>18.5±0.2</td>
<td>17.5±0.07</td>
</tr>
<tr>
<td>2</td>
<td>Total Flavonoid (mg/g)</td>
<td>3.64±0.04</td>
<td>3.51±0.04</td>
<td>4.39±0.04</td>
<td>2.510±0.03</td>
<td>2.875±0.02</td>
</tr>
<tr>
<td>3</td>
<td>( \beta )-Carotene (pmoles/g)</td>
<td>Tr</td>
<td>Tr</td>
<td>2474±0.04</td>
<td>921.4±0.08</td>
<td>1765.6±0.02</td>
</tr>
</tbody>
</table>

### Table- 9: Phenolic content of *Termitomyces* species in water and methanol extraction.

<table>
<thead>
<tr>
<th>Sl no</th>
<th>Standard Name</th>
<th>Water Extract (% phenolics)</th>
<th>Methanol Extract (% phenolics)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>T. mammiformis</em></td>
<td><em>T. globulus</em></td>
</tr>
<tr>
<td>1</td>
<td>Gallic acid/Tannic acid</td>
<td>9.00</td>
<td>8.72</td>
</tr>
<tr>
<td>2</td>
<td>Protocatechue acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>Gentisic acid</td>
<td>0.43</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>Chlorogenic acid</td>
<td>0.30</td>
<td>-</td>
</tr>
<tr>
<td>5</td>
<td>Caffeic acid</td>
<td>0.18</td>
<td>-</td>
</tr>
<tr>
<td>6</td>
<td>Vanillic acid</td>
<td>30.62</td>
<td>67.38</td>
</tr>
<tr>
<td>7</td>
<td>Syringic acid</td>
<td>2.94</td>
<td>22.53</td>
</tr>
<tr>
<td>8</td>
<td>P-coumaric acid</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>9</td>
<td>Ferulic acid</td>
<td>56.52</td>
<td>-</td>
</tr>
<tr>
<td>10</td>
<td>Cinnamic acid</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
Fig- 38: HPLC chromatograms of β carotene contents of selective *Termitomyces* species, A) Standard B) *T. mammiformis* C) *T. globulus* D) *T. clypeatus*.

Fig- 39: HPLC chromatograms of phenolic components of selected *Termitomyces* species, A) standard phenolics chromatogram ,B) *T. globulus* in WE, C) *T. globulus* in ME, D) *T. mammiformis* in WE, E) *T. mammiformis* in ME, F) *T. clypeatus* in WE, G) *T. clypeatus* in ME.
2.3.2.4 HPLC analysis of phenolic acids

Table- 8 showing the percentage phenolic acids present in the selected species of *Termitomyces*. The WE of *T. mammiformis* contains 7 phenolic compounds namely Ferulic acid (56.52%), Vanillic acid (30.62), Gallic acid/Tannic acid (9%), Syringic acid (2.94%), Gentiisic acid (0.43%), Chlorogenic acid (0.3%) and Caffeic acid (0.18%) respectively. *T. clypeatus* contains 4 types and *T. globulus* 3 types of phenologic acids. In methanolic extract *T. clypeatus* contains 8, *T. mammiformis* 7 and *T. globulus* 5 phenolic acids. Variations in the net antioxidant activity could be due to the total effect of different phenolic acids. Most of them showed gallic acid/tannic acid contents in addition to Caffeic acid acid and Syringic acid (Table - 8). Chromatograms of phonoloc acids of selected *Termitomyces* species is presented in Figure-39.

2.3.2.5 Free radical scavenging (FRS) DPPH assay

The antioxidant activity (%) of *Termitomyces* extract was having DPPH scavenging radical at 50 µg/ml was found to be IC$_{50}$ 12.07 µg/ml in *T. heimii*, 15.68 µg/ml in *T. indicus*, 6.23 µg/ml in *T. mammiformis*, 7.7 µg/ml *T.globulus* and 17.45 µg/ml *T. clypeatus*, whereas that of the synthetic antioxidant BHA was found to be 88.89 ± 0.05 (IC$_{50}$ 13.5 µg/ml) (Figure- 40).

2.3.2.6 Reducing power assay (RPA)

The reducing power of *Termitomyces* was concentration-dependent as exhibited in Figure- 41. Among the tested samples, *T. mammiformis* shows the maximum reducing ability followed by *T. globulus* as the least.
Fig- 40: DPPH radical scavenging activity of different *Termitomyces* species.

Fig- 41: Reducing power activity of *Termitomyces* species.
2.3.3 Purification and characterization of the protein from *T. mammiformis*

2.3.3.1 Anion exchange chromatography using DEAE column and Mono Q column

To isolate the putative bioactive protein from *T. mammiformis* a DEAE anion-exchange chromatography column was first used to fractionate the proteins in the ammonium sulfate precipitate (95% saturation) of *T. mammiformis* mycelium extract (Figure- 42). The fractions yielded around two sub fractions namely A1 and A2 which were eluted out for the further procedure. Collected fractions were pooled 1:1(v/v) and desalted for further separation of proteins using MonoQ. The purified proteins were isolated and presented in Figure- 43. Of this, A3, A4 and A7 fractions were eluted for further analysis.

2.3.3.2 Protein content of purified fractions

The results of protein estimation using Bradford method is presented in table Table - 9. Out of the five fractions analyzed A3 and A7 showed promising result for further characterization. For the other fractions the volume of the elutant was comparatively less and was not feasible for analyses.

<table>
<thead>
<tr>
<th>column</th>
<th>Fraction No.</th>
<th>Protein Concentration (µg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>DEAE</td>
<td>A1</td>
<td>32.00</td>
</tr>
<tr>
<td>DEAE</td>
<td>A2</td>
<td>11.00</td>
</tr>
<tr>
<td>Mono Q</td>
<td>A3</td>
<td>10.00</td>
</tr>
<tr>
<td>Mono Q</td>
<td>A7</td>
<td>20.00</td>
</tr>
</tbody>
</table>

2.3.3.3 Electrophoresis and periodic acid-Schiff’s (PAS) staining of purified fractions

Following each purification step, gel electrophoresis was performed to elucidate the biochemical characteristics of protein. SDS/PAGE yielded a single band with an apparent MW of approximately around 19 kDa of the active fractions of the DEAE-MonoQ purified peaks of A3 and A7 respectively (Figure- 44). Staining the polyacrylamide gel with periodic acid/Schiff reagent revealed that the protein was identified in *T. mammiformis* was not a glycoprotein which showed negative result upon PAS staining (Figure- 45).
**Fig- 42:** Chromatogram for separation of *T. mammiformis* proteins on DEAE column.

**Fig- 43:** Chromatogram for separation of elute from DEAE column on MonoQ column.
Fig- 44: SDS-PAGE (12%) showing the separation of proteins that were collected from the ion-exchange columns. Lanes M – Marker, Lanes 1 is of crude protein, Lanes 2, 3 are for the MonoQ fractions of purified protein A3 (protein 1) and A7 (protein 2) respectively. Lane 4 – Marker, Lanes 5 and 7 are for the fraction of A1 and A2 are for the DEAE fraction of crude protein, respectively.

Fig- 45: Periodic acid-Schiff’s (PAS) staining of purified fractions. Lane M – Marker, Lanes 1-2 for the DEAE fractions of A1 and A2 respectively. Lanes 3-5 are for the MonoQ fraction of A3, MonoQ fraction of A4, MonoQ fraction of A7 after desalting, respectively.
2.3.3.4 MALDI TOF MS protein characterization (Matrix Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry)

The Protein 1 and Protein 2 isolated from *T. mammiformis* was further characterized using software tool Mascot (peptide mass fingerprint). The mass spectra results (Figure- 47 and 48) revealed the existence of two proteins namely, E-3 ubiquitin protein like ligases (Protein 1, 18.13 kDa) and extracellular mutant like protein 11, ECM-11 (Protein 2, 19.18 kDa).

The functions of these proteins were also determined using European Bioinformatics Laboratory (EBI), Swissprot, Interpro and GO (gene ontology). The function of the protein 1 (E-3 ubiquitin protein like ligases) may be involved in ubiquitin-like modifier processing, activation, conjugation or deconjugation such as Ubl-activating enzymes (E1s), Ubl-conjugating enzymes (E2s), Ubl-protein ligases (E3s), some thiol proteases (Ubiquitin carboxyl-terminal hydrolases (UCH), Ubiquitin-specific processing proteases (UBP) and ubiquitin-like proteases and the ubiquitin-like modifier proteins. Besides signaling proteolysis, ubiquitination for example can be a signal for trafficking, kinase activation and other nonproteolytic fates. The protein 1 may be involved in the alteration of DNA, protein, or sometimes RNA, in chromatin, which may result in changing the chromatin structure. The protein 2 (Extracellular mutant like protein 11) may involved in a process that is carried out at the cellular level which results in the assembly, arrangement of constituent parts, or disassembly of the fungal-type cell wall.
Fig- 46: Mass spectra of protein 1 indicating 18.13 kDa.

Fig- 47: Mass spectra of protein 2 indicating 19.18 kDa.
Fig- 47: MALDI TOF Mass spectra of protein 1 indicating 18.13 kDa after fragmentation using trypsin digestion.

Fig- 48: MALDI TOF-Mass spectra of protein 2 indicating 19.18 kDa after fragmentation using trypsin digestion.
Fig- 49: Stimulated structure of protein 1 and protein 2 using pymol and protein data bank (PDB).
2.3.4 Biological activity of pure protein fraction from *T. mammiformis*

2.3.4.1 Free radical scavenging (FRS) DPPH assay

The antioxidant activity of the proteins (Protein 1 and Protein 2) isolated from *T. mammiformis* showed a good DPPH scavenging mechanisms. The antioxidant activity of protein 1 to scavenge DPPH radical was IC$_{50}$ 30.34 µg/ml (Figure- 50) and protein 2 was IC$_{50}$ 35.87 µg/ml respectively, when compared to that of synthetic antioxidant BHA (IC$_{50}$ 13.5 µg/ml).

2.3.4.2 Reducing power assay RPA

The results for reducing power of protein 1 and 2 exhibited a promising result with the protein 2 showing maximum in less concentration compare to protein 1. The reducing power of the above proteins increased with concentration. The reducing power of protein 1 and 2 at 50 µg/ml was higher than 0.8, in the order protein 1 > protein 2, wearers reducing power of BHA at 10 µg/ml was 0.36. The results of the same are presented in Figure- 51. This activity also corresponds to lipid peroxidation assay (Huang *et al.*, 2005).
Fig- 50: The graph showing the DPPH radical scavenging activity of protein 1 and 2 isolated from *T. mammiformis*.

Fig- 51: The graph showing the reducing power of protein 1 and 2 isolated from *T. mammiformis*.
2.3.4.3 Anticancer activity

Cell division or cell proliferation is a physiological process occurs in almost all tissues and under many circumstances. Normally the balance between proliferation and programmed cell death is tightly regulated to ensure the integrity of organs and tissues. Mutations in DNA that lead to cancer disrupt these orderly processes. The antitumor activity of protein 1 and protein 2 isolated from *T. mammiformis* against HT 29 which was tested on colon cancer cell line and exhibited with IC$_{50}$ value of 10.54 µg/ml and 13.84 µg/ml respectively (Figure- 53) whereas Paclitaxol showed IC$_{50}$ of 6.43 µg/ml.

2.3.4.4 DNA fragmentation studies

The DNA fragmentation studies against HT 29 cell line showed the potent DNA fragmentation in the cancer cell line (Figure- 54). The cell line growth was inhibited directly because of the DNA damage and fragmentation leading to the death of the cell line.
**Fig- 52:** HT 29 colon cancer cell line. A) Untreated HT 29 colon cancer cell line (Control), B) HT 29 cell line treated with Protein 1 and C) HT 29 cell line treated with protein 2 showing cytotoxicity.

**Fig- 534:** DNA fragmentation studies in HT-29 cell line. Lane 1 – DNA ladder, Lane 1 - Protein 1 showing DNA fragmentation in HT 29 cell line, Lane 2- Protein 2 showing DNA fragmentation in HT 29 cell line and Lane C – Control.
2.4 Discussion

The chemical constituents of edible mushrooms are not necessarily good indicators of nutritional value due to their inherent characteristics such as intrinsic physiological and biochemical characteristics, genetic nature of a given strain and typical heterotrophic metabolism which would result in difference in compositional data. The nutritional value of mushrooms is well documented (Buyck, 1994; Flegg et al., 1985; Taylor, 1998). Mushrooms contain about twice the protein of most fresh vegetables, apart from beans, peas and lentils. According to Ghorai et al. (2009) these are: (a) a good source of digestible proteins (20–30% of dry matter) having all the essential amino acids (especially enriched in lysine) b) chitinous wall to act as a source of dietary fibre which has potential in lowering cholesterol (Sadler, 2003), (c) high vitamin B content, (d) low in fat, and (e) virtually free of cholesterol. They are easily cultivable and are consumed either in fresh or processed form.

In general, the fruiting bodies of mushrooms on dry weight basis, the carbohydrate contents of some wild edible mushrooms from nitrogen free extracts were found to be between 41 and 65%, protein content ranging from 8.9 to 33.8%, low fats content 5.7% and ash content ranging from 2.1 to 16.6% for the tropical species (Aletor, 1995). The low dry matter and lipid contents results in the low energy value of mushrooms (Ouzouni and Riganakos, 2007). The values of 87–155 kJ/ 100 g of fresh mushrooms were reported by Barros et al. (2007). The mineral content of wild edible mushrooms has been found higher than cultivated ones (Aletor, 1995; Rudawska and Leski, 2005).

The result of the current study revealed that protein contents of Termitomyces species varied between 30.99 to 32.62%. This is in agreement with the initial reports of Parent and Thoen (1977) for the study on a comparison of the edible mushrooms of the Upper-Shab and they reported that Termitomyces had the highest protein content.

Olila et al. (2007) has conducted experiments and have found that, only 25.8% protein content was in T. microcarpus. Besides, other experiments showed that nutrient contents with respect to specific nutrients can significantly differ in mushrooms of same genus as evident by a study conducted by Kansci et al. (2003) who found that,
the protein content in six *Termitomyces* species ranged from 15 - 19% on dry weight basis. Our results are in agreement with the results of four other research workers conducted research elsewhere (Chinn, 1945; Adriaens, 1953; Aletor, 1995). Adejumo (2005) has demonstrated that *T. mammiformis* from Nigeria had the highest concentration of protein (36.8%). This study could be used to explain the reason why *Termitomyces* species are the most sought protein source among the indigenous people of Kodagu district and elsewhere.

The results of the nutritional analysis of the *Termitomyces* species showed that all the species have high moisture content. Similar results were obtained by Weinheim (2006) and Kansci (2003). The high moisture content of *T. indicus* could account for their low shelf-life. The study by Opige (2006) on nutritional value of two *Termitomyces* species from Uganda showed *Termitomyces giganticus* had lower ash content (9.87%) compared to 19.7% for *Termitomyces microcarpus*. The fat content of *Termitomyces* species is however variable. Depending on the species, as content varies from 0.4 g/100 g to 5g/100. Carbohydrates are important components in *Termitomyces* with their contents varying from 43.7 g/100 g in *T. letestui* to 57.4 g/100g in *T. schimperi*, starch, and glycogen, reducing and non reducing sugars are the main components (Zakhary, 1983). The carbohydrate content of *Termitomyces* of Kodagu district varied between 31.57 to 35.11g/100g. Similar results were obtained by Oboh (2009).

Despite their high water content, mushrooms of the genus *Termitomyces* appear to be a great source of fibres and minerals also. The high content in crude fibres, the low energy value and the low lipids content made these mushrooms good food for persons of hypocaloric diet. The micronutrients in these fungi could help to relieve disorders, which range from constipation to heart disease and cancer. For example potassium in mushrooms regularizes the heartbeat and improves oxygen supply to the brain (Serunjogi, 2005). Calcium could be used by the body to build strong bones and could play an important role in the proper functioning of the nervous system. Most of the nutrients in mushroom cannot be destroyed by sunlight and therefore dried ones still serve the purpose.
The fruiting bodies of mushrooms were found to contain high levels of well assimilated mineral elements. Major mineral include K, P, Na, Ca, Mg and elements like Cu, Zn, Fe, Mo, Cd form minor constituents (Chang, 1982). K, P, Na and Mg constitute about 56 to 70% of the total ash content of the mushrooms while potassium alone forms 45% of the total ash. The present study is in agreement with earlier studies of Bahl (1998) and Oei (1991). Mushrooms are generally low in sodium concentration (Bahl, 1998). The low sodium and high potassium concentration is of significance as a Na/K ratio less than 0.6 (Nieman, 1992) suggests that the mushrooms are suitable for diet formulation. The *Termitomyces* species found in Kodagu district are great source of mineral and consequently they can replace of meat and fish diet (Mattila *et al.*, 2000). Based on the values of proximate analysis, mineral and energy values as approximate indices of nutritional quality it would appear that some of these mushrooms (mainly *T. robustus*, *T. microcarpus*) fall between most legumes and meat. Indeed, earlier studies (Suzuki and Oshima, 1976; Gruen and Wong, 1982; Zakhary *et al.*, 1983) indicate that edible mushrooms are highly nutritious and comparable to meat, egg and milk. Several studies have confirmed that fungi make a useful contribution to vitamin intake, particularly the B vitamins and vitamins D and K, and in some cases vitamins A and C (Sadler, 2003; Sanmee *et al.*, 2003). Thus, for some groups, for example vegetarians or individuals allergic to fish, fungi can be an important dietary source of vitamin D.

Natural antioxidants are characterized by their ability to scavenge free radicals. Proton-radical scavenging action is an important attribute to antioxidants which is measured by DPPH radical scavenging assay. DPPH, a protonated radical, has a characteristic absorbance maximum at 517 nm. The absorbance of DPPH decreases in the presence of antioxidant due to the scavenging of the proton radical (Yamaguchi *et al.*, 1998). Hydrogen-donating ability of the antioxidant molecule contributes to its free radical scavenging potential (Chen and Ho, 2007). The extracts of *Termitomyces* showed potent DPPH radical scavenging assay with IC\(_{50}\) of 6.23 µg/ml in *T. mammiformis* (Figure- 40). It is believed that antioxidant activity and reducing power are related (Duh *et al.*, 1999). Reductions in these inhibit lipid peroxidation by donating hydrogen atom and thereby terminating the free radical chain reaction (Huang *et al.*, 2005). The *Termitomyces* water extract exhibited good reducing power (Figure- 41). Phenols, flavonoids present in the mushrooms potentially protect the
body cells against (‘anti-’) the damage caused by reactive oxygen species (ROS). The total antioxidant activity of fruits and vegetables is indeed related to their phenolic content, not only to their vitamin C content. Research reports have suggested that many polyphenol are more potent antioxidants than vitamins C and E (Ola and Oboh, 2004). Moreover, Pietta et al. (2000) had earlier reported that the phenolic compounds are responsible for most of the antioxidant activity in plants, while some studies have shown that the effective bioactive substance in mushrooms are mainly phenolic compounds with strong antioxidant activity (Mau and Chao, 2001). Reducing power can be a novel antioxidation defence mechanism; the two mechanisms that are available to affect this reducing power are by electron transfer and hydrogen atom transfer. The reducing power of the extracts was assessed based on their ability to reduce Fe (III) to Fe (II). Many peptides and protein hydrolysates can lower the pace of lipid auto oxidation process (Pena-Ramos and Xiong 2001). They also play a role of the heavy metal acceptors and scavenge free radicals. The maillard reaction products have also the properties to bind heavy metals and form inactive oxidative complexes. Protein hydrolysates can be potentially applied as additives to many of food products although their darkening and low fat solubility make it impossible to apply them as the antioxidative additives to fats and oils (Dziuba, 1997).

The main components of the antioxidative peptides derived from different proteins are mainly the amino acids known for their antioxidative properties such as histidine or tyrosine. Similar properties are also displayed by methionine, lysine and tryptophan. The composition of amino acids, their sequence and configuration also affect the antioxidative properties of peptides (Pena-Ramos and Xiong 2001). The antioxidant activity increases due to the presence of proline residues and the presence of hydrophobic residues fosters the interaction of peptides with linoleic acid (Chen et al., 1996).

On the other hand, the presence of free or bound thiols might be responsible for the anti-oxidant activity. It has been proposed that in living cells the protein thiols could be directly involved in the cellular defense mechanism against oxidants. New reports confirm that most protein SHs equivalents are found in the reduced state and that protein thiols are directly involved in the cellular defense mechanism against oxidants. Proteins are somewhat unique in this way compared to other food antioxidants, in that they can potentially act as multifunctional antioxidants.
(inactivation of reactive oxygen species, scavenging free radicals, chelation of prooxidative transition metals, and reduction of hydroperoxides) that can inhibit several different lipid oxidation pathways. The antioxidant activity of proteins in radical-mediated oxidation reactions may be due to their ability to act as radical trapping devices (Neuzil et al., 1993; Ostdal et al., 2002). The antioxidant activity of Termitomyces species could be regarded as the promising candidate for vital bioactive molecules. Since the nutritional content and antioxidant activity was more in T. mammiformis when compared to other species same also have been selected further for the isolation, purification and characterization of bioactive proteins.

The antioxidant activity of Ubiquitin ligase like ligase (protein 1) and extracellular mutant protein (ECM-II protein 2) extracted from T. mammiformis in respected to reducing power assay and DPPH assay are also might be due to the above menthioned mechanisms.

The anticancer activity of protein 1 and protein 2 showed very good inhibition of colon cancer cell line HT 29 with the IC₅₀ value 10.54 and 13.84 µg/ml respectively. Cancer is a multistep process, wherein mutations in DNA that lead to cancer, disrupt these orderly processes. The uncontrolled and often rapid proliferation of cells could lead to either a benign tumor or a malignant tumor (cancer). Benign tumors do not spread to other parts of the body or invade to other tissues, and they are rarely a threat to life unless they extrinsically compress vital structures. Malignant tumors can invade other organs, spread to distant locations include (metastasis) and become life threatening. Metastasis is a complex series of steps in which cancer cells leave the original tumor site and migrate to other parts of the body via the bloodstream or the lymphatic system. To do so, malignant cells break away from the primary tumor and attach to and degrade proteins that make up the surrounding extracellular matrix (ECM), which separates the tumor from adjoining tissue. By degrading these proteins, cancer cells are able to reach the ECM and escape. When oral cancers metastasize, they commonly travel through the lymph system to the lymph nodes in the neck. The body resists metastasis by a variety of mechanisms through the actions of a class of proteins known as metastasis suppressors, of which about a dozen are known (Yoshida et al., 2000). Series of molecules have been depicted to play multiple roles against metastasis. During the interplay of cancer metastasis, biomolecular interaction
between normal cell and that of cancer cell is essential to make the event possible. Several molecules such as COX₂, NF-kB, PGE₂, Interleukins and VEGF are known to be involved in metastatic event.

From previous literature it is evident that ubiquitin is targeted for cancer therapy. The ubiquitin system is a network of proteins dedicated to the ubiquitylation of cellular targets and the subsequent control of numerous cellular functions. The deregulation of components of this elaborate network leads to human pathogenesis, including the development of many types of tumor. Alterations in the ubiquitin system that occur during the initiation and progression of cancer are now being uncovered, and this knowledge is starting to be exploited for both molecular diagnostics and the development of novel strategies to combat cancer (Daniela Hoeller et al., 2009).