MEDICINAL AND NUTRACEUTICAL POTENTIAL OF GIANT MUSHROOM

(*Macrocybe gigantea* (Massee) Pegler & Lodge)

By

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THESIS

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DECLARATION

I, hereby declare that the thesis entitled **"MEDICINAL AND NUTRACEUTICAL POTENTIAL OF GIANT MUSHROOM (*Macrocybe gigantea* (Massee) Pegler & Lodge)"** is a bonafide record of research done by me during the course of research and that the thesis has not previously formed the basis for the award of any degree, diploma, fellowship or other similar title, of any other University or Society.

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Thomas A. Edison

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LIST OF ABBREVIATIONS

cm	Centimeter
CTAB	Cetyl trimethyl ammonium bromide
dl	Decilitre
DNA	Deoxy ribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
et al.	And others
Fig	Figure
g	Gram
h	Hour
HCl	Hydrochloric acid
i.p.	Intra peritoneal
ITS	Internal transcribed spacer
KA	King Armstrong
kg	Kilogram
l	Litre
M	Molar
mg	Milligram
min	Minute
ml	Milliliter
mM	Millimolar
N	Normal
NaOH	Sodium hydroxide
NaCl	Sodium chloride

LIST OF ABBREVIATIONS CONTINUED

ng	Nanogram
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
p.o.	Per oral
ppm	Parts per million
rpm	Revolution per minute
s	Second
s.c.	Subcutaneous
SDA	Sabouraud dextrose agar
S.E.	Standard error
sp.	Species
Taq	*Thermus aquaticus*
TBE	Tris-borate EDTA buffer
TE	Tris- EDTA buffer
U/L	Enzyme unit per litre
V	Volt
viz.	Namely
v/v	Volume/volume
w/v	Weight/volume
X	Times
µg	Microgram
µl	Microlitre
°C	Degree Celsius
%	Per cent
±	Plus or minus

Introduction

1. INTRODUCTION

Mushrooms have been part of human diet since time immemorial. They are considered as rich food because they contain protein, sugar, glycogen, lipid, vitamins, amino acids and crude fiber. The number of different kinds of mushrooms in the earth is estimated to be around 1, 40,000. Estimatively only 10 per cent of the species have already been described and about 2,000 of them are edible. Less than 25 species are largely used as food, being produced in commercial scale (Soares *et al.*, 2013). Mushrooms also represent major and largely untapped source of potent pharmaceutical products.

Medicinal mushrooms have a long history of use in traditional oriental therapies. Mushroom extracts have been reported to have hematological, antiviral, antitumor, hypotensive and hepatoprotective effects (Miles and Chang, 1997). The fruiting body contain many bioactive substances such as polysaccharides, phenolics, proteins (fungal immunomodulating proteins - FIPs, lectins, glycoproteins and non-glycosylated proteins and peptides), polysaccharide - protein complexes, lipid components (ergosterol) and terpenoids, alkaloids, small peptides and amino acids, nucleotides and nucleosides. Edible mushrooms having medicinal properties can be found in health tonics, tinctures, teas, soups and herbal formulas. Studies have demonstrated that the regular consumption of mushrooms or the consumption of isolated bioactive constituents present in mushrooms is beneficial to health. They are usually considered as functional foods or nutraceutical products (Lakhanpal *et al.*, 2005; Preeti *et al.*, 2012).

Macrocybe gigantea (Massee) Pegler & Lodge also known as *Tricholoma giganteum*, is a wild edible mushroom. It belongs to the family Tricholomataceae. It has high economic and medicinal values. Its fruiting bodies are rich in protein, polysaccharides, dietary fiber, mineral salts, vitamins and other healthful substances (Wang *et al.*, 2005). This new edible mushroom is pure white in colour resembling the morphology of *Calocybe indica,* was reported growing widely in summer in Indo-Gangetic plains of Howrah District, Hooghly in India

(Chakravarty and Sarkar 1982). It has been reported that *Macrocybe gigantea* has high levels of mineral elements such as Ca, Mg and Zn (Liu *et al.*, 2012). *In vitro* evaluation of antioxidant activities of *Macrocybe gigantea* showed significant inhibition of lipid peroxidation and super oxide radical scavenging activity (Banerjee *et al.*, 2007). A laccase with a novel N-terminal sequence isolated from fresh fruiting bodies of this mushroom inhibited HIV-1 reverse transcriptase with an IC_{50} of 2.21 M (Wang and Ng, 2004). Antifungal protein trichogin, isolated from *Macrocybe gigantea* exhibited antifungal activity against *Fusarium oxysporum*, *Mycosphaerella arachidicola* and *Physalospora piricola* (Guo *et al.*, 2005). *Macrocybe gigantea* has received considerable attention in recent years because of the nutritional and health protective value.

Considering these facts the present study was conducted with the objective of studying the medicinal and nutraceutical potential of *Macrocybe gigantea* with special emphasis on immunomodulation, hepatoprotection and diversity evaluation.

Review of Literature

2. REVIEW OF LITERATURE

2.1 GENERAL MORPHOLOGY OF *MACROCYBE GIGANTEA*

Macrocybe gigantea (Massee) Pegler & Lodge has a large pileus 30-35 cm diameter, conicoconvex then expanding; surface initially white, soon gray with a glaucous tint, paler towards the margin, glabrous and silky smooth but cracking on drying; margin slightly incurved, curfy, often cracking (Plate 1). Lamellae emarginate, sinuate, straw yellow, ventricose, densely crowded, with lamellulae of four lengths. Stipe 15-18 × 6 cm, cylindrical, often elongate, solid finally fistulose; surface concolorous with pileus, fibrillose-striate. Spores 5.7-7.5 × 4.0-5.3 (6.70 ± 0.90 × 4.60 ± 0.38) µm, Q = 1.46, ovoid to short ellipsoid, hyaline, inamyloid, thinwalled. Basidia 25-37 × 5-8 µm, narrowly clavate to subcylindrical, bearing four sterigmata, with basal clamp connection (Pegler *et al.*, 1998).

Tricholoma lobayense Heim, a related species of *Macrocybe gigantea* reported from West Bengal during summer season has a very large pileus 8-22 cm in diameter, upper surface convex at the beginning which gradually flatten with age, smooth, appressed scales being present at the centre, margin thin regular, gills decurrent, white, alternate, free towards the margin of the pileus, flesh white and fibrous, gill trama regular, consisting of parallel thin- walled hyphae, stipe 14-28 cm in length, unequal tapering towards the apex, smooth, fibrillose, solid sub-bulbous base, spore print milky white (Chakravarthy and Sarkar, 1982).

2.2 CULTURAL STUDIES OF *MACROCYBE GIGANTEA*

2.2.1 Growth of mushrooms under different solid and liquid media

Balakrishnan (1994) tested four different solid media *viz.* PDA, oats agar, carrot agar, and modified oats agar medium for the growth of different *Pleurotus* species. He found that the oats agar blended with 40 per cent coconut milk (modified oats agar) supported maximum mycelia growth for all the species of *Pleurotus* tested, followed by common oats agar medium.

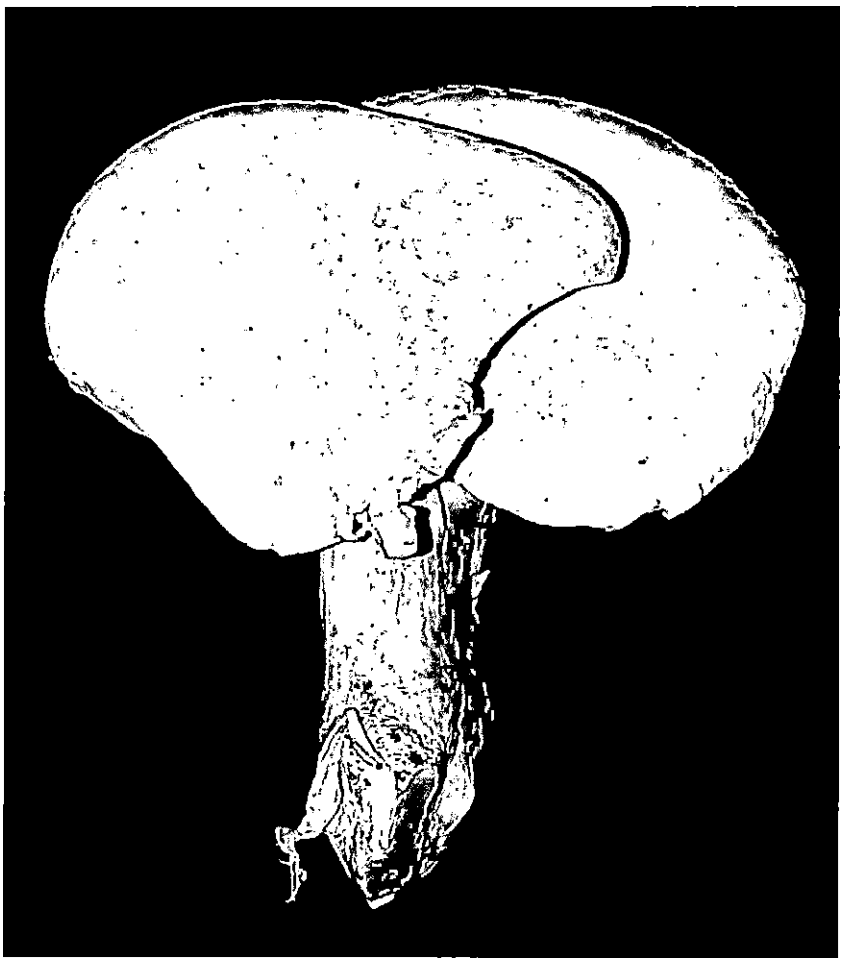


Plate 1. ***Macrocybe gigantea***

Cultural characterization of *Lentinus* in various solid and liquid media revealed woods extract agar as the best solid media followed by potato dextrose agar and glucose asparagines solution, the best liquid medium (Kaur and Lakhanpal, 1999). Out of the eleven culture media evaluated by Rafique *et al.* (1999) potato dextrose agar (PDA) was found to be the optimum medium for the growth of *Pleurotus*.

Nasrin *et al.* (2001) reported that the mycelia growth of *Pleurotus ostreatus, P. sajor-caju* and *Volvariella volvacea* were maximum in medium plates containing malt extract medium. Ling *et al.* (2005) reported potato dextrose agar as an excellent medium for the growth of *Auricularia* species. Kinjo and Miyagi (2006) reported that the mycelial growth of *Tricholoma giganteum* was superior on the Hennerberg medium of synthetic liquid medium, and the GCMY (glucose, casamino acid, malt extract and yeast extract) medium of natural liquid medium.

Studies conducted by Garasiya *et al.* (2007) revealed that *Auricularia polytricha* grows well in malt extract agar medium. Xiao *et al.* (2008) reported that PDA No. 4 medium composed of potato (20 %), glucose (2 %), yeast powder (0.5 %), KH_2PO_4 (0.3 %), Mg SO_4 (0.2 %) and agar (2 %) at pH 7 and an incubation temperature of 28-30° C was found to be best for the mycelia growth of *Tricholoma giganteum.*

Jayasinghe *et al.* (2008) used ten different culture media to screen the optimal mycelia growth of eight different strains of *Ganoderma lucidum.* The result showed that hamada, glucose peptone, yeast-malt extract, mushroom complete and lilly media were the most suitable. Chen and Yang (2009) reported that the growth period of *Tricholoma lobayense* mycelium in the mixed PDA medium was 30 days shorter than that in the conventional PDA medium.

Johnsy and Kaviyarasan (2013) investigated the effect of different semi-synthetic growth media *viz.* potato-dextrose agar, malt-yeast agar, cepeck-dox agar, modified Melin Norkrans medium, carrot potato agar, yeast-peptone broth, modified cepeck-dox agar, malt dextrose agar, czapek yeast agar medium and

glucose-malt extract salt medium on the vegetative growth of wood rot fungus *Neolentinus kauffmanii* (A.H. Smith). The best mycelial extensions were observed on malt-yeast agar and potato-dextrose agar with average growth per day of 20 and 10-15 per cent respectively.

Kumar and Anil (2013) studied the significant effect of different media on mycelial growth of *Ganoderma lucidum* strains. The highest mycelial growth was observed with malt extract agar medium, followed by sugarcane bagasse extract agar medium. Among the liquid media, malt extract broth proved to be best medium for mycelial growth, followed by sugarcane bagasse extract broth, potato dextrose broth, sawdust extract broth and maize straw extract broth. Atri and Lata (2013) studied the vegetative growth of *Lentinus cladopus* Lev. on twelve solid media and eleven liquid media. Malt extract agar medium (MEA) supported the best mycelial growth (4.88 cm) from amongst the solid media evaluated while the vegetative growth on malt broth (MB) was maximum (8.63 mg ml^{-1}) amongst the liquid media screened.

2.2.2 Effect of temperature on the growth of mushrooms

Shim *et al.* (2003) reported that the mycelial growth of *Paecilomyces fumosoroseus* had been expedited gradually in proportion to the rise of temperature and was the most suitable at 25° C. Sharma *et al.* (2004) observed that for the cultivation of *Agrocybe aegerita* maximum growth was recorded at 25° C and there was no growth at 35° C.

Veena and Pandey (2006) observed that for the cultivation of *Ganoderma lucidum* primordial initiation was fast at 30 ± 2° C and it is delayed by another week at 24 ± 2° C. Kinjo and Miyagi (2006) reported that *Tricholoma giganteum* prefer a temperature of 30° C for mycelia growth. Studies conducted by Garasiya *et al.* (2007) reported that *Auricularia polytricha* grows well in 25-30° C. Jayasinghe *et al.* (2008) observed that the minimum and maximum cardinal temperatures for the mycelial growth and density of *Ganoderma lucidum* were 15 and 35° C, respectively.

Nwokoye *et al.* (2010) reported that maximal mycelial growth of *Pleurotus ostreatus* can be achieved by cultivating the fungus at temperature of 28° C. Kibar and Peksen (2011) investigated the effects of different temperatures (15, 20, 25 and 30°C) on mycelial growth of *Tricholoma terreum*. The optimum temperature for the best mycelial growth was found to be 25° C.

After inoculation of *Pleurotus ostreatus* and *Pleurotus florida* in the potato dextrose broth separately in conical flasks and incubated at different temperatures (25-55° C) for seven days; maximum growth was observed at temperatures of 25° C for *Pleurotus ostreatus* and 30° C for *Pleurotus florida* (Neelam *et al.*, 2013).

2.2.3 Effect of pH on the growth of mushrooms

Kaur and Lakhanpal (1999) observed the mycelial growth of *Lentinus edodes* at different pH levels ranging from 3.5 to 8.5 and concluded that acidic pH of 4.5 supported maximum growth. The optimum pH for the growth of *Pleurotus* sp. was found to be 5.5 (Rafique *et al.*, 1999). Singh *et al.* (2000) studied the effect of pH on different edible mushrooms like *Lentinus edodes, Agaricus bisporus, Pleurotus ostreatus, Auricularia polytricha, Morchella esculanta* etc. and found suitable pH as 6.0 for the *Auricularia polytricha.*

Kinjo and Miyagi (2006) reported that the optimum pH value for mycelia growth of *Tricholoma giganteum* is 5.0. Jayasinghe *et al.* (2008) observed that *Ganoderma lucidum* has a broad pH range (5-9) for its mycelial growth and most favorable growth was found at pH 5.

Kim *et al.* (2010) observed the mycelia growth of *Tricholoma matsutake* at different acidity (uncontrolled pH and controlled pH of 6) and concluded that pH control does not affect mycelia growth. Nwokoye *et al.* (2010) reported that maximal mycelial growth of *Pleurotus ostreatus* can be achieved by cultivating the fungus at pH of 9.0.

The media with pH 6.5 and 7.0 were optimal for the growth of strain Mel-28 of *Tuber melanosporum* (Kamal, 2011). Kibar and Peksen (2011) reported that the

suitable pH range for mycelial growth of *Tricholoma terreum* was 4.5 to 6.0. The optimum pH for growth of *Pleurotus ostreatus* and *Pleurotus florida* was 5.5 (Neelam *et al.*, 2013).

2.3 CUTIVATION OF *MACROCYBE GIGANTEA* ON DIFFERENT SUBSTRATES

Krishnamoorthy and Muthusamy (1997) utilized several agro - wastes namely paddy straw, sorghum stalks, sugarcane bagasse, palmrosa grass, vetiver grass, groundnut haulms, soyabean hay and paddy straw compost for the cultivation of *Calocybe*. Higher yield and higher biological efficiency was observed in paddy straw followed by maize stalk, sorghum stalk and vetiver grass. Paddy straw compost was not suitable for the cultivation of *Calocybe indica.* Suharban *et al.* (1998) reported pseudostem of red banana as a better substrate for the oyster mushroom production when compared to pseudostem of nendran, red banana, palayamkodan, robusta and rasakadali.

Pandey and Tewari (2002) reported successful cultivation of *Tricholoma giganteum* with paddy straw giving biological efficiency of 92 per cent. Sherin *et al.* (2004) conducted experiment to study the suitable substrate for *Calocybe indica* cultivation among retted coir in combination with 75 per cent paddy straw, followed by 50 per cent combination of non retted coir pith and spent mushroom substrate. Milky mushroom can be grown on wide range of substrates such as paddy straw, wheat straw, stalks of maize, bajra, cotton etc. Straw is chopped into small pieces (2 - 4 cm) and soaked in fresh water for 8 to 16 h (Tewari, 2004).

Pramod *et al.* (2005) observed red banana pseudostem as the most efficient substrate for the cultivation of oyster mushroom. Thirumalvalavan *et al.* (2005) reported that sorghum and sorghum plus kudhiraivali were the most suitable substrate for *Pleurotus florida.* Veena and Pandey (2006) observed that the best locally available substrate for the cultivation of *Ganoderma lucidum* was 90 per cent sawdust and 10 per cent rice bran. Kinjo and Miyagi (2006) reported that saw dust media supplemented with wheat bran and hannoki (*Alnus japonica*) gave

highest yield of *Tricholoma giganteum.* Li *et al.* (2006) conducted a study on culturing of *Tricholoma giganteum* with rape seed coat and the result indicated that fresh yield of *Tricholoma* in rape seed coat mixed with cotton seed hull increased by 2.56 times.

Kumari and Achal (2008) studied the effect of five different substrates viz. paddy straw, wheat straw, mixture of paddy and wheat straw (in the ratio of 1 : 1), bamboo leaves and lawn grasses on the production of *Pleurotus ostreatus*. The highest yield of *P. ostreatus* was recorded on wheat straw (29.27 g fresh weight/kg substrate).

Mondal *et al.* (2010) evaluated the performance of *Pleurotus florida* in different substrate compositions *viz.* banana leaves, rice straw, banana leaves: rice straw (1: 3) banana leaves : rice straw (1:1), banana leaves : rice straw (3:1). Highest biological yield and economic yield (164.4 g and 151.1 g) was obtained from rice straw. Amin *et al.* (2010) reported that rice straw was the best substrate for commercial cultivation of *Calocybe indica.* Buah *et al.* (2010) reported that corn cob used as a substrate for *Pleurotus ostreatus* cultivation performs better than saw dust with biological efficiency of 91.21per cent whereas sawdust had 85.69 per cent biological efficiency.

Gurung *et al.* (2012) investigated the effects of various kinds of sawdust of *Alnus nepalensis*, *Shorea robusta* and *Dalbergia sisoo* and supplements of rice bran, wheat bran, corn flour and gram flour on *Ganoderma lucidum* cultivation. *Alnus nepalensis* sawdust supplemented with gram flour showed higher yield among all treatments. Samuel and Eugene (2012) evaluated the growth performance of *Pleurotus ostreatus* on some locally available substrate material compositions *viz* palm cones (100 %), corn cobs (100 %), corn cobs and palm cones (1:1), corn cobs and palm cones (1:3), corn cobs and palm cones (3:1)). The highest biological yield (146.1g) was obtained from corn cobs.

Sawdust of different woods which included kikar, mango, simbal and kail used as substrates were investigated for the cultivation of oyster mushroom.

Among all substrates, sawdust of kikar proved the best substrates for the effective cultivation of oyster mushroom with biological efficiency of 70.56 per cent (Khan *et al.*, 2012). Oseni *et al.* (2012) reported that 15 per cent wheat bran supplementation of fermented pine sawdust proved to be a viable option for *Pleurotus ostreatus* cultivation.

Ashraf *et al.* (2013) evaluated the growth of three *Pleurotus* species viz. *Pleurotus sajor - caju, Pleurotus ostreatus* and *Pleurotus djmor* on three different substrates i.e. cotton waste, paddy straw and wheat straw. Among all the treatments cotton waste was found most favourable for mushroom cultivation. Mixture of river sand and fermented sawdust substrate is recommended as the best substrate for the production of *Pleurotus tuberregium* mushrooms while mixture of corn waste and fermented sawdust substrate is recommended for sclerotia production (Olufokunbi and Chiejina, 2013).

2.3.1 Temperature preference for substrates

Bano and Rajarathnam (1982) observed maximum yield of oyster mushroom (*Pleurotus sajor - caju*) during rainy seasons, when the temperature was nearly 20-26° C and relative humidity 70-90 per cent. According to Das *et al.* (1987, 1991) variations in season seriously affected the number, weight and crop production period of mushroom. They reported that favorable temperature and moisture condition enhanced the production of fruiting bodies of mushroom. The highest number of effective fruiting body was produced in December to February for selected *Pleurotus* species. During this time the average temperature and relative humidity of culture house were respectively, 14 - 27° C and 70 - 80 per cent.

Upadhyay *et al.* (2002) reported the maximum yield of *Pleurotus membranaceus* in October to January and minimum yield in June to September. Tripathi (2005) suggested that *Pleurotus ostreatus* and *Pleurotus florida* gave very poor yields during summer season.

Uddin *et al.* (2011) investigated the production of four species of oyster mushroom: *Pleurotus ostreatus, P. florida, P. sajor - caju* and *P. high king* cultivated in every season (January to December) in Bangladesh. In all of the selected species, the minimum days required for primordial initiation, and the maximum number of fruiting bodies, biological yield and biological efficiency were found during December to February (14-27° C, 70-80 % relative humidity). The production was found minimum during the cultivated time August to October.

2.3.2 Effect of substrate sterilization on the growth of mushrooms

The method of disinfection affects the mycelium development, date of fructification and yield (Diana *et al.*, 2006).

The highest yields of *Pleurotus ostreatus* (868 and 772.5 g) were obtained from substrates treated with formalin 750 ppm + Bavistin 75 ppm and one hour laboratory autoclaving, respectively (Hussain *et al.*, 2002).

Different techniques of pasteurization including control, hot water treatment, steam pasteurization and chemical sterilization with formalin were applied to cotton waste to evaluate the optimum method for best mycelia growth of three species of oyster mushroom. Results showed that steam pasteurization gave maximum mycelia growth which completed in shortest period of time (Ali *et al.*, 2004).

Diana *et al.* (2006) reported that good alternatives to the disinfection by boiling the materials are scalding with hot water (100° C) and chemical disinfection with diluted fungicide solutions. These alternative methods assure high productions and are easier to realize and much cheaper.

Ali *et al.* (2007) evaluated the influence of different pasteurization methods namely, pasteurization with steam, hot-water treatment and chemical sterilization with formalin on cotton waste substrate on yield of three species *Pleurotus* i.e. *Pleurotus florida, Pleurotus pulmonarius* and *Pleurotus ostreatus.* Steam

pasteurization produced the best results as for the performances of individual species are concerned, *Pleurotus pulmonarius* completed the mycelial growth in the shortest time. *Pleurotus florida* behaved better in all the treatments than other species.

Khan *et al.* (2011) recommended lab autoclave as the best substrate sterlization method for the yield improvement of *Pleurotus ostreatus.* Caral *et al.* (2013) reported that autoclaving method of sterilization is more efficient than the chemical sterilization method in both the substrates (sawdust and straw) for the cultivation of *Pleurotus ostreatus.* Jaramillo and Alberto (2013) proved that immersion in hot water treatment of substrate reduced yield of *Pleurotus ostreatus* at least 20 per cent when compared with other straw treatments such as steam, chemical or untreated wheat straw.

2.4 MUSHROOMS AS IMMUNOMODULATORS

Polysaccharides or polysaccharide–protein complexes from mushrooms are able to stimulate the non-specific immune system and to exert antitumor activity through the stimulation of the host's defence mechanism. The drugs activate effector cells like macrophages, T lymphocytes and NK cells to secrete cytokines like TNF-a, IFN-g, IL-1b, etc., which are antiproliferative and induce apoptosis and differentiation in tumor cells.(Chihara *et al.,*1969; Mizuno, 1999; Wasser and Weis, 1999; Reshetnikov *et al.,* 2001).

The immunomodulating action of two mushroom antitumor polysaccharides, polysaccharide-protein complex (PSPC) and lentinan, was elucidated through analysing the expression profile of cytokines during a time course (0 h to 48 h) after their administration. Among the five cytokine genes, the induction of a marked increase in the mRNA levels of IL-la, IL-lp, TNF-a, IFN-I and M-CSF by PSPC and lentinan was observed in the peritoneal exudate cells and splenocytes (Liu *et al.,* 1999).

Bioactive polysaccharides or polysaccharide protein complexes from mushroom-type fungi have application as host defense potentiators or biological response modifiers for inhibiting tumour growth (Ooi and Liu 2000). The immunomodulatory effect of aqueous extracts of *Inonotus obliquus*, called as Chaga was tested on bone marrow cells from chemically immunosuppressed mice. The results suggested the great potential of the aqueous extract from *Inonotus obliquus* as immune enhancer during chemotherapy (Kim, 2005).

Wu *et al.* (2007) reported that increased intake of white button mushrooms may promote innate immunity against tumors and viruses through the enhancement of a key component, NK activity. This effect might be mediated through increased IFN - γ and TNF - α production.

Israilides *et al.* (2008) demonstrated the cytotoxic and cell growth inhibitory (cytostatic) effect of aqueous extracts of *Lentinula edodes* on MCF-7 human breast adenocarcinoma cell line using an MTT cytotoxicity assay and immunostimulatory properties in terms of mitogenic and co-mitogenic activity *in vitro*. Jung *et al.* (2008) studied the immunomodulating activities of water-soluble exopolysaccharides (LL-EX) obtained from submerged mycelial culture of *Lentinus lepideus* and their effectiveness was compared with lipopolysaccharide (LPS).

Han *et al.* (2009) evaluated inhibitory effects against lung metastasis and promotion of splenocytes by water extracts from various mushrooms including *Armillaria mellea, Grifola frondosa, Ganoderma frondosa, Codyceps militaris, Hericium erinaceus, Coriolus versicolor, Agaricus blazei* with *Lycium chinense Miller.* Roy *et al.* (2009) studied an immune-enhancing water soluble glucan isolated from hot water extract of an edible mushroom, *Pleurotus florida*, cultivar Assam Florida and found that the glucan stimulates macrophages, splenocytes and thymocytes.

Refaie *et al.* (2009) characterized the hot water protein-bound polysaccharides (polysaccharopeptides) from the mycelial culture broth of

Pleurotus ostreatus, and studied their toxicity in mice. The immunomodulatory effect of the polysaccharopeptides was also assessed at various doses by monitoring the serum IFN-α level in mice. Yoo *et al.* (2009) evaluate the inhibitory effects against lung metastasis and promotion of splenocytes by water-soluble components from five mushrooms extracts (WEFM): *Garnoderma frondosa, Corious versicolor, Codyceps militaris, Hericium erinaceus* and *Lentinula edodes*. Oral administration of WEFM to BALB/c mice resulted in a significant inhibition of lung metastasis after intravenous injection of colon 26-L5 cells in a dose dependent manner. There was also a significant increase in T cell and B cell mitogenic stimuli and production of IFN - γ by splenocytes stimulated with concanavalin A compared to untreated controls.

A heteroglycan was isolated from an aqueous extract of an edible mushroom, *L. squarrosulus* and analyzed for its structural characterization with immune-enhancing activity by Bhunia *et al.* (2010) which showed macrophage as well as splenocyte and thymocyte activation.

Dey *et al.* (2010) analyzed an immunoenhancing water soluble polysaccharide of an edible mushroom, *Pleurotus florida* blue variant and they found that the molecule activated macrophages, splenocytes and thymocytes. Shukla *et al.* (2010) evaluated the immunomodulatory effects of roots of *Gmelina arborea* Linn. on humoral and cell-mediated immune response using animal models like cyclophosphamide-induced myelosuppression, delayed-type hypersensitivity (DTH) response and humoral antibody (HA) titre.

Singh *et al.* (2010) investigated the immunomodulatory property of ethanolic extract of stem bark of *Balanites roxburghii* Planch. The assessment of immunomodulaotory activity were carried out by carbon clearance test for phagocytic index, haemagglutination antibody titre for humoral immune response and delayed type hypersensitivity for cell mediated immune response.

Morris *et al.* (2011) examined the immunomodulating effects of *Pleurotus* species fruiting bodies powder on cyclophosphamide (CY) treated mice. An *in*

vitro lymphoproliferative-stimulating response evaluated in mice spleen cells was also demonstrated with aqueous and ethanolic extracts obtained from *Pleurotus* powder. A new immunomodulatory protein from *Trametes versicolor*, named TVC was purified by ammonium sulfate precipitation, ion-exchange chromatography and gel filtration chromatography. TVC markedly increases the proliferation of human peripheral blood lymphocytes in a dose-dependent manner and enhances the production of both nitric oxide and tumor necrosis factor-alpha by lipopolysaccharide-induced murine macrophages (Feng *et al.*, 2011).

A structural and biological study of a heteropolysaccharide from aqueous extract of an edible mushroom, *Pleurotus ostreatus* has been conducted by Maity *et al.* (2011) and revealed that the heteroglycan stimulates macrophages, splenocytes and thymocytes.

The toxicological and immunodulatory properties of some selected *Ganoderma* species was investigated using animal bioassay. The result of the haematological analysis showed that there was no significant difference in the total white blood cell (WBC) count value between the control animal and those fed with *Ganoderma* extract, while there was increase in the packed cell volume (PCV) of the rat fed with the extract. Histopathological analysis revealed haemorrhage, reduction and detachment of the glomerulus of the kidney while the intestinal section showed degeneration and erosion of the crypts of villi and cellular infiltration. There was necrosis in the heart, expansion and waxy contraction. Thus *Ganoderma* extract contain immunodulatory constituents that are toxic (Obameso *et al.*, 2011).

Mushroom sclerotial polysaccharides extracted from *Pleurotus tuberregium* and *Polyporus rhinocerus* activated the innate immune cells and T-helper cells in normal and athymic BALB/c mice (Wong *et al.*, 2011). *Agaricus bisporus* polysaccharides, ABP-1 and ABP-2 had the ability to inhibit the growth of human breast cancer MCF-7 cells but had little effect on the growth of human colon, prostate, gastric cancer and murine Sarcoma 180 cells as assessed by a tetrazolium

dye [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]-based assay. However, when murine Sarcoma 180 cells exposed to ABP-1 or ABP-2 were implanted subcutaneously into mice, a reduction in tumor growth was observed compared with that observed in control mice (Jeong *et al.*, 2012).

Kyakulaga *et al.* (2013) determined the immunomodulatory effect of aqueous extracts of *Auricularia* sp. and *Pleurotus* sp. mushrooms using an immunosuppression animal model. The methanolic and hexane extracts of *Musa acuminata* peel (plantain peel) were pharmacologically validated for immunomodulatory activity at dose (100, 300, 500 mg kg^{-1} body weight p.o.) by carbon clearance method, neutrophil adhesion and footpad swelling method on Wistar albino rats. Results suggested that both hexane and methanolic extracts of *Musa acuminata* was found to be potent immuo-stimulant in a dose dependent manner when compared with control group (Singhal and Ratra, 2013).

2.5 MUSHROOMS AS HEPATOPROTECTIVE AGENTS

Hepatoprotective effect was found in the extract of the Maitake mushroom (*Grifola frondosa*) when given to rats (300 mg kg^{-1}) in a hepatitis model (paracetamol – induced hepatotoxicity) (Lee *et al.*, 1992; Ooi *et al.*, 1993). In a clinical report from MARA Institute of technology (Malaysia), a lyophilized extract of Reishi mushroom was beneficial in alleviating the symptoms of patients suffering from hepatitis B by significantly reducing the SGOT and SGPT levels and leading to seroconversion after three months of administration (Soo, 1994).

Treatment with the water extract of *Ganoderma lucidum, Ganoderma formosanum* and *Ganoderma neo-japonicum* caused a marked decrease in the CCl_4-induced toxicity in rat liver, made evident by their effect on the levels of glutamic oxaloacetic transaminase (GOT) and lactic dehydrogenase (LDH) in the serum. The scavenging potency of the water extracts of the crude drugs were evaluated in terms of their ability to reduce the peaks of spin adducts using electron spin resonance (ESR) spin-trapping techniques (Lin *et al.*, 1995).

The ethyl acetate extract of *Phellinus rimosus* showed potent antihepatotoxic activity against carbontetrachloride-induced acute toxicity in rat liver. The amelioration of liver toxicity by the ethyl acetate extract was evident from its significant effect on the levels of serum glutamate pyruvate transaminase (SGPT), serum glutamate oxaloacetate transaminase (SGOT) and serum alkaline phosphatase (ALP) (Ajith and Janardhanan, 2002).

Hsiao *et al.* (2003) evaluated the ability of *Antrodia camphorata* extracts to protect against oxidative stress *in vitro* and against carbon tetrachloride (CCl_4)-induced hepatic injury *in vivo*. Mroueh *et al.* (2004) evaluated the hepatoprotective activity of methanol extract of the leaves of *Centaurium erythraea* L. (Gentianaceae) against acetaminophen-induced liver toxicity in rats. An oral dose of 300 mg kg^{-1} day^{-1} for six days or a single dose of 900 mg kg^{-1} for 1 day exhibited a significant protective effect by lowering serum glutamate oxaloacetate transaminase (SGOT), glutamate pyruvate transaminase (SGPT) and lactate dehydrogenase (LDH). The activity of the extract was supported by histopathological examination of liver sections.

Wu *et al.* (2004) evaluated the hepatoprotective and anti-fibrotic actions of crude extracts of *Ganoderma tsugae* (GTE) on chronic liver injury induced by carbon tetrachloride (CCl_4) in rats. GTE showed reducing actions on the elevated levels of glutamate-oxalate-transaminase (GOT) and glutamate-pyruvate-transaminase (GPT) caused by CCl_4. The CCl_4-induced liver fibrosis may prolong the prothrombine time and increase albumin/globulin (A/G) ratio. GTE significantly decreased the prothrombine time and A/G ratio. Liver fibrosis induced by CCl_4 markedly increased the weight of the spleen, hepatic water and hydroxyproline contents in rats, while GTE decreased the rat's spleen weights, hepatic water and hydroxyproline contents.

Watanabe *et al.* (2006) examined the protective effect of *Lentinus edodes* mycelium (L.E.M.) in GalN-induced liver injury model in rats. L.E.M. suppressed

the leakage of AST and ALT into the serum, indicating that hepatocyte death was protected by L.E.M. treatment *in vivo*.

Chang *et al.* (2007) investigated the hepatoprotective and antioxidant capacities of ethanol extract of *Phellinus merrillii* (PM) on carbon tetrachloride-induced hepatotoxicity. Treatment with PM (0.5, 1 and 2 g kg^{-1}) significantly prevented the increased serum alanine aminotransferase (SGOT) and serum aspartate aminotransferase (SGPT) in a dose-dependent manner. The incidences of ballooning degeneration, necrosis and portal triaditis were lowered in the group pretreated with PM. The hepatoprotective effects of the mycelia of *Antrodia camphorata* and *Armillariella tabescens* were evaluated *in vivo* using acute ethanol-intoxicated rats as experimental model. Administration of *Antrodia camphorata* and *Armillariella tabescens* markedly prevented ethanol-induced elevation of levels of serum AST, ALT, ALP, and bilirubin comparable with standard drug silymarin (Lu *et al.*, 2007).

El *et al.* (2009) investigated the protective effects of an aqueous extract of *Pleurotus cornucopiae* (PC) on carbon tetrachloride (CCl_4) - induced hepatotoxicity and the possible mechanism involved in this protection including cytochrome P_{450} (CYP) 2E1. Pretreatment of Wistar rats with PC mushroom extract significantly prevented the increased serum enzyme activities of alanine and aspartate aminotransferases in a dose-dependent manner, and suppressed the expression of CYP2E1.

Nada *et al.* (2010) investigated the effect of mushroom insoluble non-starch polysaccharides (MINSP) on carbon tetrachloride induced hepatic damage in rats. Oluba *et al.* (2010) investigated the hepatoprotective effect and antioxidant property of aqueous extract from *Ganoderma lucidum* (GLE) on carbon tetrachloride induced hepatotoxicity in rats. *G. lucidum* extract (200 mg kg^{-1}) prevented liver damage and suppressed the leakage of ALT, AST and γ-GT through cellular membrane.

Sasidharan *et al.* (2010) suggested that *Lentinula edodes* extract could protect liver cells from paracetamol-induced liver damage by its antioxidative effect on hepatocytes, hence diminishing or eliminating the harmful effects of toxic metabolites of paracetamol. Sumy *et al.* (2010) observed the hepatoprotective effect of oyster mushroom (*Pleurotus florida*) against paracetamol induced liver damage in Wistar albino rats. This study reveals that oyster mushroom (*Pleurotus florida*) which is excellently edible and nutritious, may have some hepatoprotective role.

Chatterjee *et al.* (2011) investigated the hepatoprotective activity of ethanolic extract of a wild edible mushroom (*Calocybe indica*) against carbon tetrachloride (CCl_4) induced hepatic damage in mice. Administration of ethanolic extract of *Astraeus hygrometricus* (150 mg kg^{-1} body weight day^{-1}) orally protected the CCl_4 mediated elevation of serum transaminase such as glutamate pyruvate transaminase (GPT) and glutamate oxaloacetate tansaminase (GOT) and of serum alkaline phosphatase (ALP), total bilirubin and direct bilirubin. The hepatic antioxidant status such as superoxide dismutase and catalase activities were reduced in the CCl_4 alone treated animals. Administration of extract to CCl_4 challenge restored the hepatic antioxidant status. The findings thus suggested ethanolic extract of *A. hygrometricus* protects CCl_4 induced chronic hepatotoxicity in mice by restoring the liver antioxidant status (Biswas *et al.*, 2011).

The hepatoprotective potential of antrosterol (ergostatrien-3b-ol, ST1) from *Antrodia camphorata* (AC) against carbon tetrachloride (CCl_4)-induced liver damage was evaluated in preventive models in mice. Results showed that ST1 can inhibit lipid peroxidation, enhance the activities of antioxidant enzymes, decreases the TNF-α level, nitric oxide production and inducible nitric oxide synthase (iNOS), and cyclooxygenase- 2 (COX-2) expressions (Huang *et al.*, 2012). Wong *et al.* (2012) investigated the hepatoprotective effects of *P. giganteus* against thioacetamide (TAA) induced liver injury in *Sprague-Dawley* rats. Rats administered with *P. giganteus* showed lower liver body weight ratio, restored

levels of serum liver biomarkers and oxidative stress parameters comparable to treatment with the standard drug silymarin.

Aqueous extract *Volvariella volvacea* (500, 1000 mg kg^{-1} p.o) showed significant hepatoprotective activity against carbontetrachloride induced hepatotoxicity in rats by normalizing the levels of serum AST, ALT, ALP, LDH and total bilirubin. The extract improved the activity of catalase (CAT), superoxide dismutase (SOD), and hepatic glutathione (GSH) content and depleted the lipid peroxidation levels in a dose dependent manner (Kalava and Menon 2012).

The hepatoprotective activity of cultured mycelium of morel mushroom *Morchella esculenta* against CCl_4 and ethanol induced chronic hepatotoxicity was investigated (Nitha *et al.*, 2013). Wu *et al.* (2013) evaluated the hepatoprotective effects of *Ganoderma lucidum* aqueous extracts (GLEs) on liver injury induced by α-amanitin (α-AMA) in mice and analyzed the possible hepatoprotective mechanisms related to radical scavenging activity. The results demonstrated that GLE induces hepatoprotective effects on acute liver injury induced by α-AMA. These protective effects may be related in part to the antioxidant properties of GLE.

Ravikumar and Kalidoss (2014) evaluated the antioxidant and hepatoprotective activity of the ethanolic extract of the mycelium of *Morchella esculenta* against carbon tetrachloride induced acute hepatotoxicity in rats.

2.6 NUTRITIVE VALUE

Many genera of mushrooms are edible and are rich in essential nutrients such as carbohydrates, proteins, vitamins, mineral, fat, fibers and various amino acids (Okwulehie *et al.*, 2004). Barros *et al.* (2008) reported that the wild mushrooms were richer sources of protein and had a lower amount of fat than commercial mushrooms. Wild mushroom protein contains considerable amounts

of non-essential amino acids such as alanine, arginine, glycine, glutamic acid, aspartic acid, proline and serine.

Ell-kattan and Salma (1999) studied the nutrient composition of *Pleurotus ostreatus* and *P. florida* on rice straw and supplemented with legume waste. They observed that the dry matter and protein content of *P. ostreatus* and *P. florida* increased with a higher percentage of legume waste. *P. ostreatus* was more responsive to supplementation than *P. florida*. The highest beneficial effect on yield was achieved when rice straw was enriched with 50 per cent legume waste.

Nutritive content of *Agaricus* consisted of 90.10 per cent moisture, 3.75 per cent protein, 0.53 per cent crude fibre and 4.59 per cent carbohydrate (Singh *et al.*, 1999). Patrabansh and Madan (1999) studied the mineral content of *Pleurotus sajor - caju* grown on different substrates. There was an increase in mineral content when mushrooms were grown on substrates rich in mineral content. Among the eight minerals determined the potassium content was the highest followed by phosphorous, magnesium and sodium. The mineral content of the fruiting bodies per 100 g ranged as follows; Ca (25.1 mg to 35.3 mg), P (448 mg to 602 mg), K (2146 mg to 2350 mg), Na (139 mg to 229 mg), Mg (153 mg to 224 mg), Fe (9.74 mg to 20.75 mg), Mn (2.5 mg to 4.0 mg) and Zn (2.2 mg to 3.1 mg).

Mushrooms contain essential amino acids such as Leucine, Isoleucine, Valine, Tryptophan, Lysine, Threonine, Phenylalanine, Methionine and Histidine. They can thus supplement diets that lack protein (Jiskani, 2001). Manzi *et al.* (2001) reported that the total carbohydrates of commonly consumed mushrooms in Italy (*Agaricus bisporus, Pleurotus ostreatus* and *Boletus* species) varied between 60 and 70 per cent of dry weight.

Anandh (2001) reported nutritive value of *Calocybe indica* with 88.37 per cent moisture, 11.63 per cent dry matter, 26.5 per cent protein, 36.5 per cent fibre and 8.8 per cent carbohydrate. He also stated the proximate constituent composition of *Tricholoma lobayense* with 85.2 per cent moisture, 14.8 per cent dry matter, 33.2 per cent protein, 23.74 per cent fibre and 11.38 per cent

carbohydrate. Keun Yang *et al.*, (2002) reported that *Auricularia polytricha* contained 77.5 per cent carbohydrate and 22.5 per cent protein.

Rathore and Thakore (2004) studied the effect of different substrates on nutrient composition of *Pleurotus florida* and found that the sporophores contain protein 35 per cent, carbohydrates 44.23 per cent, fat 2.20 per cent, fibre 9.85 per cent, ash 8.72 per cent and moisture 89 per cent. Ram (2004) reported that the dried milky mushroom contain protein 32.3 per cent, fat 4.5 per cent fibre 41 per cent and carbohydrate 64.26 per cent, minerals, ash and had a good delicious flavour. Agrahar-murugkar and Subbulakshmi (2005) analyzed the essential amino acid of seven wild edible mushrooms from the Khasi hills of Meghalaya and found average ranges between 16.3 per cent (lysine) and 45.8 per cent (methionine).The vitamin C content (mg g^{-1}) these mushrooms were found to be 14.9, 41.8, 41.9, 28.0, 19.6, 25.8, 18.1 vitamin C present in *C. gigantea, C. cinerea, C. cibarius, R. brevispora, R. integra, G. floccosus* and *L. quieticolor respectively.*

Goyal *et al.* (2006) reported that the fat and ash content were significantly higher in *Agaricus bisporus*, whereas, crude fibre and crude protein contents were significantly higher in *Pleurotus sajor - caju.* No significant differences were found in the energy, carbohydrates and non-protein nitrogen contents of both varieties of mushrooms.

Liu *et al.* (2007) studied the nutrient content of *Tricholoma giganteum* and *Pleurotus eryngii* cultivated with cotton seed hull compost and found that contents of protein, fat, total sugar and crude fibre in *T. giganteum* and *P. eryngii* were 35.28, 2.91, 53.74, 8.76 per cent and 15.40, 0.55, 52.10, 5.40 per cent respectively. Prakasam *et al.*, (2011) reported that *Tricholoma giganteum* contain 86.20 per cent moisture, 32.90 per cent crude protein, 11.80 per cent carbohydrate, 0.91 per cent crude fat, 20.71 per cent crude fibre, 8.32 per cent ash, 5.60 per cent iron, 1.18 per cent manganese, 1.38 per cent zinc and 1.10 per cent copper.

Pandey and Budhathoki (2007) collected thirty three species of wild mushroom from different altitude (200 m – 4200 m), phytogeographical habitat of central Nepal and two species *Agaricus bisporous* and *Pleurotus sajor - caju* cultivated sample from Balambu farm. The protein of these 35 species was determined by Bradford's method. The highest amount of protein 1.576 mg ml^{-1} in *Cantharellus subscibarius* and least 0.131 mg ml^{-1} in *Cordycep sinensis* were found.

Alam *et al.*, (2008) determined the nutritional values of dietary mushrooms- *Pleurotus ostreatus, Pleurotus sajor - caju, Pleurotus florida* and *Calocybe indica.* The mushrooms were rich in proteins (20 - 25 %) and fibers (13 - 24 % in dry samples) and contained a lower amount of lipid (4 to 5 %). The carbohydrate contents ranged from 37 to 48 per cent (on the basis of dry weight). These were also rich in mineral contents (total ash content is 8 - 13 %). The pileus and gills were protein and lipid rich and stripe was carbohydrate and fiber-rich. The moisture content of mushrooms ranged from 86 to 87.5 per cent.

Kavishree *et al.* (2008) have analyzed twenty three species of naturally grown and collected mushroom fruiting bodies from different geographic locations of India for their total fat and fatty acid contents and mushroom species were found to contain 0.6 - 4.7 per cent total fat. These mushroom species were also high in unsaturated fatty acids (52 - 87 %), compared to saturated fatty acids. Khan *et al.* (2008) determined the nutritional composition of six species of oyster mushrooms such as *Pleurotus sajor - caju, P. ostreatus, P. florida, P. cystidiosus, P. highking 51* and *P. geestaranus.* The highest protein (24.5 g/100 g of dry weight), lipid (5.5 g/100 g dry sample), carbohydrate (45.9 g/100 g dry sample), fiber (30.3 g/100 g dry sample), ash (8.3 g/100 g dry sample) contents were found in *P. sajor-caju, P. cystidiosus, P. geestaranus, P. highking51* and *P. florida* respectively.

Jagadeesh *et al.* (2010) reported that 34.75 and 38.90 per cent of carbohydrate content present in mycelia and fruit body of *V. bombycina.* Pushpa

and Purushothama (2010) evaluated the composition of wild, commercial and medicinal mushrooms (*Calocybe indica, Agaricus bisporous, Russula delica, Pluerotus florida, Lyophyllum decastes*) collected from Bangalore, India. The total protein, total carbohydrate, total lipid, crude fiber and ash content of each mushroom were studied on dry weight basis. They range from 18.32 - 41.06, 28.38 - 49.20, 1.54 - 4.96, 13.20 - 29.02 and 7.01 - 17.92 per cent respectively.

Ayaz *et al.* (2011) studied the nutritional content of eight edible mushrooms (*Boletopsis leucomelaena, Hydnum repandum, Laetiporus sulphureus, Boletus edulis, Armillaria mellea, Macrolepiota procera* var. *procera, Lactarius piperatus* and *L. quietus*) collected from East Black Sea region in Turkey. The average ash, moisture, carbohydrate, fat, nitrogen and protein contents of mushrooms were 6.72, 11.70, 56.86, 3.64, 3.07 and 19.19 g/100 g dry weight, respectively. An average of 8.57, 0.99 and 15.20 g kg^{-1} dry weight of malic, ascorbic and citric acids were determined. As for the energy value, it was averaged 377 kcal/100 g dry weight.

Brauer *et al.* (2011) reported the starch concentrations in shiitake mushrooms are influenced by the spawn source, the characteristics of the environment and the interaction between fungal phenotypes and environment. Johnsy *et al.* (2011) analyzed the nutritional value of 10 edible mushroom species such as *Pleurotus roseus, Pleurotus ostreatus, Pleurotus sajor - caju, Termitomyces microcarpus, Termitomyces heimii, Auricularia auricular, Volvariella volvacea, Lentinus squarrosulus, Lentinus tuberegium, Grifola frondosa*, which forms a part of the food culture of the Kani tribal community settled in the forests of Kanyakumari District in Tamil Nadu.

Manjunathan and Kaviyarasan (2011) reported a comparative evaluation of the nutritional values of *Lentinus tuberregium* cultivated on paddy straw and isolated from the wild. The cultivated mushroom accumulated higher concentrations of carbohydrates (58.05 %), protein (25.0 %), moisture (12.51 %), total ash (5.14 %), crude fibre (14.69 %), fat (1.54 %), potassium (90.80 mg g^{-1}),

calcium (87 mg g^{-1}), magnesium (30.40 mg g^{-1}), sodium (37.30 mg g^{-1}), iron (6.50 mg g^{-1}), copper (1.0 mg g^{-1}), zinc (4.90 mg g^{-1}), manganese (1.70 mg g^{-1}), energy (338 kcal). Where as the wild mushroom proximate value such as carbohydrates (55.80 %), protein (25.0 %), moisture (9.40 %), total ash (4.70 %), crude fibre (3.60 %), fat (1.60 %), potassium (7.53 mg g^{-1}), calcium (2.66 mg g^{-1}), magnesium (2.45 mg g^{-1}), sodium (1.20 mg g^{-1}), iron (0.53 mg g^{-1}), copper (0.11 mg g^{-1}), zinc (0.41 mg g^{-1}), manganese (0.08 mg g^{-1}), energy (331 kcal).

Manjunathan *et al.* (2011) reported the proximate compostion of four wild mushrooms from Tamil Nadu, India in which *A. polytricha* had the highest concentration of protein (37 %) and *Clitocybe* sp. had the least (24.8 %). Atri *et al.* (2012) studied the vitamin content (vitamin A, vitamin B1, B2 and vitamin C) in seven wild edible termitophilous and lepiotoid mushrooms *viz. Termitomyces reticulatus, T. heimii, T. mammiformis, T. radicatus, Macrolepiota dolichaula, M. rhacodes* and *Lepiota humei* collected from different localities of North- West India. In the samples evaluated, vitamin A ranged from 0.01- 0.16 mg/100 g, vitamin B1 0.021- 0.80 mg/100 g, vitamin B2 0.13- 0.23 mg/100 g and vitamin C ranged from 0.18- 1.45 mg/100 g.

Giri *et al.* (2013) evaluated the different nutritional parameters, i.e. protein, carbohydrate, fat and crude fiber contents of three wild edible mushroom namely *Lentinus squarrosulus, Tricholoma giganteum* and *Russula albonigra.* Results showed that these mushrooms had significant amount of carbohydrate, protein, free amino acids and crude fiber where as low amount of fat.

Singdevsachan *et al.* (2013) reported the nutrient values of two wild mushrooms (*Lentinus sajor - caju* and *Lentinus torulosus*) from Similipal Biosphere Reserve, Odisha, India where highest protein content (28.36 %) was found in *L. sajor - caju* and lowest (27.31 %) in *Lentinus torulosus.*

2.7 DIVERSITY ANALYSIS USING INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCE

The internal transcribed spacer (ITS) region of rDNA constitutes one of the most widely applied molecular markers in phylogenetic studies and species differentiation. The ITS region is part of the rDNA cistron, which consists of 18S, ITS1, 5.8S, ITS2, and 26S, and is present in several hundred copies in most eukaryotes (Hyosig and Renner, 2005). In the transcribed region, ITS are found on either side of 5.8S rRNA gene and are described as ITS1 and ITS2. In eukaryotes, each repeat is composed of a transcription unit that codes for three RNAs: a small subunit RNA (SSU), a large subunit RNA (LSU) and 5.8S RNA. The three genes are separated by two transcribed spacers, the ITS 1 and ITS 2. Each repeat is separated by a non-transcribed spacer, also called an intergenic spacer (IGS). The length and sequences of ITS regions of rDNA repeats are believed to be fast evolving and therefore may vary.

Moncalvo *et al.* (1995) used sequencing of ITS region and domain D2 of 25S rDNA of 14 species of *Ganoderma* and identified four phylogenetically related clusters of strains of laccate *Ganoderma* species, two of which represented members of *G. lucidum* complex. Sequences of the internal transcribed spacer region 1 (ITS1) of the ribosomal DNA were used to determine the phylogenetic relationships of species of *Trichoderma* sect. *Pachybasium* (Kindermann *et al.*, 1998).

Gottlieb (2000) adopted rDNA analysis (ITS I and II of 5.8S rDNA) to identify South American isolates of *Ganoderma* and *Elfvingia* and found that agreement between molecular and morphological data was clear at the subgeneric level, however this relationship was difficult to visualize at the species level. James *et al.* (2001) describes rDNA spacer variation in *Schizophyllum commune* throughout the species' entire geographic range. Phylogenetic relationships among wild edible *Russula* in north eastern Thailand were demonstrated on the

basis of the sequence polymorphisms in the internal transcribed spacer (ITS) region of the nuclear ribosomal genes (rDNA) (Manassila *et al.*, 2005).

Wang and Yao (2005) tried ITS heterogeneity at the intra strainal level in *Ganoderma.*Tao *et al.* (2007) investigated that the genetic diversity of *Tricholoma matsutake*, studied ITS and IGS1 sequences and PCR polymorphism of a retrotransposon in 56 fruit bodies collected from 13 counties of 9 regions in Yunnan Province. Rai *et al.*, (2007) clearly distinguished *Tyromyces*, *Ganoderma* and *Trametes* from each other through direct sequencing using ITS-I and ITS-4 primers.

Imtiaj *et al.* (2010) used DNA sequences of the ITS (Internal Transcribed Spacer) region to analyze the genetic diversity of *Pleurotus* strains. Ro *et al.* (2007) investigated the genetic relationship of the 22 most cultivated strains of *P. eryngii* in the Korean mushroom industry using both ITS1–5.8S rDNA–ITS2 sequence analysis and RAPD analysis. Park *et al.* (2012) analysed the ITS rDNA region and partial beta tubulin gene sequences of *Ganoderma* species in order to clarify their genetic relationships and found that Korean *G. lucidum* strains were different from those of China, Taiwan and Canada. Bazzicalupo *et al.* (2013) investigated the suitability of ITS1 vs. ITS2 sequence clusters in characterizing hyperdiverse fungal communities and suggested that ITS2 may be more variable and recovers more of the molecular diversity.

Materials and Methods

3. MATERIALS AND METHODS

The present study entitled, 'Medicinal and nutraceutical potential of giant mushroom (*Macrocybe gigantea* (Massee) Pegler & Lodge)' was carried out at the Ethnomedicine and Ethnopharmacology Division and Microbiology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute, Palode, during 2013 – 2014. The details of materials and methods are presented in this chapter.

3.1 SAMPLE COLLECTION

Macrocybe gigantea (Massee) Pegler & Lodge cultures were obtained from Directorate of Mushroom Research (ICAR), Solan, Himachal Pradesh.

3.2 CULTURAL STUDIES

3.2.1 Growth under different solid media

Potato dextrose agar (PDA) and sabouraud dextrose agar (SDA) were used to find out the best medium for the growth of *Macrocybe gigantea.* The composition of the media is given in the Appendix I and II. The media were prepared and sterilized by autoclaving at 1.06 kg cm^{-2} pressure for 15-20 min. After cooling it was poured to sterile petridishes of 9 cm diameter and allowed to solidify. Culture disc of 5 mm diameter cut from the actively growing culture of fungus was inoculated into petridish and was incubated at room temperature (28 ± 2° C). Each test was replicated three times and colony diameter was measured after ten days.

3.2.2 Growth under different liquid media

Potato dextrose broth (PDB) and sabouraud dextrose broth (SDB) were used for the study. The composition of the media is given in the Appendix I and II. One hundred ml of each liquid medium was prepared in 250 ml conical flask and was autoclaved at 1.06 kg cm^{-2} pressure for 15 – 20 min. The media after cooling were then inoculated with 5 mm culture disc of actively growing culture under aseptic condition. The flasks were kept at room temperature for ten days. After ten days,

the mycelia were filtered through a Whatman No. 1 filter paper and dried in an oven at 60° C. The dry weights were recorded.

3.2.3 Effect of different temperature on the growth of *Macrocybe gigantea*

The temperature requirement for the optimal growth of *Macrocybe gigantea* was investigated by inoculating a 5 mm culture disc of actively growing culture of this fungus was placed at the centre of petridishes containing solidified PDA media. The cultures were then incubated at 25, 30 and 35° C. Three replications were maintained for each treatment. The mycelial diameter was measured after ten days.

3.2.4 Effect of different pH on the growth of *Macrocybe gigantea*

To find out optimum pH for the favorable mycelial growth of *Macrocybe gigantea* PDA media was prepared and pH was adjusted to 5, 7 and 9 by using 0.1 N sodiumhydroxide or 0.1 N hydrochloric acid. The prepared media were sterilized 1.06 kg cm^{-2} pressure for 15-20 min. Twenty ml of medium was then poured to steriIe petridishes and allowed to solidify. Culture discs of 5 mm cut from actively growing culture of this fungus was inoculated into petridish and incubated at room temperature (28 ± 2° C). Three replications were maintained for each treatment. The mycelial diameter was measured after ten days.

3.3 CULTIVATION OF *MACROCYBE GIGANTEA* ON DIFFERENT SUBSTRATES

The *Macrocybe gigantea* culture obtained from DMR which was maintained on PDA slants in the laboratory. This culture was inoculated in grain based spawn material for further study.

3.3.1 Preparation of grain spawn

Spawn was prepared as per the method described by Sinden (1934). The paddy grains were cooked for one hour in boiling water. The excess water was drained off and grains were spread on a clear area. Polypropylene bags and

glucose drip bottles were filled with cooked grains after mixing with calcium carbonate at the rate of 50-60 g/kg of seeds. The filled bags and bottles were sterilized at 1.06 kg cm^{-2} for two hours in an autoclave. The mycelial bits from seven day old actively growing pure culture of *Macrocybe gigantea* was inoculated aseptically and incubated at room temperature (28 ± 2° C). The spawn thus obtained as the mother spawn is used for further spawn production and also to raise beds.

3.3.2 Substrate sterilization

The different substrates used for the cultivation of mushroom included paddy straw, bamboo leaves and neopeat. The substrates were soaked in a solution containing formalin and Bavistine (for 100 l water, 60 ml formalin and 5 g Bavistine) for 18 h. After draining excess water and air drying, they were used for mushroom bed preparation.

The polybag method as described by Baskaran *et al.* (1978) was adopted for bed preparation. The polythene bags of 60 x 30 cm were used. The paddy straw made into twists was placed in polybag as a layer. Spawn laid in the centre over which paddy straw twists were laid and spawning was done. This was repeated three times. The same method was applied for bamboo leaves and neopeat substrate bed preparation. Polythene bags were made compact tied at the top and provided with few holes for air circulation. After completion of spawn run the bags were kept for fruiting in room with relative humidity of 80-85 per cent. After 20 days of spawn run casing was done. Regular sprinkling of water was done. The best substrate was selected based on the criteria *viz.* time taken for complete colonization, time taken for fruit body formation and biological efficiency.

3.3.3 Casing

Casing material used for the experiment was a mixture of sand (25 %), neopeat (75 %) and calcium carbonate (2 %) after sterilization. Uniform 3-4 cm thick casing layer was put on top of *Macrocybe* beds. Care was taken to keep the

casing layer wet by giving regular water spraying. Observations were made during the time the mycelia colonized the casing layer and during pinning stage, when the primodial developed.

3.3.4 Effect of sterilization method on the growth of *Macrocybe gigantea*

To assess the effect of sterilization method, paddy straw substrate was sterilized by chemical and boiling methods of sterilization.

3.3.4.1 Chemical method

The substrates were soaked in a solution containing formalin and Bavistine (for 100 l water, 60 ml formalin and 5 g Bavistine) for 18 h. After draining excess water and air drying, they were used for mushroom bed preparation.

3.3.4.2 Boiling method

The substrates were soaked in water overnight, taken out, excess water removed and the substrates were sterilized by boiling for about one hour. They were then air dried and used for bed preparation. The biological yield of *Macrocybe gigantea* was determined.

3.3.5 Temperature preference for substrates

In order to find out the temperature preference for substrates *Macrocybe gigantea* was cultivated during 2013-2014 at three time periods *viz.* October - November, December - January and March - April. The biological yield obtained during the three time periods were recorded.

3.4 IMMUNOMODULATORY AND HEPATOPROTECTIVE ACTIVITY OF *MACROCYBE GIGANTEA*

3.4.1 Experimental animals

The immunomodulatory and hepatoprotective studies of *M. gigantea* were carried out in Wistar albino rats (150-250 g) and Swiss albino mice (20-30 g), obtained from the Animal House of JNTBGRI. They were grouped and housed in

poly-acrylic cages with three animals per cage and maintained under standard laboratory conditions (temperature 24-28° C). Experiments were carried out according to NIH guidelines, after getting the approval of Institute's Animal Ethics Committee.

3.4.2 Extract Preparation

The fruiting bodies were dried in an oven at 45° C for 48 h and powdered. The powder (100 g) was extracted with 1.0 l ethanol, overnight at room temperature with constant stirring. The ethanol extract was filtered using filter paper. The filtrate was then concentrated and the solvent was then evaporated completely at low temperature under reduced pressure in a rotary evaporator (Buchi, Switzerland). The yield of the extract was found to be 12 per cent. The extract was then dried in a dessicator. This crude extract was referred to as MG. For administration, the crude extract was suspended in 0.5 per cent Tween-80, to required concentrations and used for experiments.

3.4.3 Immunomodulation Studies

Immunomodulatory potential of *M. Gigantea* was determined by the following experiments;

- Delayed type hypersensitivity (DTH) test
- Active paw anaphylaxis test
- Inhibition of cyclophosphamide induced myelosuppression
- Carbon clearance assay
- Mast cell study

3.4.3.1 Delayed type hypersensitivity test (DTH)

Delayed type hypersensitivity (DTH) test was performed by modified method of Saraf *et al.* (1989).

3.4.3.1.1 Preparation of Sheep Red Blood Cells (SRBC) suspension

The blood was collected from a healthy sheep from the animal house, College of Agriculture, Vellayani, Thiruvananthapuram, India, in a mixture of 0.49 per cent EDTA and 0.9 per cent of sodium chloride solution. It was prepared at a temperature of 2-8° C on the day of immunization, the blood was centrifuged at 5000 rpm for ten min and then washed three times to remove plasma with 0.9 per cent sodium chloride solution. The SRBC (20 % v/v) suspension was then prepared in 0.9 per cent sodium chloride solution (Jayathirtha and Mishra, 2004).

Wistar rats were divided into five groups of six each. The animals were immunized on day 0 by i.p. administration of 0.1 ml of 20 per cent of fresh sheep red blood cell suspension and were challenged by injecting 20 µl of SRBC suspension intradermally to the left hind foot pad on day +14. The MG extract at different doses (50, 100, 150 mg kg^{-1} body weight) was administered orally from day -14 until day +13. The right hind paw volume was measured (before challenge) using plethysmometer on the 14^{th} day. Foot pad reaction was assessed after 24 h on 15^{th} day, in terms of increase in the paw volume as a result of hypersensitivity reaction due to oedema. The footpad reaction was expressed as the difference in the paw volume (in ml).

3.4.3.2 Active paw anaphylaxis test

Active paw anaphylaxis test was performed by modified method of Ghooi and Bhide (1981).

Swiss albino mice were divided in four groups each having six animals. Group 1-Vehicle (0.5 % Tween-80), Group 2 – standard, DSCG 5 mg kg^{-1} (disodium chromoglycate), Group 3 and 4 were administered with MG extract at different doses (25 and 50 mg kg^{-1}). Animals were sensitized by injecting 0.25 µg ovalbumin adsorbed on 6 mg aluminium hydroxide gel on the back of mice subcutaneously on day 0. The test drug was fed from day 1 to day 11. On the 11^{th} day, animals were challenged with 10 µg (0.05 ml of 200 µg ml^{-1}) ovalbumin in

normal saline s.c. in the plantar region of hind paw. The contra lateral paw, received an equal volume of saline. The paw volume was measured using plethysmometer (Plate 2) 1, 3 and 24 h after challenge. The difference in paw volume reflects the edema due to the antigen-antibody reaction.

3.4.3.3 Inhibition of cyclophosphamide-induced myelosuppression

Inhibition of cyclophosphamide-induced myelosuppression was carried out as per the protocol described by Yadav *et al.* (2011).

Wistar rats were divided into five groups of six animals each. Group 1 (Normal control group) and Group 2 (cyclophosphamide-treated group) received the vehicle (0.5 % Tween-80) for a period of 13 days. Group 3, 4 and 5 were given different doses of the MG extract (100, 200, 300 mg kg^{-1} body weight), p. o., daily for 13 days. The animals of groups 2 - 4 were injected with cyclophosphamide (30 mg kg^{-1}, i.p.) on the 11^{th}, 12^{th} and 13^{th} day, and 1 h after the administration of the respective drug treatments. On the 14^{th} day of the experiment, the animals were sacrificed and blood samples were collected by cardiac puncture. Total and differential white blood cells were determined.

3.4.3.4 Carbon clearance assay

Carbon clearance assay was done by modified method of Yadav *et al.* (2011).

To evaluate the phagocytic activity of the reticulo-endothelial system *in vivo*, Wistar rats were divided in five groups each having six animals. Group 1- Vehicle (0.5 % Tween-80), Group - 2, 3 and 4 were treated with MG at different doses (100, 200, 300 mg kg^{-1} body weight). Group 5, Standard (levamisol) 50 mg kg^{-1}, was administered orally. The carbon clearance test was performed after completion of the drug treatment. On day 14, the treated rats, received an intravenous injection (tail vain) of carbon suspension (1:50 dilution of Indian ink, Camel) in a dose of 0.5 ml 100 g^{-1} body weight. Blood was withdrawn from the retro-orbital venous plexus at five min and 15 min after injection of the carbon

Plate 2. Plethysmometer

suspension. Blood (0.05 ml) was lysed with 4 ml of 0.1 per cent Na_2CO_3 and the optical density was measured spectrophotometrically at 650 nm wavelength.

3.4.3.5 Mast cell study

Mast cell study was performed by the method of Gupta *et al.* (1995).

Wistar rats were divided into five groups of six animals each. The animals were treated with the ethanolic extract of MG at different doses (100, 200, 300 mg kg^{-1} body weight) and DSCG (10 mg kg^{-1} i.p) for four days prior to the collection of mast cells. After four days of treatment, 10 ml of normal saline was injected into the peritoneal cavity and after gentle massage; the peritoneal fluid was collected and was transferred into siliconized test tubes containing 7-10 ml of RPMI-1640 (pH 7.2 to 7.4). The collected mast cells were washed three times by centrifugation at low speed (400-500 rpm), discarding the supernatant and taking the pellet of the mast cells. The mast cells from normal or sensitized groups of control and treated groups were incubated with egg albumin (1 mg ml^{-1}) respectively, at 37° C in water bath for ten min. After ten min, the mast cells were stained with 0.1per cent toluidine blue, and percentage of protection against granulation was counted under compound microscope.

3.4.4 Hepatoprotective studies of *M. gigantea*

Hepatoprotective activity of *M. gigantea* was studied by acetaminophen induced hepatotoxicity study and carbontetrachloride induced liver damage

3.4.4.1 Acetaminophen induced hepatotoxicity study

Acetaminophen induced hepatotoxicity study was performed using the method described by Suja *et al.* (2003).

Wistar rats were divided into six groups, each containing six rats. Groups 1 and 2, the normal control and toxin control groups respectively and both received 0.5 per cent Tween 80 (1 ml, p.o), for six days. Groups 3, 4 and 5 were administered ethanolic extract of MG at different doses (100, 200, 300 mg kg^{-1}

body weight). Group 6, was given Silymarin, the known standard drug for six days. Paracetamol (2.5 g kg^{-1} in 0.5 % Tween 80, 1. ml p.o) was administered to group 2 to 6 on the fifth day of the experiment. The animals were sacrificed 48 h after paracetamol administration by carbondioxide inhalation. The blood samples were collected for evaluating the bio chemical parameters like AST, ALT, ALP and serum bilirubin and liver samples were fixed in 10 per cent neutral formalin for histopathological analysis.

3.4.4.2 Carbontetrachloride induced liver damage

Carbontetrachloride induced liver damage was carried out using the method described by Suja *et al.* (2004).

Wistar rats were divided into six groups (six per groups). Group 1, the normal control group received a single daily dose of 0.5 per cent Tween-80 (1ml) on all five days and olive oil (1ml kg^{-1}) s.c. on days 2 and 3. Group 2, the carbontetrachloride control group, received a single daily dose of 0.5 per cent Tween-80 (1ml) p.o. on all five days and on the second and third day, they were administered s.c. 2 ml kg^{-1} of CCl_4: olive oil (1:1). Group 3, 4 and 5 were administered MG (100, 200, 300 mg kg^{-1} body weight) p.o. for all five days and a single dose of CCl_4: olive oil mixture (2 ml kg^{-1}) s. c. on days 2 and 3, 30 min after MG administration. Group 6 was administered silymarin, the standard drug, at a dose of 100 mg kg^{-1}, p. o. on all five days and a single dose of CCl_4: olive oil mixture (2 ml kg^{-1}) s.c. on days 2 and 3, 30 min after silymarin administration. On the 6^{th} day, all the animals were sacrificed using carbondioxide chamber. Blood samples were collected from carotid artery for evaluating the biochemical parameters and liver tissue slices were collected for histopathological studies and antioxidant assays.

3.4.4.3 Biochemical estimations

The collected blood was allowed to coagulate for 1h at room temperature. The blood was centrifuged at 1500 rpm for 15 min to separate the serum. The

serum was then used for the assay of marker enzymes namely, alanine transaminase (ALT), aspartate transaminase (AST), serum bilirubin (SB) and alkaline phosphatase (ALP) and the level of serum cholesterol was also determined.

3.4.4.3.1 Determination of alanine transaminase (ALT) activity

Alanine transaminase activity was determined according to the method of Reitman and Frankel (1957) using kits procured from Crest Biosystems, Goa, India.

Principle

ALT converts L-Alanine and α Ketoglutarate to Pyruvate and Glutamate. The Pyruvate formed reacts with 2, 4-Dinitrophenyl hydrazine to produce hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured.

The reaction can be represented as:

L-Alanine + α Ketoglutarate $\xrightarrow[\text{pH 7.4}]{\text{ALT}}$ Pyruvate + L- Glutamate

Pyruvate + 2, 4-DNPH $\xrightarrow{\text{Alkaline medium}}$ 2, 4-Dinitrophenyl Hydrazone (Brown coloured complex)

3.4.4.3.2 Determination of aspartate transaminase (AST) activity

Aspartate transaminase activity was determined according to the method of Reitman and Frankel (1957) using kit procured from Crest Biosystems, Goa, India.

Principle

AST converts L-aspartate and α Ketoglutarate to Oxaloacetate and Glutamate. The Oxaloacetate formed reacts with 2, 4-Dinitrophenyl hydrazine to

produce hydrazone derivative, which in an alkaline medium produces a brown coloured complex whose intensity is measured.

The reaction can be represented as:

$$\text{L- Aspartate} + \alpha\text{ Ketoglutarate} \xrightarrow[\text{pH 7.4}]{\text{AST}} \text{Oxaloacetate} + \text{L-Glutamate}$$

$$\text{Oxaloacetate} + \text{2, 4-DNPH} \xrightarrow{\text{Alkaline medium}} \text{2, 4-Dinitrophenyl hydrazone (Brown coloured complex)}$$

3.4.4.3.3 Determination of serum alkaline phosphatase (ALP) activity

Serum alkaline phosphatase activity was determined by Modified King and King's method (1954) using kit obtained from Crest Biosystems, Goa, India.

Principle

Alkaline phosphatase at an alkaline pH hydrolyses disodium phenyl phosphate to form phenol. The phenol formed reacts with 4-aminoantipyrine in the presence of potassium ferricyanide, as an oxidizing agent to form red coloured complex. The intensity of colour formed is directly proportional to the activity of ALP present in the sample

The reaction can be represented as:

$$\text{Disodiumphenylphosphate} + H_2O \xrightarrow[\text{pH 10}]{\text{ALP}} \text{Phenol} + \text{Disodiumhydrogen phosphate}$$

$$\text{Phenol} + \text{4-aminoantipyrine} \xrightarrow[K_3Fe(CN)_6]{\text{Alkaline medium}} \text{Red coloured complex}$$

3.4.4.3.4 Determination of serum bilirubin

Serum bilirubin was determined by Modified Jendrassik and Grof's method (1983) using kit obtained from Crest Biosystems, Goa, India.

Principle

Bilirubin reacts with diazotized sulphanilic acid to form a coloured azobilirubin compound. The unconjugated bilirubin couples with sulphanilic acid in the presence of caffeine – benzoate accelerator. The intensity of the colour formed is directly proportional to the amount of bilirubin present in the sample.

The reaction can be represented as:

Bilirubin + Diazotized sulphanilic acid ⟶ Azobilirubin compound

3.4.4.3.5 Determination of serum cholesterol

Serum cholesterol was measured by CHOD/ PAP method of Meiattini *et al.* (1978) using using kit obtained from Crest Biosystems, Goa, India.

Principle

Cholesterol esterase hydrolyses esterified cholesterols to free cholesterol. The free cholesterol is oxidized to form hydrogen peroxide which further reacts with phenol and 4-aminoantipyrine by the catalytic action of peroxidase to form red coloured quinonemine dye complex. Intensity of the colour formed is directly proportional to the amount of cholesterol present in the sample.

The reaction can be represented as:

Cholesterol esters + H_2O $\xrightarrow{\text{Cholesterol Esterase}}$ Cholesterol + Fatty acids

Cholesterol + O_2 $\xrightarrow{\text{Cholesterol Oxidase}}$ Cholestenone + H_2O

H_2O_2 + 4-aminoantipyrine + phenol $\xrightarrow{\text{Peroxidase}}$ Red Quinonemine dye + H_2O

3.4.4.4 Histopathological examination

The liver tissues were dissected out and fixed in 10 per cent formalin. The paraffin sections were prepared and stained with haematoxylin and eosin and examined using light microscopy. The sections obtained were observed

microscopically for assessment of necrosis, fatty infiltration, fibrosis, lymphocyte infiltration etc.

3.4.5 Statistical analysis

All data are expressed as mean ± S.E. Data were statistically analysed using one-way analysis of variance (ANOVA) using the SPSS software package. The Dunnett's test was applied for the detection of significance between different groups.

3.5 NUTRITIVE ANALYSIS

3.5.1 Estimation of protein

The protein content of *Macrocybe gigantea* was estimated using the method described by Bradford (1976)

One gram of dried mushroom powder was weighed and ground in 10 ml 0.05 M Tris pH 7.1. The sample was centrifuged at 5000 rpm for 15 min at 4° C. The supernatant was collected for protein analysis. The reaction mixture consisting of 0.5 ml of sample extract, 0.5 ml of distilled water and 5 ml of Coomassie brilliant blue G-250 was used. The color developed was read at 595 nm using spectrophotometer against reagent blank. A standard graph was prepared by using different concentrations of Bovine Serum Albumin and the protein content in the sample was calculated by comparing with the graph.

3.5.2 Estimation of total carbohydrates

Total Carbohydrate content was estimated by Phenol-Sulphuric acid method described by Dubois *et al.* (1956)

One hundred mg of dried mushroom powder was weighed. The sample was hydrolysed by keeping it in a boiling water bath for 3 h with 5 ml of 2.5 N HCl and cooled to room temperature. It was neutralised with solid sodium carbonate until the effervences ceases. The volume was made up to 100 ml and centrifuged at 5000 rpm for 15 min. The supernatant was collected for further analysis. The

reaction mixture consisting of 0.5 ml of sample extract, 0.5 ml of distilled water, 1 ml of 5 per cent phenol solution, 5 ml of sulphuric acid was used. While adding sulphuric acid, the tubes were kept in a water bath at 25-30° C for 20 min. The blank solution was prepared by taking 1ml distilled water. The colour developed was read at 490 nm using a spectrophotometer. The amount of total carbohydrate present in the sample solution was calculated from the standard graph prepared using glucose.

3.5.3 Estimation of glycogen

The glycogen content was estimated by method described by Morales *et al.* (1973).

Dried mushroom powder (0.2 g) was ground in a mortar with 5 ml 3 per cent KOH and the homogenate was heated over a boiling water bath for 1 h. The homogenate was centrifuged at 5000 rpm for 20 min. To 1 ml supernatant 3 ml of ethanol (100 %) was added. Mixture was shaken well and incubated over night in refrigerator. The precipitated glycogen was centrifuged and the residue was collected, washed twice with ethanol and finally dissolved in 5 ml distilled water. An aliquot of 0.2 ml was analyzed for glycogen (Dubois *et al.*, 1956). The reagent alone served as control. Glucose solution was used as the standard.

3.6 DIVERSITY ANALYSIS USING INTERNAL TRANSCRIBED SPACER (ITS) SEQUENCE

3.6.1 DNA isolation

DNA was isolated from dried mushroom samples by modified CTAB extraction method (Moller *et al.*, 2010).

Day 1

Dried mushroom (2 g) was manually ground in pestle and mortar adding 20 ml of pre warmed (60° C) TES lysis buffer (Appendix III). Proteinase K (2 mg) was added to the ground material, incubated in 60° C for 60 min. To the

suspension, 5.6 ml of 5 M NaCl and 2.5 ml of 10 per cent (W/V) of CTAB (Appendix IV) were added and incubated at 65° C for 10 min. DNA was extracted by adding 3 ml of phenol: chloroform: isoamylalcohol (25:24:1), centrifuged at 12000 rpm for 15 min. Equal volume of chloroform : isoamylalcohol (24:1) was added and centrifuged at 14000 rpm for 10 min. DNA was precipitated by adding 0.6 volume of cold isopropanol and 0.1 volume of 3 M sodium acetate pH 5.2 and maintained at -20° C overnight.

Day 2

The precipitate was then centrifuged at 12,000 rpm for 15 min and washed twice with ethanol (70 %) and the pellet was dried and suspended in 100 µl of TE buffer (Appendix V). RNA was digested by adding 10 mg ml^{-1} of RNAse A and incubating at 37° C for 45 min and stored in -20° C for further use.

3.6.2 Agarose gel electrophoresis

Both Genomic DNA and PCR amplicons were confirmed by horizontal gel electrophoresis. Gel was run at 5 V cm^{-1}. Electrophoresis of genomic DNA was done at 0.8 per cent Low EEO agarose made in 1X TBE buffer (Appendix VI), whereas for PCR amplicons 1.2 per cent agarose gel was used. The DNA samples were mixed with required volume of 6X gel loading buffer (Appendix VII). Each well was loaded with 6 µl of sample and run for about one hour. After electrophoresis, gel was visualized using gel documentation unit (Gel Doc – IT™) and data was recorded using 'Launch Vision World' software.

3.6.3 Quantification of DNA

DNA quantification was carried out with the help of UV – spectrophotometer (Cary – 100, Agilent Technologies). The spectrophotometer was calibrated at 260 nm and 280 nm wavelength using TE buffer. The optical density (O.D.) of 5 µl DNA dissolved in 2 ml of TE buffer was recorded at both 260 nm and 280 nm. Since the O.D. of 1.0 at 260 nm represent 50 µg ml^{-1} of

DNA, the quantity of DNA in the sample was estimated by employing the following formula:

Concentration of DNA ($\mu g\ ml^{-1}$) = A260 x 50 x Dilution factor (where, A260 is absorbance at 260 nm).

The quality of DNA could be judged from the ratio of the O.D. values recorded at 260 nm and 280 nm. The ratio 1.8 indicates good quality DNA.

3.6.4 PCR amplification

The DNA was amplified using ITS 4 (5'TCCTCCGCTTATTGATATGC-3') and ITS 5 (5'GGAAGTAAAAGTCGTAAC-3') primers. Reaction mixture (25 µl - 50 µl) was set in a Programmable Thermal Cycler (Sure cycler 8800 Agilent technologies). The details of PCR master mix and the program for PCR is as follows:

Component	Volume (µl)
DNA sample (25-50 ng)	4 µl
10X PCR buffer	2.5 µl
dNTP mix (25 mM)	0.2 µl
ITS 4 primer	0.7 µl
ITS 5 primer	0.7 µl
Taq DNA polymerase	0.4 µl
Sterile distilled water	16.5 µl
Total volume	25 µl

Thermocycler program was set for 39 PCR cycles with initial denaturation at 97° C for 5 min followed by repeated cycles of denaturation at 97° C for 1 min, annealing at 48° C for 1 min and extension at 72° C for 2 min. Final extension was done for 5 min at 72° C.

3.6.5 Sequencing

Amplified DNA samples were sent to the Rajiv Gandhi Centre for Biotechnology, Thiruvananthapuram, Kerala for sequencing.

3.6.6 *In silico* analysis

The sequences were verified with the chromatogram file and edited with bioedit. The sequence was converted into FASTA (Fast Alignment) format and saved in notepad.

The BLAST (Basic local Alignment Search tool) analysis of the ITS sequences were performed with the nucleotide database of NCBI (National Centre for Biotechnology Information).

Sequences of different strains and related species of *Macrocybe gigantea* were retrieved from GenBank and aligned using multiple alignment software program ClustalW. Phylogenetic analysis was done by Neighbor-Joining method in MEGA4.

Results

4. RESULTS

4.1 CULTURAL STUDIES

4.1.1 Culture characteristics

The mat was white coloured spreading uniformly with even margin. In the advancing zone, hyphae were generative, branched and septate with clamp connections. Pale white colour was observed in the colony reverse.

4.1.1 Growth under different solid media

The growth of *Macrocybe gigantea* in potato dextrose agar (PDA) and sabouraud dextrose agar (SDA) was observed. The radial growth of *Macrocybe gigantea* after ten days in PDA and SDA were 7.12 cm and 7.37 cm respectively.

Results showed that SDA is as effective as PDA favouring the mycelial growth (Table 1). The nature of mycelia growth was dense in sabouraud dextrose agar as compared to potato dextrose agar (Plate 3).

4.1.2 Growth under different liquid media

The effect of liquid media on the mycelia growth of *Macrocybe gigantea* is shown in Table 2. The biomass produced by sabouraud dextrose broth and potato dextrose broth were 0.52 g /100 ml and 0.43 g/100 ml respectively.

4.1.3 Effect of different temperature on the growth of *Macrocybe gigantea*

Three different temperature conditions 25, 30, 35° C were tested to find out the optimum growth of *Macrocybe gigantea.* The maximum and minimum mycelial growth of 8.20 cm and 3.12 cm were observed at temperature 35 and 25°C respectively (Table 3 and Plate 4).

4.1.4 Effect of different pH on the growth of *Macrocybe gigantea*

Three different pH *viz.* 5, 7 and 9 were tested to determine the favourable mycelial growth of *Macrocybe gigantea.* Results showed that pH 7 was found to be best for *Macrocybe* producing radial growth of 8.64 cm. pH 5 and pH 9 was on par with each other producing 5.67 and 5.78 radial growth (Table 4 and Plate 5)

Table 1. Growth of *Macrocybe gigantea* in different solid media

Media	Growth of *Macrocybe* after 10 days (cm)*
Potato dextrose agar (PDA)	7.12
Sabouraud dextrose agar (SDA)	7.37

Table 2. Growth of *Macrocybe gigantea* in different liquid media

Media	Dry weight of mycelia (g/100 ml)*
Potato Dextrose Broth (PDB)	0.43
Sabouraud Dextrose Broth (SDB)	0.52

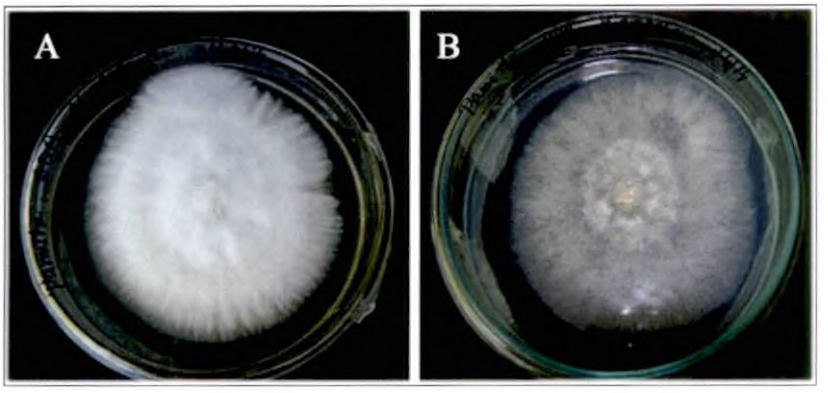
Table 3. Growth of *Macrocybe gigantea* at different temperature

Temperature (° C)	Growth of *Macrocybe* after 10 days (cm)*
25	3.12
30	6.82
35	8.20

Table 4. Growth of *Macrocybe gigantea* culture in different pH level

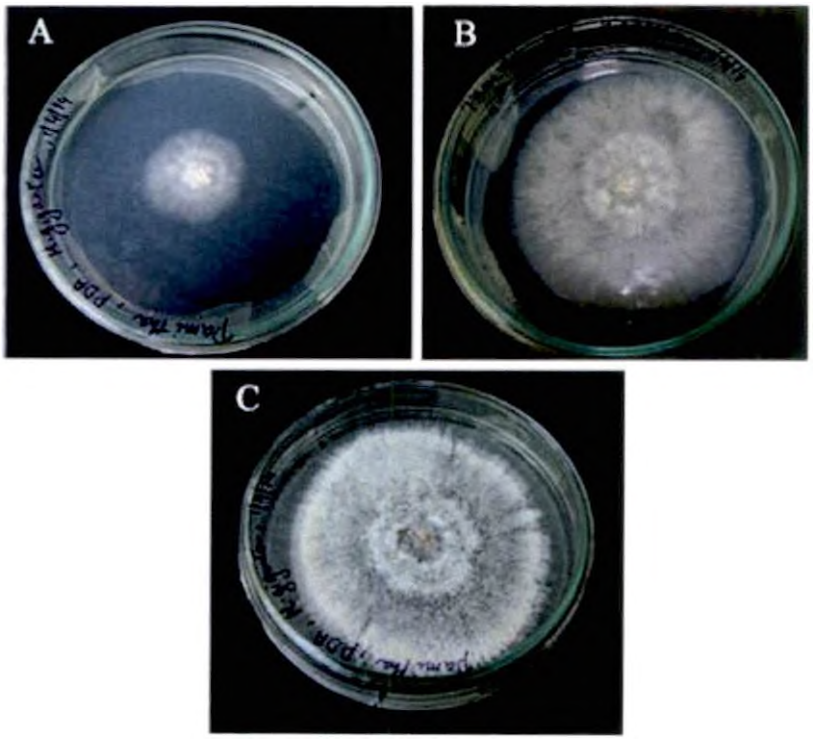
pH	Growth of *Macrocybe* (cm) after 10 days*
5	5.67
7	8.64
9	5.78

*** Mean value of three replications**



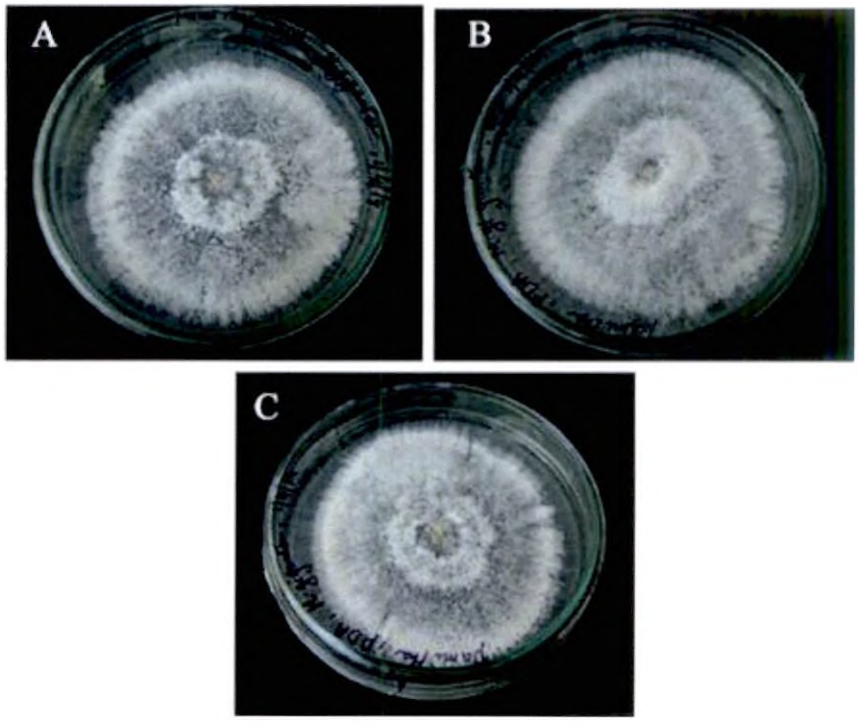
A - SDA B – PDA

Plate 3. Growth of *Macrocybe* in different solid media



A - 25° C, B - 30° C, C - 35° C

Plate 4. Growth of *Macrocybe* culture at different temperature



A - pH 5, B - pH 7, C - pH 9

Plate 5. Growth of *Macrocybe* culture in different pH level

4.2 GROWTH OF *MACROCYBE GIGANTEA* ON DIFFERENT SUBSTRATES

The growth of *Macrocybe gigantea* on three different substrates such as paddy straw, bamboo leaves and neopeat were studied. The yield parameters like number of days taken to colonize mycelium, number of days taken to fruit, nature of fruiting bodies and biological efficiency (%) were recorded (Table 6 and Plate 5).

The number of days taken to colonize mycelium for different substrates ranged from 15 – 21 days. The least colonization time was observed for neopeat (15.75 days) followed by paddy straw (17.25 days). The longer period (21 days) was observed in bamboo leaves.

The days for fruit formation in neopeat were 24 days followed by paddy straw (28.50) and bamboo leaves (36.50). The fruiting bodies formed in all the substrates were healthy.

The biological efficiency was highest in paddy straw with 72.40 per cent and the least in neopeat (54.60 %).

4.2.1 Effect of sterilization

The paddy straw substrate was sterilized by two methods namely boiling and chemical method. No contaminations were observed in both treatments and the average yield appears to be same for both boiling and chemical sterilization.

4.2.2 Temperature preference for paddy straw substrate

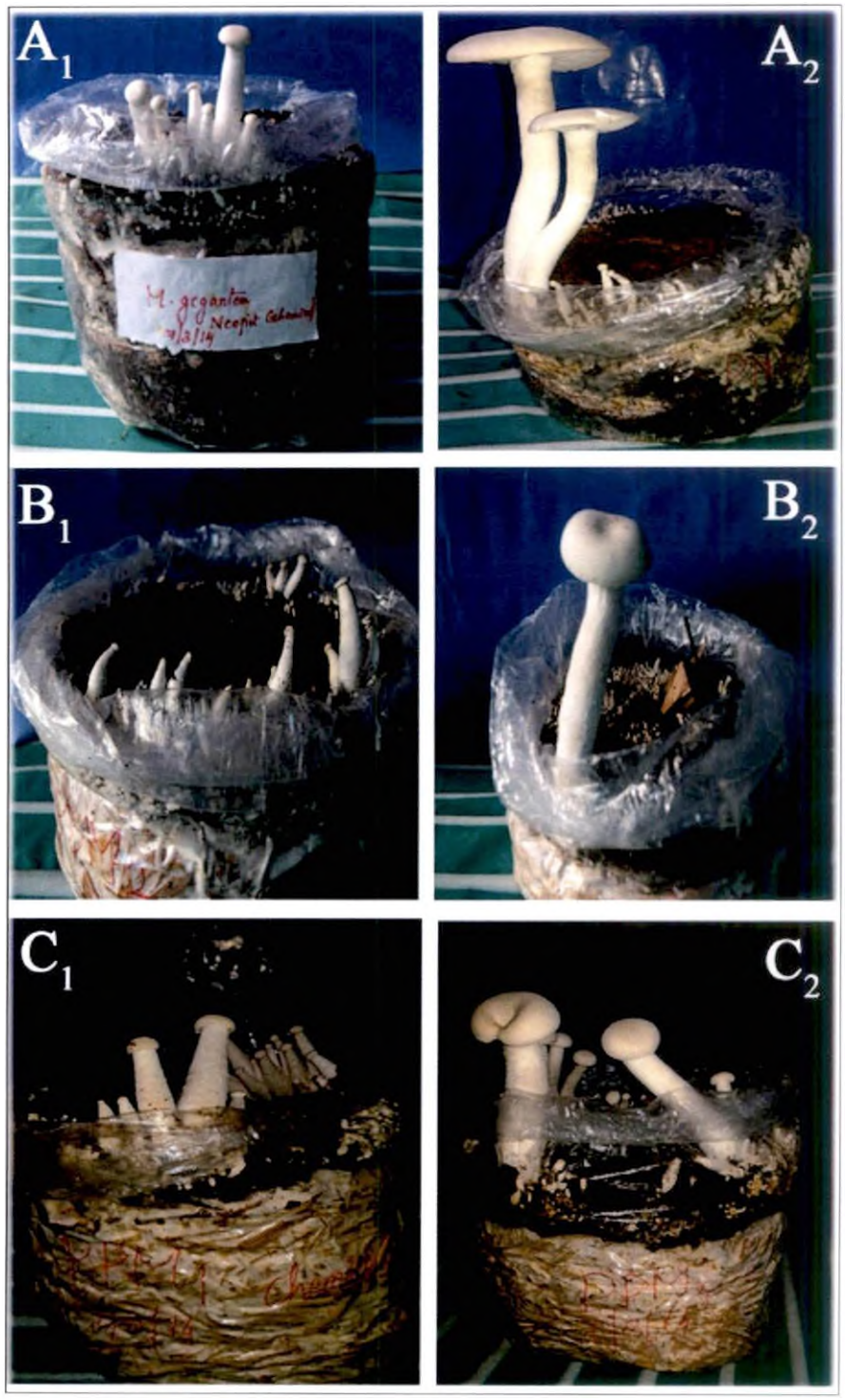
The temperature preference for paddy straw substrate was determined by cultivating *M. gigantea* during three different time periods viz. October – November, December – January, March – April. The biological yield throughout the year is shown in table 6. The maximum yield was obtained during March – April with 724 g/kg substrate.

Table 5. Growth of *Macrocybe gigantea* on different bed substrates

Treatments	No of days taken to colonize mycelium	Number of days taken to fruit	Nature of fruiting bodies	Biological efficiency (%)
Paddy straw	17.25	28.50	Healthy	72.40
Bamboo leaves	21.00	36.50	Healthy	56.75
Neopeat	15.75	24.00	Healthy	54.60

Table 6. Temperature preference for paddy straw substrate

Experimental time	Total yield per bed (g kg^{-1})
October – November	592.50
December – January	488.50
March – April	724.00



A_1, A_2 – Neopeat; B_1, B_2 - Bamboo leaves; C_1, C_2 - Paddy straw

Plate 6. Growth of *Macrocybe gigantea* on different bed substrates

4.3 IMMUNOMODULATORY AND HEPATOPROTECTIVE ACTIVITY OF *MACROCYBE GIGANTEA*

4.3.1 Immunomodulation Studies

4.3.1.1 Delayed type hypersensitivity test (DTH)

The effect of 50 mg kg^{-1}, 100 mg kg^{-1} and 150 mg kg^{-1} of MG on delayed type hypersensitivity test is depicted in Figure 1. Administration of MG (100 and 150 mg kg^{-1} body weight p.o.) increased the delayed type hypersensitivity responses significantly ($p<0.05$) in terms of increase in the mean difference of paw volume when compared with control group. The maximum increase in paw volume of 0.36 ± 0.024 ml was observed in animals treated with MG at 100 mg kg^{-1}.

4.3.1.2 Active paw anaphylaxis test

The results of active paw anaphylaxis test in mice showed that treatment with MG at 50 mg kg^{-1} significantly reduced the paw volume to 0.110 ± 0.0010 ml as compared to control. Animals treated with MG at 25 mg kg^{-1} showed no significant activity as compared with normal control group (Figure 2).

4.3.1.3 Inhibition of cyclophosphamide-induced myelosuppression

Treatment with cyclophosphamide at a dose of 30 mg kg^{-1} significantly reduced the total white blood cell count to 3005 ± 126.46 cells mm^{-3}as compared to normal control group. MG at 100 mg kg^{-1}, 200 mg kg^{-1} and 300 mg kg^{-1} p.o. increased the total white blood cell count to 6003.33 ± 138.12 cells mm^{-3}, 4999.17 ± 129.82 cells mm^{-1} and 4100.00 ± 142.43 cells mm^{-3} respectively (Figure 3).

Animals treated with cyclophosphamide showed significant decrease in neutrophils (N), lymphocytes (L), eosinophils (E) and monocytes (M) as compared to control group. Treatment with MG at 100 mg kg^{-1}, 200 mg kg^{-1} and 300 mg kg^{-1} p.o. significantly increased the percentage of neutrophils and lymphocytes and in significantly increased the percentage of eosinophils and monocytes compared with cyclophosphamide treated group (Table 7).

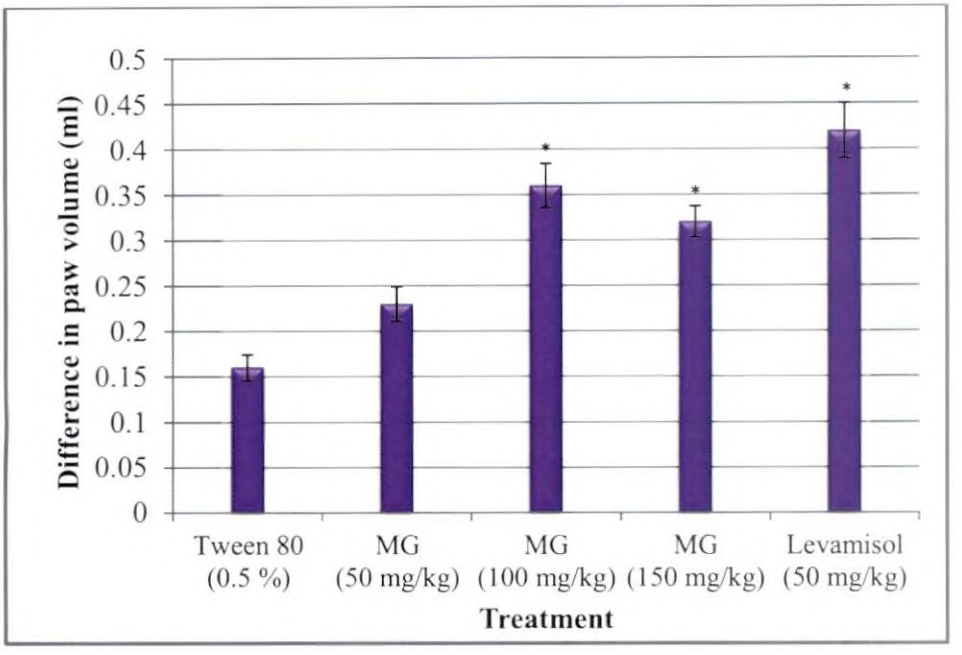


Fig. 1. Delayed type hypersensitivity test using MG

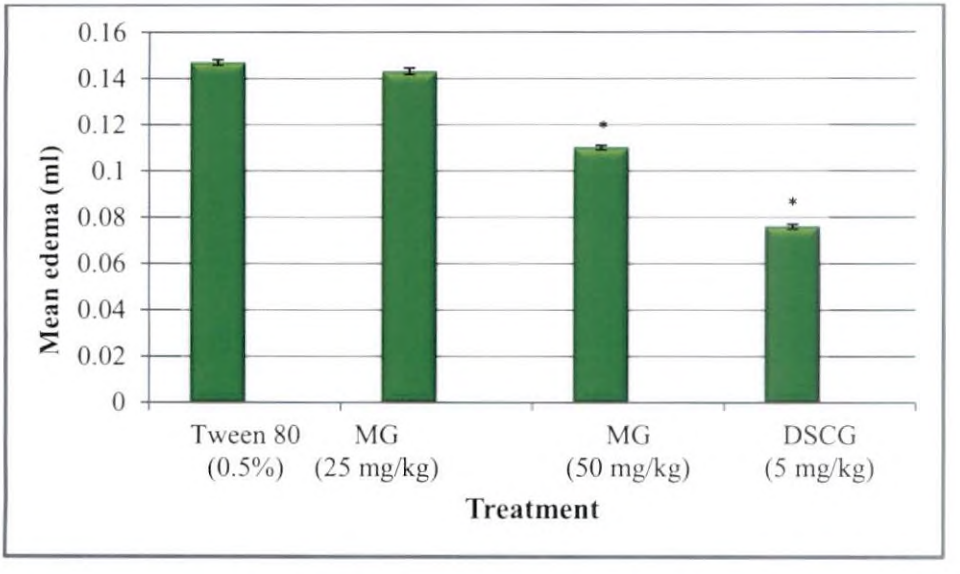


Fig. 2. Active paw anaphylaxis using MG

Values are expressed as mean ± S.E., n = 6

*$p<0.05$ as compared to normal control group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.

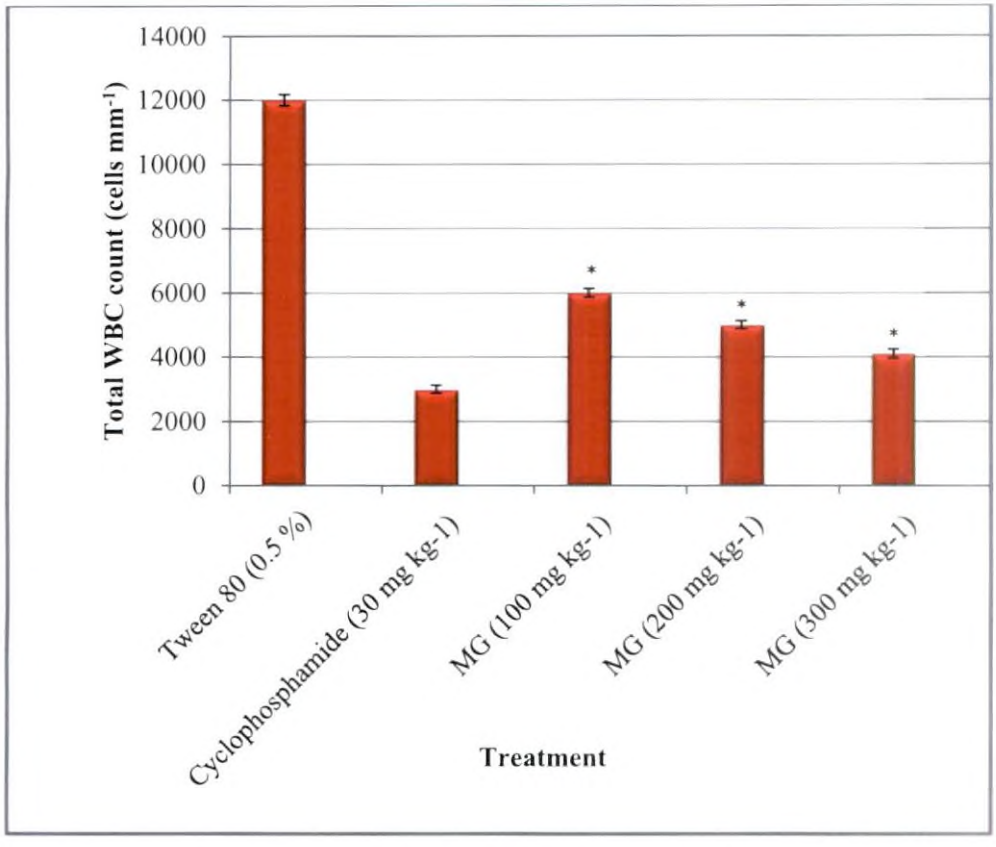


Fig. 3. Cyclophosphamide induced myelosuppression using MG

Values are expressed as mean ± S.E., n = 6

*$p<0.05$ as compared to toxin treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.

Table 7. Differential leukocyte counts

Groups	Treatment	Neutrophil (%) Mean ± S. E.	Lymphocyte (%) Mean ± S.E.	Eosinophil (%) Mean ±S.E.	Monocyte (%) Mean ± S.E.
1	Control (0.5 % tween 80)	23.83 ± 1.40	73.67 ± 0.88	1.83 ± 0.17	1.17 ± 1.17
2	Cyclophosphamide (30mg/kg)	6.33 ±0.67	62.50 ± 0.92	0.67 ± 0.21	0.17 ± 0.17
3	Cyclophosphamide(30mg/kg) + MG (100mg/kg)	22.17±0.91*	69.83±0.87*	1.67 ± 0.21	0.67 ± 0.21
4	Cyclophosphamide (30mg/kg) + MG (200mg/kg)	19.83±0.95*	67.00±1.06*	1.50 ± 0.22	0.50 ± 0.22
5	Cyclophosphamide (30mg/kg) + MG(300mg/kg)	10.17 ±0.83*	66.33 ± 0.67*	1.17 ± 0.31	0.50 ± 0.22

Values are expressed as mean ± S.E., n = 6

*$p<0.05$ as compared to toxin treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.

4.3.1.4 Carbon clearance assay

The results of carbon clearance assay are shown in table 8. There was a significant increase in the mean phagocytic index with 6.6 x 10^{-3} ± 0.00016, 0.9 x 10^{-3}± 0.00001 and 0.8 x 10^{-3} ± 0.00001 in animals treated with MG at 100 mg kg^{-1}, 200 mg kg^{-1} and 300 mg kg^{-1} respectively as compared to normal control group.

4.3.1.5 Mast cell study

The results of mast cell study showed that MG significantly prevented the degranulation of mast cells at 100 mg kg^{-1}. The percentages of granulated mast cells were 80.00 ± 4.49 per cent, 40.00 ± 2.71 per cent and 70.00 ± 4.04 per cent at 100 mg kg^{-1}, 200 mg kg^{-1} and 300 mg kg^{-1} respectively (Table 9). The rat mast cell degranulation caused by egg albumin and inhibition of mast cell degranulation by MG is shown in Plate 7.

Table 8. Carbon clearance assay using MG

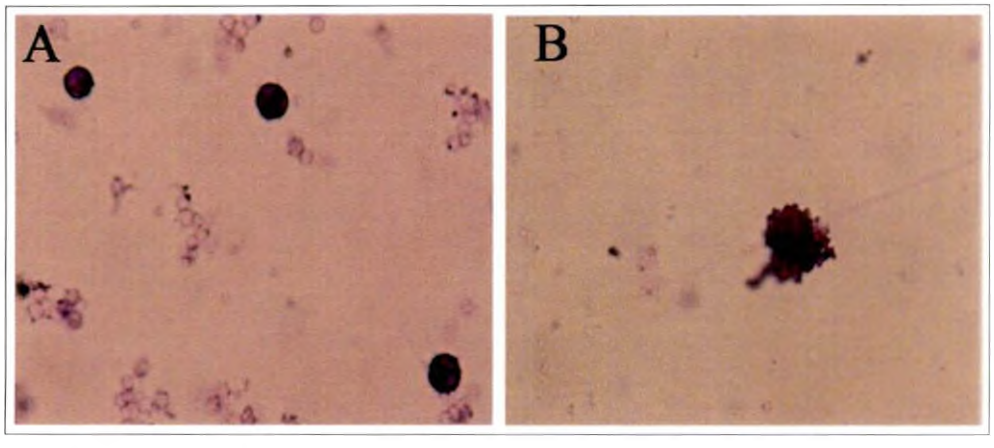
Groups	Treatment	Phagocytic Index (Mean ± S.E.)
1	Normal Control (0.5 % tween 80)	$0.4 \times 10^{-3} \pm 0.0001$
2	MG (100 mg kg^{-1})	$6.6 \times 10^{-3} \pm 0.00016$*
3	MG (200 mg kg^{-1})	$0.9 \times 10^{-3} \pm 0.00001$*
4	MG(300 mg kg^{-1})	$0.8 \times 10^{-3} \pm 0.00001$*
5	Standard (Levamisol 50 mg kg^{-1})	$7.1 \times 10^{-3} \pm 0.00014$*

Table 9. Mast cell study using MG

Groups	Treatment	Degranulated mast cells (%) (Mean ± S.E.)	Granulated mast cells (%) (Mean ± S.E.)
1	Egg albumin (1 mg ml^{-1}) Control	70.94 ± 4.24	29.06 ± 3.15
2	Egg albumin + MG (100 mg kg^{-1})	20 ± 2.26*	80.00 ± 4.49*
3	Egg albumin + MG (200 mg kg^{-1})	30 ± 3.18*	70.00 ± 4.04*
4	Egg albumin + MG (300 mg kg^{-1})	60 ± 3.59	40.00 ± 2.71
5	Egg albumin +DSCG (10 mg kg^{-1})	21.8 ± 2.56*	78.2 ± 3.62*

Values are expressed as mean ± S.E., n = 6

*$p<0.05$ as compared to normal control group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.



A - Rat mast cell degranulation caused by egg albumin

B - Inhibition of mast cell degranulation by MG

Plate 7. Mast cell study

4.3.2 Hepatoprotective studies

The results of hepatoprotective effects of MG on acetaminophen and CCl_4 induced hepatic injury in rats are shown on tables 10 and 11. The levels of serum ALT, AST, ALP, bilirubin and cholesterol were elevated significantly after administration of acetaminophen and carbontetrachloride. The ALT, AST, ALP, bilirubin and cholesterol levels were 191.92 ± 5.73 U L^{-1}, 383.33 ± 5.26 U L^{-1}, 91.17 ± 4.87 KA Units, 2.03 ± 0.25 mg dl^{-1} and 109.83 ± 5.64 mg dl^{-1} respectively in acetaminophen treated rats and 171.00 ± 5.11 U L^{-1}, 395.08 ± 5.30 U L^{-1}, 40.63 ± 4.92 KA Units, 1.58 ± 0.22 mg dl^{-1} and 95.08 ± 4.62 mg dl^{-1} respectively in carbontetrachloride treated rats. Treatment with MG (100, 200, 300 mg kg^{-1}body weight) reduced the elevated serum enzyme activities, bilirubin and cholesterol in a dose dependent manner. Treatment with MG at a dose of 300 mg kg^{-1} significantly reduced the levels of ALT, AST, ALP, bilirubin and cholesterol to 31.50 ± 3.45 U L^{-1}, 139.33 ± 2.89 U L^{-1}, 18.42 ± 2.28 KA Units, 0.543 ± 0.017 mg dl^{-1} and 51.83 ± 3.54 mg dl^{-1} in acetaminophen treated animals and 47.50 ± 3.76 U L^{-1}, 190.08 ± 4.05 U L^{-1}, 19.45 ± 1.99 KA Units, 0.480 ± 0.047 mg dl^{-1} and 74.10 ± 3.38 mg dl^{-1} in CCl_4 treated animals when compared to toxin control group.

4.3.2.1 Histopathological observations

Histopathological observations showed extensive damage characterized by necrosis, degenerated nuclei, fatty infiltration in acetaminophen treated animals and centrilobular necrosis, fatty infiltration, extensive vacuolization in CCl_4 treated animals as compared to normal control group. Treatment with MG (100, 200, 300 mg kg^{-1}body weight) showed significant reduction in progression of these toxic cellular effects of hepatotoxicants (Plates 8 and 9).

Table 10. Effect of *M. gigantea* on APAP induced hepatotoxicity in wistar rats

Groups	ALT (U L^{-1})	AST (U L^{-1})	ALP (KA Units)	Bilirubin (mg dl^{-1})	Cholesterol (mg dl^{-1})
Normal Control (0.5 % Tween -80)	23.33 ± 4.38	103.17 ± 4.87	15.75 ± 2.61	0.272 ± 0.036	41.63 ± 3.65
APAP Control (2.5 g kg^{-1})	191.92 ± 5.73	383.33 ± 5.26	91.17 ± 4.87	2.03 ± 0.25	109.83 ± 5.64
APAP(2.5 g kg^{-1}) + MG (100 mg kg^{-1})	44.5 ± 4.49*	218.00 ± 4.49*	21.33 ± 3.76*	0.650 ± 0.202*	81.63 ± 4.66*
APAP (2.5 g kg^{-1}) + MG (200 mg kg^{-1})	38.33 ± 3.82*	168.42 ± 3.67*	19.67 ± 3.04*	0.570 ± 0.024*	70.83 ± 3.84*
APAP (2.5 g kg^{-1}) + MG (300 mg kg^{-1})	31.50 ± 3.45*	139.33 ± 2.89*	18.42 ± 2.28*	0.543 ± 0.017*	51.83 ± 3.54*
APAP (2.5 g kg^{-1}) + Silymarin (100 mg kg^{-1})	27.67 ± 5.12*	116.00 ± 3.65*	16.23 ± 2.92*	0.480 ± 0.019*	46.33 ± 4.06*

Values are expressed as mean ± S.E., n = 6

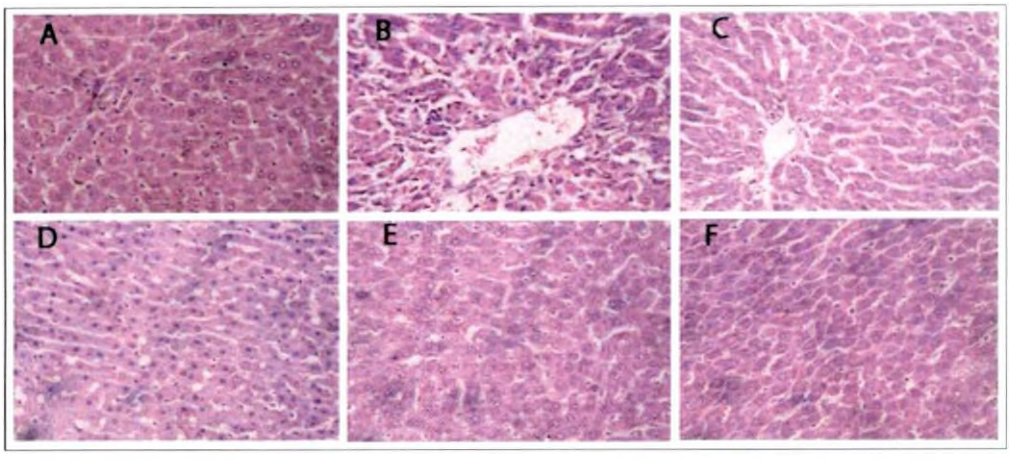
*$p<0.05$ as compared to toxin treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test

Table 11. Effect of *M. gigantea* on CCl_4 induced liver damage in wistar rats

Groups	ALT (U L^{-1})	AST (U L^{-1})	ALP (KA Units)	Bilirubin (mg dl^{-1})	Cholesterol (mg dl^{-1})
Normal Control (0.5 % Tween -80)	25.50 ± 2.86	106.33 ± 3.99	14.48 ± 2.06	0.290 ± 0.017	43.42 ± 2.70
CCl_4 Control (2 ml kg^{-1})	171.00 ± 5.11	395.08 ± 5.30	40.63 ± 4.92	1.58 ± 0.22	95.08 ± 4.62
CCl_4 (2 ml kg^{-1}) + MG (100 mg kg^{-1})	71.67 ± 4.65*	305.25 ± 4.49*	26.62 ± 3.88*	0.563 ± 0.024*	81.42 ± 3.85*
CCl_4 (2ml kg^{-1}) + MG (200 mg kg^{-1})	58.83 ± 3.49*	274.50 ± 3.22*	24.33 ± 3.20*	0.527 ± 0.024*	79.83 ± 3.61*
CCl_4 (2 ml kg^{-1}) + MG (300 mg kg^{-1})	47.50 ± 3.76*	190.08 ± 4.05*	19.45 ± 1.99*	0.480 ± 0.047*	74.10 ± 3.38*
CCl_4 (2 ml kg^{-1}) + Silymarin (100 mg kg^{-1})	44.58 ± 3.14*	183.42 ± 4.75*	17.85 ± 2.50*	0.420 ± 0.020*	65.08 ± 2.70*

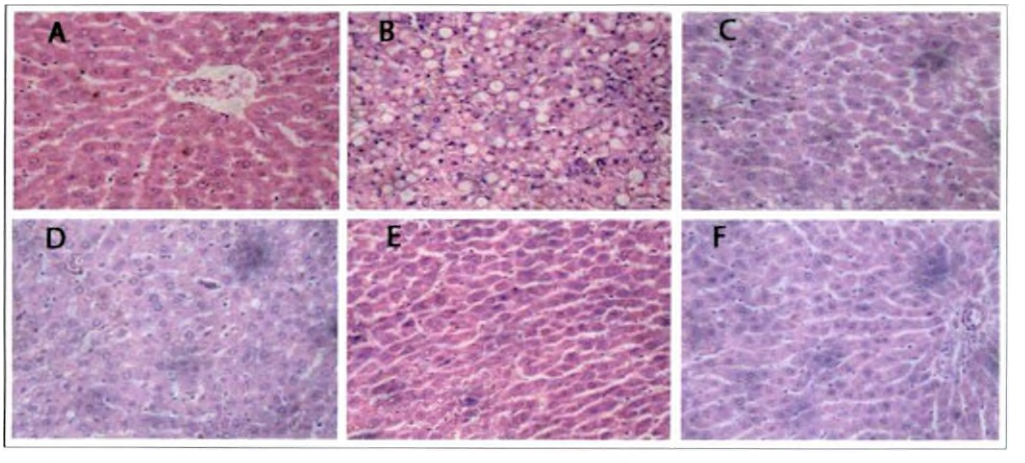
Values are expressed as mean ± S.E., n = 6

*$p<0.05$ as compared to toxin treated group. (Statistically analysed by One-way analysis of variance (ANOVA) followed by (Dunnett) multiple comparison test.



A - Normal control
B - APAP group
C - APAP + MG (100 mg kg^{-1})
D - APAP + MG (200 mg kg^{-1})
E - APAP + MG (300 mg kg^{-1})
F - APAP + Silymarin (100 mg kg^{-1})

Plate 8. Histopathological architecture (APAP)



A - Normal control
B - CCl_4 group
C - CCl_4 + MG (100 mg kg^{-1})
D - CCl_4 + MG (200 mg kg^{-1})
E - CCl_4 + MG (300 mg kg^{-1})
F - CCl_4 + Silymarin (100 mg kg^{-1})

Plate 9. Histopathological architecture (CCl_4)

4.4 NUTRITIVE VALUE

The nutritive value of *Macrocybe gigantea* was determined using standard protocols. The protein, carbohydrate and glycogen contents were 24.10 per cent, 11.20 per cent and 5.6 per cent respectively (Figure 4).

4.5 DIVERSITY ANALYSIS USING ITS

Internal transcribed spacer (ITS) sequence was used to study the genetic diversity among *Macrocybe gigantea* isolates/strains.

4.5.1 DNA Isolation

Dried samples of *Macrocybe gigantea* obtained from DMR, Solan and wild isolate from Venjaramood were used for DNA isolation. On agarose gel electrophoresis (0.8 %) the isolated genomic DNA showed intact bands (Plate 10). The absorbance of the isolated genomic DNA was measured using spectrophotometer, revealed good quality and quantity of DNA (Table 12).

4.5.2 PCR analysis of genomic DNA with ITS primers

The genomic DNA of two samples was amplified using ITS 4 and ITS 5 primers. Agarose gel electrophoresis (1.2 per cent) of the PCR products showed intact bands (Plate 11).

4.5.3 Sequence analysis

The sequence of two samples Venjaramood (PAM–W-ITS) and DMR strain (PAM-C-ITS) is given in appendix VIII. Homology search was done with BLASTN program of NCBI. The phylogenetic tree was constructed using the ITS sequences of two samples (Venjaramood and DMR strain), similar and related sequences retrieved from NCBI by MEGA 4 (Fig 5).

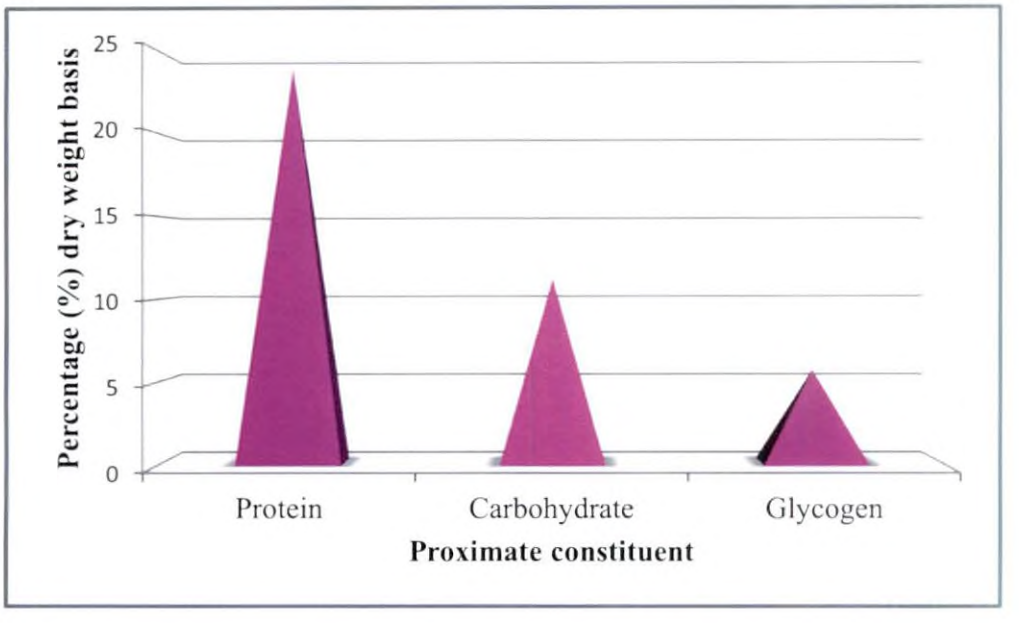
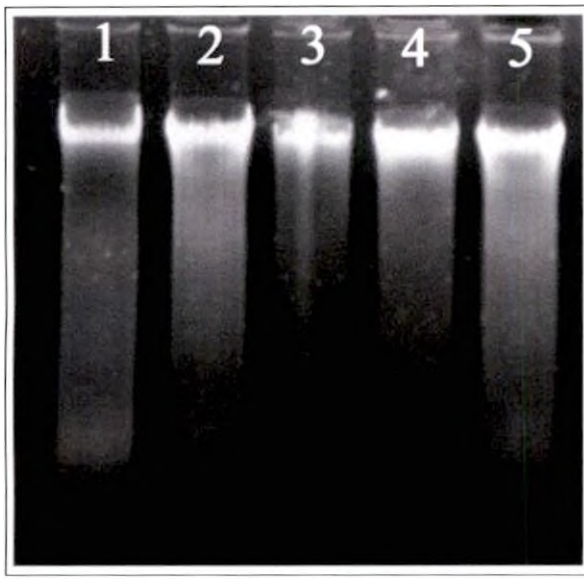


Fig 4. Nutritive analysis of *Macrocybe gigantea*

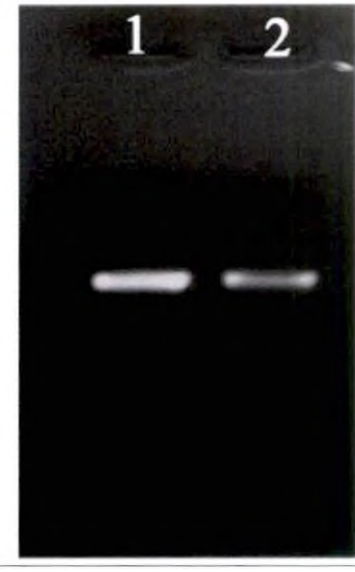
Table 12. Quality and quantity of isolated genomic DNA

Sl. No.	Sample	Absorbance (A_{260} nm)	Absorbance (A_{280} nm)	A_{260}/A_{280}	DNA Yield (ng μl^{-1})
1	Solan strain	0.480	0.258	1.86	9600
2	Venjaramood isolate	0.252	0.140	1.80	5040



1, 2, 3 - Venjaramood strain
4, 5 - Solan strain

Plate 10. Genomic DNA of *M. gigantea*



1 - Venjaramood strain
2 - Solan strain

Plate 11. PCR product of ITS region

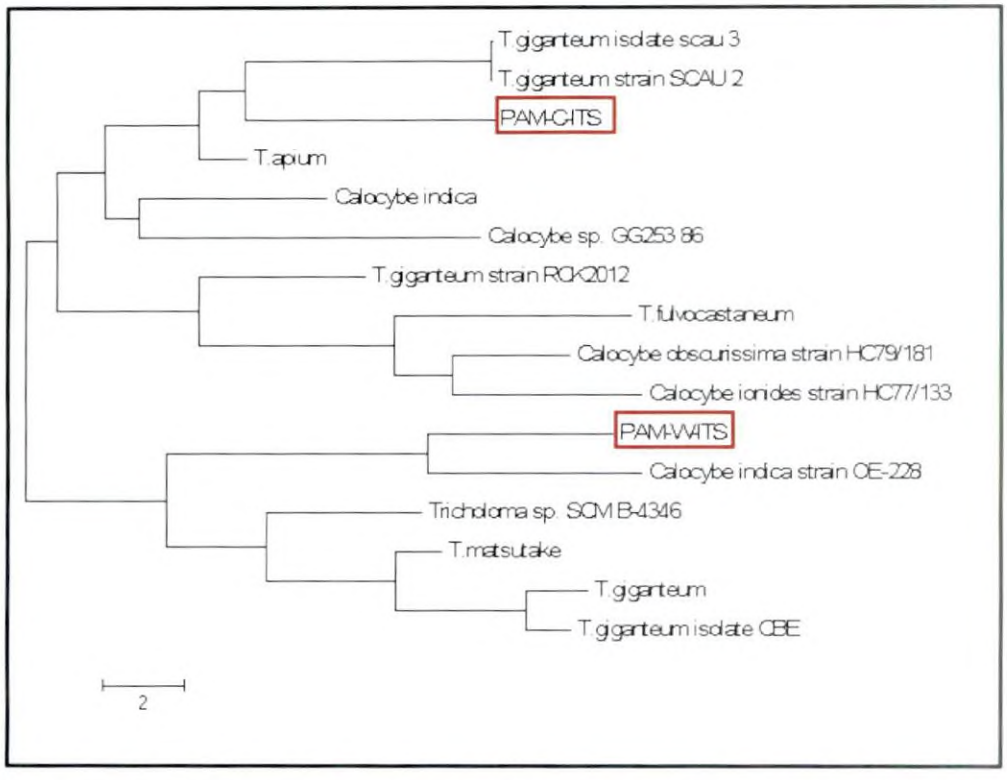


Fig 5. Phylogenetic tree showing relationship of DMR and Venjaramood strain with other species

Discussion

5. DISCUSSION

5.1 CULTURAL STUDIES

The mycelia growth of *Macrocybe gigantea* on SDA was on par with PDA. This indicates SDA is as effective as PDA for the growth of mycelia. Dense and fluffy nature of mycelia was observed in SDA. No work of this nature is available on *Macrocybe gigantea*. But Kinjo and Miyagi (2006) reported that the mycelial growth of *Tricholoma giganteum* was superior on the Hennerberg medium and GCMY (glucose, casamino acid, malt extract and yeast extract) medium. Chen and Yang (2009), reported that the growth period of *Tricholoma lobayense* mycelium in the mixed PDA medium was 30 days shorter than that in the conventional PDA medium.

The biomass production of *Macrocybe gigantea* was more in SDB with 0.52 g /100 ml as compared to PDB.

The mycelium of *Macrocybe gigantea* grew optimally at neutral pH (7). This is contrary to Kinjo and Miyagi (2006), reported that the optimum pH value for mycelia growth of *Tricholoma giganteum* is 5.0.

The results obtained when *Macrocybe gigantea* was grown in a different temperature range revealed that maximum mycelia growth was observed was at 35° C. Decrease of temperature resulted in decrease of mycelia growth. But Kinjo and Miyagi (2006) reported that *Tricholoma giganteum* prefer a temperature of 30° C for mycelia growth.

5.2 GROWTH OF *MACROCYBE GIGANTEA* ON DIFFERENT SUBSTRATES

The growth of *Macrocybe gigantea* on three different substrates such as paddy straw, bamboo leaves and neopeat were studied. Highest yield of mushroom was reported in paddy straw with biological efficiency of 72.4 per cent. Pandey and Tewari (2003) observed similar results. They reported cultivation of *Tricholoma giganteum* in paddy straw yielded biological efficiency of 92 per cent.

5.2.1 Effect of sterilization

Boiling water sterilisation observed to be good for initiating mycelial growth and prevention of rate of fungal (*Coprinus*) contamination. Chemical sterilisation observed to have high percent of fungal contaminations especially during winter and rainy days. The present study revealed that the average yield appears to be same for both boiling and chemical sterilization and there was no contamination in both treatments. Caral *et al.* (2013) reported that autoclaving method of sterilization is more efficient than the chemical sterilization method in both the substrates (sawdust and straw) for the cultivation of *Pleurotus ostreatus*.

5.2.2 Temperature preference for paddy straw substrate

The yield of *M. gigantea* obtained in paddy straw substrate was different during three different time periods. According to Das *et al.* (1987, 1991) variations in season seriously affected the number, weight and crop production period of mushroom. They reported that favorable temperature and moisture condition enhanced the production of fruiting bodies of mushroom. The maximum yield was obtained during March - April i.e., when the temperature is 30 - 35° C. This was similar to the findings of Kinjo and Miyagi (2006). They reported that *Tricholoma giganteum* prefer a temperature of 30° C for mycelia growth.

5. 3 IMMUNOMODULATORY ACTIVITY OF *M. GIGANTEA*

Immunomodulation is a process which can alter the immune system of an organism by interfering with its functions, if it results in an enhancement of immune reactions it is named as an immunostimulative drug which primarily implies stimulation of specific and non specific system, i.e. granulocytes, macrophages, complement, certain T-lymphocytes and different effector substances. Immuno-suppression implies mainly to reduce resistance against infections, stress and may occur on account of environmental or chemotherapeutic factor (Makare *et al.*, 2001). Whether certain compounds enhance or suppress immune responses can depend on a number of factors, including dose, route of administration, and timing of administration of the compound. The type of

activity, these compounds exhibit can also depend on their mechanism of action or the site of activity (Tzianabos, 2000). The immune responses through stimulation or suppression may help in maintaining a disease-free state. Agents that activate host defense mechanisms in the presence of an impaired immune responsiveness can provide supportive therapy to conventional chemotherapy (Wagner, 1983).

M.gigantea extract possess immunostimulation property, stimulating specific as well as non specific immunity. In the present study SRBC induced delayed type hypersensitivity test was used to assess effect of MG on cell mediated immunity.

Cell-mediated immunity (CMI) involves effector mechanisms carried out by T lymphocytes and their products (lymphokines). DTH requires the specific recognition of a given antigen by activated T lymphocytes, which subsequently proliferate and release cytokines. These in turn increase the vascular permeability, induce vasodilatation, macrophage accumulation and activation, promoting increased phagocytic activity and increased concentrations of lytic enzymes for more effective killing. When activated TH1 cells encounter certain antigens, viz. SRBCs they secrete cytokines that induce a localized inflammatory reaction called delayed type hypersensitivity. DTH comprises of two phases, an initial sensitization phase after the primary contact with SRBC antigen. A subsequent exposure to the SRBCs antigen induces the effectors phase of the DTH response, where TH1 cells secrete a variety of cytokines that recruits and activates macrophages and other non specific inflammatory mediators. The delay in the onset of the response reflects the time required for the cytokines to induce the recruitment and activation of macrophages (Dashputre and Naikwade, 2010).

Therefore, increase in DTH reaction in rats in response to T cell dependent antigen revealed the stimulatory effect MG on T cells. DTH response was determined by increased paw volume using plethysmometer. There was a

significant increase in the paw volume in animals treated with MG at 100 mg kg^{-1} and 150 mg kg^{-1} which indicates the immunostimulatory activity of the extract.

The active paw anaphylaxis in mice model is type – I IgE mediated anaphylactic reaction by using ovalbumin as an allergen (Agarwal *et al.*, 1999). Increased vascular permeability and release of histamine from mast cells was associated with allergic reaction. Treatment with MG at 50 mg kg^{-1} and DSCG (standard drug) significantly reduced the paw volume as compared to control group which revealed the mast cell stabilizing effect.

Bone marrow is a source of cells involved in immune activity and is the sensitive organ most affected during any immunosuppression therapy with cytotoxic drugs. Stem cells degeneration and inability of bone marrow to regenerate new blood cells will give rise to thrombocytopenia and leucopenia (Pelczar *et al.*, 1990). Cyclophosphamide suppresses humoral, cellular, non specific and specific cellular immune response. When animals were treated with cyclophosphamide, then haemoglobulin (Hb), RBC counts, WBC count, lymphocyte per cent and platelet count all were reduced significantly (Rastogi *et al.*, 2008).

MG at the doses (100, 200 and 300 mg kg^{-1} body weight) p. o. increased the total WBC count and differential leukocyte count compared with cyclophosphamide treated group. These results clearly indicated the protection of suppressive effect of cyclophosphamide by administration of ethanolic extract of *M. gigantea*.

The carbon clearance assay was used to evaluate the effect on reticuloendothelial cell mediated phagocytosis (Jayathirtha and Mishra, 2004). When ink containing colloidal carbon is injected intravenously, the macrophages engulf the carbon particles of the ink. The rate of clearance of carbon particles (ink) from blood is known as phagocytic index which is determined by the exponential equation. Treatment with MG at doses (100, 200, 300 mg kg^{-1} body weight) enhanced the rate of carbon clearance from the blood when compared to

the control group. This shows the enhancement of the phagocytic activity of mononuclear macrophage and nonspecific immunity, which includes opsonisation of the foreign particulate matter with antibodies and complement C3b, leading to a more rapid clearance of foreign particulate matter from the blood (Furthvan and Bergvanden, 1991).

The results of mast cell study showed that MG has potent anti allergic activity. MG at doses (100, 200 mg kg^{-1} body weight) and DSCG (standard drug) significantly inhibited mast cell degranulation by preventing the release of inflammatory mediators from mast cells and enhancing their membrane stability.

Mast cells are known to be the primary responders in allergic reactions, most of which are triggered by cross-linking of high affinity Ig E receptors (Gohil and Mehta, 2011). Agents that stimulate intracellular calcium level have been shown to induce mast cell degranulation (Tasaka *et al.*, 1986). In the study egg albumin was used to induce mast cell degranulation. Histamines which play a major role in allergic reactions are released from mast cells during degranulation. Hence, the antiallergic activity of MG is due to its mast cell stabilizing effect.

5.4 HEPATOPROTECTIVE ACTIVITY OF *M. GIGANTEA*

The results of hepatoprotective studies revealed that ethanolic extract of *M. gigantea* possess significant protective effect against acetaminophen and CCl_4 induced chronic hepatotoxicity. The extent of hepatic damage is assessed by the level of increased cytoplasmic enzymes (SGPT, SGOT and ALP), serum bilirubin content in circulation and by histopathological examinations (Sallie *et al.*, 1991).

Acetaminophen or paracetamol is widely used as analgesic and antipyretic. However, paracetamol overdosing causes severe hepatotoxicity that leads to liver failure in both humans and experimental animals (Kaplowitz, 2005; Ghanem *et al.*, 2009; Chen *et al.*, 2009). At therapeutic doses acetaminophen is rapidly metabolized in the liver principally through glucuronidation and sulfation reactions and only a small portion is oxidized by cytochrome P-450 into highly reactive and cytotoxic intermediates (Figure 6). These molecules, N-acetyl-p-

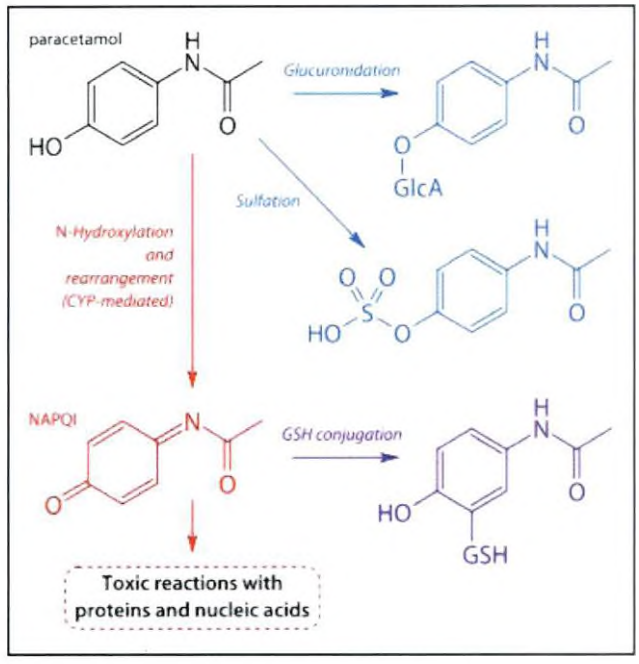


Fig.6. Paracetamol metabolism

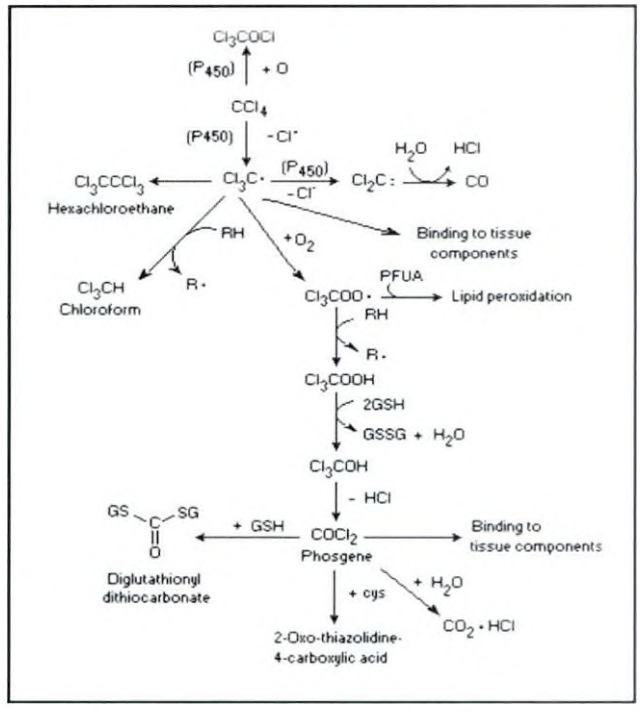


Fig.7. Biotransformation of carbontetrachloride (Harris and Anders, 1981; Anders and Jakobson, 1985; Mcgregor and Lang 1996)

benzoquinone imine (NAPQI) or N-acetyl-*p*-benzosemiquinone imine (NAPSQI) can be quickly conjugated to hepatic glutathione (Mahesh *et al.*, 2009; Mitchell *et al.*, 1973). Both NAPQI and NAPSQI are chemically very active and they are capable of taking part in free radical reactions. Consequently, paracetamol overdosing can lead to a number of unfavorable consequences, especially those affecting the liver (Kurtovic and Riordan, 2003). A large dose of the drug causes depletion of the hepatic cellular glutathione (GSH). NAPQI reacts rapidly with GSH, a phenomenon that exacerbates oxidative stress in conjunction with mitochondrial dysfunction. The GSH depletion, occurring in acute hepatotoxicity, gives free way to the highly reactive intermediates, whose actions on structural and functional molecules affects the liver functions and leads to massive hepatocyte necrosis, liver failure or death (Oz *et al.*, 2004; Masubuchi *et al.*, 2005; Marzullo, 2005). Since oxidative stress and GSH depletion contributes to the acetaminophen induced liver injury, the agents with antioxidant properties and/or GSH preserving ability may provide a preventive action against hepatocellular injury (Hsu *et al.*, 2008).

The involvement of free radicals in the pathogenesis of liver injury has been investigated for many years (Poli, 1993). In the liver, CCl_4 is metabolically activated by cytochrome P450-dependent mixed oxidases in the endoplasmic reticulum to form the CCl_3 radical (Figure 7) that combines with cellular lipids and proteins in the presence of oxygen to induce lipid peroxidation by hydrogen abstraction (Kadhska *et al.*, 2000; Lim *et al.*, 2000). This results in structural changes in the endoplasmic reticulum and other membranes and losses in metabolic enzyme activations with the consequent impairment of liver functions.

Many studies have shown that the hepatoprotective activities may be associated with an antioxidant capacity to scavenge reactive oxygen species (ROS) (Purmova and Opletal, 1995). Previous studies reported that *M. gigantea* possess strong *in vitro* free radical scavenging activity and inhibition of lipid peroxidation (Banerjee *et al.*, 2007).

The antioxidant and hepatoprotective activities may be due to the presence of flavonoid and phenolic compounds (Di Carlo *et al.,* 1999). Phenolic compounds possess antioxidant, radical scavenging, anti-mutagenic and anti-carcinogenic properties (Jayaprakasha *et al.,* 2007). Previous reports on phytochemical analysis of ethanolic extract of *M gigantea* have shown that it contains high concentration of phenolic compounds and flavonoids (Acharya *et al.,* 2012). These results support the findings on hepatoprotective activity of the MG.

Serum AST, ALT, ALP and bilirubin are the most sensitive markers employed in the diagnosis of hepatic damage, because these are cytoplasmic in location and are released into the circulation after cellular damage (Sallie *et al.,* 1991).

Administration of both acetaminophen and CCl_4 markedly increased serum levels of ALT, AST, ALP, serum bilirubin and cholesterol. This can be attributed to the altered structural integrity of hepatic cells. The increased levels of serum enzymes, bilirubin and cholesterol were considerably reduced by pre-treatment with ethanolic extract of *M. gigantea* to acetaminophen and CCl_4 treated rats. This indicates that MG possesses the ability to preserve the structural integrity of the liver from the adverse effects of acetaminophen and CCl_4.

The histopathological studies revealed that there was improvement in the liver architecture on treatment with MG, suggesting the protective effects of the extract. These results can be correlated with data obtained from evaluation of the biochemical parameters.

Several workers reported the hepatoprotective potential of various extracts and purified compounds mushrooms. Ooi, (1996) investigated the hepatoprotective activities of aqueous extracts of *Volvariella volvacea*, *Lentinula edodes*, *Flammulina velutipes*, *Auricularia auricular*, *Tremella fuciformis*, *Grifola frondosa* and *Tricholoma lobayense* were screened against paracetamol-induced liver injury. Carbontetrachloride induced chronic hepatotoxicity in rats is

protected by ethyl acetate extract of *Phellinus rimosus* (Ajith *et al.*, 2006). *In vitro* and *in vivo* protective effects of *Ganoderma lucidum* proteoglycan were exhibited on CCl_4 induced hepatic tissue damage (Yang *et al.*, 2006).

5.5 NUTRITIVE VALUE.

The nutrient composition *Macrocybe* was found to be protein (24.10 %), carbohydrate (11.20 %) and glycogen (5.6 %).

Similar findings on the nutrient content in various mushrooms were reported by several workers. Liu *et al.*, (2007) studied the nutrient content of *Tricholoma giganteum* and *Pleurotus eryngii* cultivated with cotton seed hull compost and found that contents of protein, fat, total sugar and crude fibre in *T. giganteum* and *P. eryngii* were 35.28, 2.91, 53.74, 8.76 per cent and 15.4, 0.55, 52.10, 5.40 per cent respectively. Prakasam *et al.* (2011) reported that *Tricholoma giganteum* contain 86.20 per cent moisture, 32.9 per cent crude protein, 11.8 per cent carbohydrate, 0.91 per cent crude fat, 20.71 per cent crude fibre, 8.32 per cent ash, 5.60 per cent iron, 1.18 per cent manganese, 1.38 per cent zinc and 1.10 per cent copper.

5.6 DIVERSITY ANALYSIS USING ITS

The sequences were analyzed by multiple sequence alignment (ClustalW) to check similarity amongst the species. Search for sequence identity in the GenBank DNA database was carried out using BlastN (NCBI) (Altschul *et al.*, 1997), which revealed that ITS region of *M. gigantea* (Solan strain–PAM–C-ITS) showed 92 per cent similarity with *Tricholoma giganteum* and Venjaramood isolate (PAM-W-ITS) showed 93 per cent similarity with the *Calocybe indica* sequences of NCBI database.

Phylogenetic tree constructed using MEGA 4 revealed that the wild species obtained from Venjaramood was morphologically similar to *M.gigantea* but on sequence analysis it was found to be *Calocybe indica.*

Summary

6. SUMMARY

The study entitled "Medicinal and nutraceutical potential of giant mushroom (*Macrocybe gigantea* (Massee) Pegler & Lodge)" was conducted at the Ethnomedicine and Ethnopharmacology Division and Microbiology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, during the year 2013 to 2014. The objectives of the study was to assess the medicinal and nutraceutical potential of *Macrocybe gigantea* with special emphasis on immunomodulation, hepatoprotection and diversity evaluation.

Macrocybe gigantea (Massee) Pegler & Lodge culture obtained from Directorate of Mushroom Research (ICAR), Solan was used for the study.

The growth of *M. gigantea* was tested in two different solid media *viz.* PDA and SDA. Maximum growth was observed in SDA. The biomass production in liquid broth revealed that SDB was superior to PDB in biomass production. Studies on the effect of pH (5, 7 and 9) on radial growth of *M. gigantea* revealed that pH 7 was optimum for the growth of mycelia. The effect of temperature (25, 30, 35° C) on radial growth was tested and found that temperature of 35° C was effective in supporting the growth of *M. gigantea.*

Studies on the growth of *M. gigantea* on three different substrates namely paddy straw, bamboo leaves and neopeat revealed that beds prepared with paddy straw produced maximum biological efficiency. No contaminations were observed on both methods of substrate (chemical and boiling) sterilization. The temperature preference for paddy straw substrate was compared by cultivating *M. gigantea* during three different time periods *viz.* October - November, December - January, March - April and found that the maximum yield was obtained during March - April.

The immunomodulatory effect of *M. gigantea* was evaluated by different experiments such as Delayed type hypersensitivity (DTH) test, Active paw

anaphylaxis test, Inhibition of cyclophosphamide induced myelosuppression, Carbon clearance assay and Mast cell study.

In the DTH test, significant increase in the paw volume in animals treated with MG at 100 mg kg^{-1} and 150 mg kg^{-1} was observed which indicates the immunostimulatory activity of the extract. Treatment with MG at 50 mg kg^{-1} significantly reduced the paw volume as compared to control group in active paw anaphylaxis test which revealed the mast cell stabilizing effect. MG at the doses (100, 200 and 300 mg kg^{-1} body weight) p. o. increased the total WBC count and differential leukocyte count compared with cyclophosphamide treated group indicated the protection of suppressive effect of cyclophosphamide. Treatment with MG at doses (100, 200, 300 mg kg^{-1} body weight) enhanced the rate of carbon clearance from the blood with significant increase in the mean phagocytic index when compared to the control group. This shows the enhancement of the phagocytic activity. MG at doses (100, 200 mg kg^{-1} body weight) significantly inhibited mast cell degranulation by preventing the release of inflammatory mediators from mast cells and enhancing their membrane stability which indicates the anti allergic activity of the extract.

The hepatoprotective effects of MG on acetaminophen and CCl_4 induced hepatic injury in rats was tested at three different doses i.e. 100, 200, 300 mg kg^{-1} body weight. Treatment with MG at above doses was found to significantly lower the serum hepatic enzyme levels *viz.* AST, ALT, ALP and serum bilirubin and cholesterol of acetaminophen and CCl_4 treated rats. The histopathological architecture of liver sections of rats treated with MG showed more or less normalized lobular pattern almost comparable to normal and silymarin treated animals with well preserved cytoplasm and prominent nucleus, suggesting the protective effects of the extract

In the nutritive analysis, the protein, carbohydrate and glycogen contents of *Macrocybe* were found to be 24.10 per cent, 11.20 per cent and glycogen 5.6 per cent respectively.

Sequence analysis revealed that ITS region of *M. gigantea* (Solan strain) showed 92 per cent similarity with *Tricholoma giganteum* and Venjaramood isolate showed 93 per cent similarity with the *Calocybe indica* sequences of NCBI database.

Phylogenetic tree constructed using MEGA 4 revealed that the wild species obtained from Venjaramood was morphologically similar to *M. gigantea* but on sequence analysis it was found to be *Calocybe indica.*

Based on the results obtained during the investigation it can be concluded that *Macrocybe gigantea* can be cultivated in Kerala during March – April to get maximum yield. Further studies are needed to explorate the correct mechanism of action to prove its therapeutic utility. Thus *M. gigantea* can be used as a best candidate for liver protection, immunomodulation and as a nutraceutical for the benefit of human kind.

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Appendices

APPENDIX I

Potato dextrose agar (PDA)

Potato	200 g
Dextrose	20 g
Agar-agar	20 g
Distilled water	1 l

In case of Potato dextrose broth (PDB) the component agar-agar is avoided

APPENDIX II

Sabouraud dextrose agar (SDA)

Dextrose	40 g
Mycological peptone	10 g
Agar-agar	20 g
Distilled water	1 l

In case of Sabouraud dextrose broth (SDB) the component agar-agar is avoided

APPENDIX III

TES lysis buffer

Tris - HCl (pH 8.0)	100 *mM*
EDTA (pH 8.0)	10 *mM*
SDS	2 %

APPENDIX IV

CTAB extraction buffer

CTAB	2.5 %
Tris- HCl (pH 8.0)	100 *mM*

EDTA	25 *mM*	
NaCl	1.5 *M*	
β-mercaptoethanol	0.2 % (v/v)	freshly added prior to DNA extraction
PVP	4 % (w/v)	

APPENDIX V

TE buffer

Tris – HCl (pH 8.0)	10 *mM*
EDTA (pH 8.0)	1*mM*

APPENDIX VI

TBE buffer (5X) for 1 liter solution

Tris base	54 g	(0.445 *M*)
Boric acid	27.5 g	(0.445 *M*)
0.5 M EDTA (pH.8.0)	20 ml	(0.01 *M*)

APPENDIX VII

Gel loading buffer (6X)

Bromophenol blue	0.25 %
Glycerol	30 %
Sterile water	70 %

APPENDIX VIII

ITS sequences

> DMR strain (PAM –C-ITS)

CAGGGGCCAACCGCCACAGAGGTCAAATGATCAAAGACAGTTAATTG
TCCTGTTAGAAAGCTGAACCACACAATATCGTGCTAAGCCACAGCGTA
GATAACTATCACACCAGGGGCTAAATCAACCATTGGTCCCACTAATGT
ATTTTAAAAAAAACTGACTCTTTGAACACAAAAAGCCCGCAACTCCCAC
ATCCCAACACTTTGAAACAAAAAAGTAACAAAGGGTGAAAATTTCAT
GACACTCAAACCGGGATGCTCCTCGGAATACCCAGGAACGCAAGGGG
CGTTCAAAGATTCCATGAATCACTGAATTCTGCAATTCACATTACTTAT
CGCAATTCCCTGCGTTCTTCATCCATGCCAGAGCCAAGAAATCCCTTGT
TGAAAGTTGTATATTAATTAAAGGCATCAAAAAAATGCCTTAAAAAAC
ATTCTTATACCTAGAGTAAATGATATGAAAAGACCTAAACCTTGGAAT
ATAAAGGCCAAGATCACTCTCGACTCAAAACTTTACCCAAAGTCTACC
AAAGGGGCACAAGTGGTTTAAGAAACAATAACCCAGCGTGCACATGC
TCTGAAAGGGCCCGCAACCACTTGACCAAATTCATTCACTAATGATCC
TTCCCCAAGGTCACCTACCGAAACCTTGTTACCACTTTTACTTCCAAAA
AA

> Venjaramood strain (PAM –W – ITS)

TTTTTAACCCTCCCACCCCCAGAGGTCAAATAATCAAAGACAGTTGTT
GTTGTCTCGTTAGAAAGCTGAACCACACAATATCGTGATGAGCCACGG
CGTAGATAACTATCACACCAGGGAGCTAATCAACGATGGGTCCCACTA
ATGTATTTCAGAGGAGCCGACTCTTTCAACAAAAGCCAGCAACCTCCA
CTTCCAGACTCTTTGAAACAAAAAAAGTAACACAGGTGGAGAATTTCT
TGACACTCTCACAGGCGTGCTCCTCGGAAAACCCCGGAGCGCACGGGG
CGTTCAAAGATACGATGATACACTGAATTCTGCGATTCACATTACTTAT
CGCATTTTTCTGCGTTCTTCATCGATGCGAGAGCCAAGAGATCCCTTGT
TGAAAGTTGTATATGATTTAAAGGCGTTAATAATGCCTTATAAAACAT
TCTTATACACACAGTAAAATGATATGAAAAAGACATATACACGGATGT

Abstract

GTAAGGGGCAAGATAACTCTCTACTCACACTCTTCCCAAGTCTACAAA
AGGTGCGCAAGTGGGTTTAAAAAAAAAAAACCCAGTGTGGACATGGT
CTTAAAAAGCCCCCAACAACTTGACCAAATTTATTTTCCAATGATCCTT
CCCCGGTTTTCCTTCTGAAAACTTGTTTCCACATTTTTCTTTCAAAAAA
A

Abstract

MEDICINAL AND NUTRACEUTICAL POTENTIAL OF GIANT MUSHROOM

(*Macrocybe gigantea* (Massee) Pegler & Lodge)

PAMITHA N. S.

(2009-09-106)

Abstract of the

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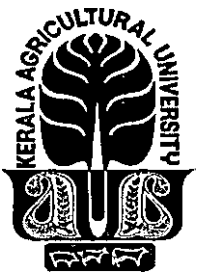

M.Sc. (INTEGRATED) BIOTECHNOLOGY COURSE

DEPARTMENT OF PLANT BIOTECHNOLOGY

COLLEGE OF AGRICULTURE

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KERALA, INDIA

2014

ABSTRACT

The study entitled "Medicinal and nutraceutical potential of giant mushroom (*Macrocybe gigantea* (Massee) Pegler & Lodge)" was conducted at the Ethnomedicine and Ethnopharmacology Division and Microbiology Division of Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI), Palode, Thiruvananthapuram, during the year 2013 to 2014. The objectives of the study was to assess the medicinal and nutraceutical potential of *Macrocybe gigantea* with special emphasis on immunomodulation, hepatoprotection and diversity evaluation.

Macrocybe gigantea cultures were procured from DMR, Solan. The culture and cultivation conditions were standardised in different substrates, media, pH and temperature. The temperature preference for substrates and the effect of sterilization methods were also studied. The immunomodulatory potential of *M. gigantea* was determined by delayed type hypersensitivity (DTH) test, active paw anaphylaxis test, inhibition of cyclophosphamide induced myelosuppression, carbon clearance assay and mast cell study. The hepatoprotective potential of *M. gigantea* was determined using acetaminophen induced hepatotoxicity study and carbon tetrachloride induced liver damage. In the nutritive analysis, the total carbohydrates, protein and glycogen contents were estimated. The diversity analysis was done using ITS sequence data.

Cultural studies conducted showed that SDA was as effective as PDA in favouring the growth of *Macrocybe gigantea.* The maximum mycelia growth of *M.gigantea* was observed at temperature 35° C (8.20 cm) and pH 7 (8.60 cm). Highest yield of mushroom was obtained in paddy straw beds with biological efficiency of 72.40 per cent and the best time period for cultivation was found to be March – April. No contaminations were observed in both methods of substrate sterilization i.e. chemical and boiling methods.

The hepatoprotection and immunomodulation studies revealed that *Macrocybe gigantea* possess significant hepatoprotective activity at 300mg kg^{-1} and immunomodulatory activity at 100mg kg^{-1}.

Analysis of the nutrient composition of *Macrocybe gigantea* indicated that the protein, carbohydrate and glycogen content were found to be 24.10 per cent, 10.20 per cent and 5.6 per cent respectively.

Sequence analysis revealed that ITS region of *M. gigantea* (Solan strain) showed 92 per cent similarity with *Tricholoma giganteum* and Venjaramood isolate showed 93 per cent similarity with the *Calocybe indica* sequences of NCBI database. The phylogenetic tree constructed using MEGA 4 revealed that the wild species obtained from Venjaramood was morphologically similar to *M. gigantea* but on sequence analysis it was found to be *Calocybe indica.*

Based on the findings of the present investigation, it is confirmed that *Macrocybe gigantea* can be cultivated in Kerala during March – April for increasing its biological efficiency. For the first time, the hepatoprotective potential and immunomodulatory potential of *M. gigantea* has been scientifically validated. Further studies are needed to explorate the correct mechanism of its action with a biomarker-based approach for upgrading *M. gigantea* from functional food to holistic medicine to utilize its therapeutic potential.