BIOPROSPECTING OF SELECTED MACROFUNGI IN TALUKS OF SHIMOGA DISTRICT

Thesis submitted to

Kuvempu University
for the award of Degree of

DOCTOR OF PHILOSOPHY
in
APPLIED BOTANY

By

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Declaration

I, **Jayashree K. Kodiyalmath,** hereby declare that the thesis entitled **"Bioprospecting of Selected Macrofungi in Taluks of Shimoga District"** embodies the results of bonafide research work carried out by me under the guidance of **Dr. M. Krishnappa,** Professor, Department of Applied Botany, Kuvempu University, Jnana Sahyadri, Shankaraghatta. I further declare that this or part thereof has not been the basis for the award of any other degree or diploma either in this or any institution or university.

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COURSE WORK COMPLETION CERTIFICATE

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- Jayashree K. Kodiyalmath

ABBREVIATIONS

%	Per cent
°C	Degree Celsius
µl	Microliter(s)
cm	Centimetre(s)
DLA	Daltons Lymphoma Ascites
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
DNSA	Dinitro Salicylic Acid
e.g.	Exempli gratia (for example)
EAC	Ehrlich Ascites Carcinoma
et al.	And others (Co-authors)
GC-MS	Gas Chromatography-Mass spectrometry
h	Hour(s)
HD	High Dose
ITS	Internal Transcribed Spacer
LD	Low Dose
m	Meter(s)
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimeter(s)
NaOH	Sodium Hydroxide
NCBI	National Centre for Biotechnology Information
rpm	Rounds Per Minute
SAM	Senescence Accelerated Mouse
sp.	Species singular
spp.	Species plural
viz.,	Namely

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Introduction

Mushrooms are the fruiting bodies of fungi belongs Basidiomycetes and even some of the Ascomycetes. Mushroom is defined as "a macro fungus with a distinctive fruiting body, which may be above ground (epigeous) or below ground (hypogeous) which has a fruiting body that can be seen by the naked eye and to be picked up by hand" (Chang and Mile, 1992). Whereas mycelia remains buried underground, around the tree roots below the leaf litters, inside the tissues of plants and on fallen wood logs (Halpern, 2007). These are omnipresent and helpful in decomposing dead and decaying organic matter like litter (Krivtsov *et al.*, 2007).

Fungi plays very important role in nature as it is a molecular disassembler which breakdown the complex compounds like cellulose, carbohydrates and plant proteins biodegrade lignin into simpler (Tuomela *et al.*, 2000). Due to this, fungi are very important in an ecosystem to maintain healthy soil. Fungi also associate with roots of some plants, these are called mycorrhizae. These fungi act as secondary roots which penetrate deep into soil to get nutrients that tree its self cannot get, in return plants provide shelter and food to fungi (Ingold, 1993), because of their ability of degrading natural recalcitrant molecules e.g., lignin used for the biodegradation of xenobiotics (Wesenberg *et al*., 2003). Though mushroom fruiting bodies are short-lived, the underlying mycelium can itself be long-lived and massive. Mushrooms are estimated to be 41,000 species of which 850 are reported from India (Manoharachary *et. al.*, 2005). According to their usage, mushrooms can be divided into four groups, Edible, Medicinal, Poisonous and Miscellaneous (Sridhar *et al.*, 2011).

Mushrooms have been used as food and traditional medicine for more than 3000 years (Fabricant and Farnsworth, 2001). Edible fungi have long been used as garnishes or as medicine in folk but mushroom represent a high quality proteinaceous food and industrially useful enzymes and biologically active principles which successfully convert organic waste materials into useful things. Mushrooms are nutritionally functional foods and a source of physiologically beneficial, non-invasive and non-toxic medicines. Many pharmaceutical substances with potent and unique health-enhancing properties have been isolated from medicinal mushrooms and distributed worldwide. Mushroom-based products obtained from mycelia or fruiting bodies are consumed in the form of capsules, tablets or extracts (Nitha *et al*., 2007).

The protein content of fresh edible mushroom is 3.7 per cent, it is double of vegetables and lower than meat and egg. It has high essential amino acids, traces of sugar and contain no cholesterol and very less fat. Mushrooms also contain vitamins like B-1, B-2 B-12 and vitamin c, with essential minerals like P, K, Fe and copper (Sohi, 1992; Kalac, 2009). Out of 10000 species of fleshy fungi about 150 are considered as prime edible mushrooms. Only 60 are cultivated commercially, and only ten have reached an industrial scale in many countries. In India *Agaricus bisporus, Pleurotus sojar kaju, Volvariella volvacea, Pleurotus florida, P. pulmonarius, P. citrinopileatus* and *Flamulina velutipes* are commonly cultivated. More than 190 species of poisonous mushrooms belonging to 58 genera and 26 families are reported from Asia, of these, 179 species are Basidiomycetes belonging to 50 genera and 20 families. More than 40 species are highly poisonous and about 30 species may result in death if consumed. More than 20 other species have slight toxicity (Mao, 1980, 2000).

After discovery of Penicillin in 1929 by Alexandra Fleming, fungi were regarded as rich source of natural antibiotics and other bioactive compounds like polysaccharides, glycoproteins, proteoglycans, terpenoids, fatty acids, proteins lectins that possess certain medicinal properties (Moradali *et al.,* 2007), not only polysaccharides, triterpinoids and also a wide range of different class of chemical compounds are produced by mushroom like glycolipids (Schizonellin), compounds derived from the shikimic acid (strobilurins and oudemansins), fatty acid derivatives (filiboletic acid, podoscyphic acid), polyketides (caloporoside, hericinones A-H), nucleosides (clitocine, nebularine), aromatic phenols (drosophilin, armillasirin, omphalone), different sesquiterpenes (protoilludanes, marasmanes, hirsutanes, caryophyllanes), diterpenes (cyathins, striatal), polyacetylenes (arocybin, xerulin), sesterterpenes (aleurodscal), and other substance of different origin (Wasser, 2002).

Mushroom represent a major and potent pharmaceutical products. A wide range of activities like antitumor cardiovascular and antimicrobial properties are reported. They are also reported to possess promising antioxidative, hypercholesterolemia, hepato-protective and anticancer effect. Besides mushroom's pharmacological feature they are important in our diet because of their nutritional value, like high protein and low fat and energy content. Mushrooms are boon for progress in field of medicine and food especially in developing countries like India (Khatun *et al.,* 2012). There are many more mushroom species of wild and cultivated, edible and non-edible species are analysed for both their nutritional and nutraceuticals components. Mushrooms species that have been analysed extensively for medicinal properties are *Cordyceps militaris (*Caterpillar fungus*), Ganoderma lucidum (*Reishi*), Lentinus edodes (*Shiitake*), Grifola frondosa*

*(*Maitake*), Pleurotus ostreatus (*Oyster mushroom*), Hericium erinaceous (*Lions mane*)* and *Agaricus blazei* (Hime-matsutake) (Lakhanpal and Rana, 2005). Mushrooms like *Agaricus bisporus, Armillaria mella, Boletus edulis, Calvatia exipuliformis, Lepiotar hacodes, Lepista nuda, Paxillus involutus, Psalliola compestris, Pleurotus sojor-caju, Pleurotus ostreatus, Rasulla delica* and *Tricoloma terreum* absorb heavy metals like Pb, Cu, Mn, Cd and Hg thus these mushroom can be used as a low cost technology to solve the problem of heavy metal pollution. In this way mushroom can be used as bioremediation for overcome pollution problems (Das, 2005).

Human immune system consists a group of mechanisms to protect against diseases, to identify and kill pathogens like bacteria fungi and viruses. Compounds derived from mushroom are good source of antimicrobial which kill a wide range of pathogens which already have become resistance to allopathic medicines (Shavit, 2009). Antimicrobial includes antibacterial, antifungal and antiviral (David, 2012; Guler, 2009). *Ganoderma lucidum* can inhibit the multidrug resistant *Staphylococcus aureus* (Prasad and Wesely, 2008), *Fomitopsis pinicola* and *Lactarius vellereus* are potent to inhibit fungi (Guler *et al*., 2009). Whereas *Pleurotus florida* and *Phellinus gilvus, Agaricus bernardii, A. arvensis, A. bisporus, A. porphyrocephalus* and *A. silvicola*, these mushroom exhibit antimicrobial nature (Ehssan and Saadabi, 2012; Bhat *et al*., 2011; Sittiwet and Puangpronpitag, 2008). Elements from sporocarp and mycelia both contain antimicrobial compounds which can be isolated for the welfare of human beings (Yamac, 2006). *Phellinus hartigii, P. swieteniae* and *P. merrillii* are antimicrobial in nature (Altuner and Akata, 2010; Belsare *et al.,* 2010). *P. ignarius* proved good antiviral agent against influenza virus (Song *et al.,* 2014). *Entoloma nubigenum* exhibited its

antimicrobial nature when tested by Reinosa *et al.* (2013), but *H. erinaceus* alone produce six different antimicrobial bioactive compounds (Thongbai, 2015). Three species of *Pleurotus* like *P. ostreatus, P. eryngii* and *P. soroj-caju* inhibited different gram +ve and gram –ve bacteria and fungi like *Aspergillus* and *Fusarium* etc. (Gregori *et al.*, 2007), *Lentinula edodes* and *Grifola frondosa* increase host immune defence against viral infections even against AIDS (Poucheret, 2006).

Mushrooms are used as antitumor, antiviral, anti-allergants (Wisitrassameewong *et al.*, 2012) and anti-inflammatory (Mouroa *et al.*, 2011). *Phellinus* is a potent mushroom as *Ganoderma*. The secondary metabolites of *Phellinus* were used to cure gonorrhoea, abdominal pain, stomach ailments and diarrhoea (Sonawane *et al.*, 2012). *Phellinus boumii* showed good hypoglycaemic effects against ob/ob mice (Cho *et al.*, 2007). *Phellinus* are wood inhibiting fungi and may be used as antioxidant and anticancerous (Khatun, 2012). *P durissimus* and *P. lintenus*are antioxidant (Lahiri *et al.*, 2010; Liang *et al.*, 2009). *Phellinus linteus* is a medicinal mushroom used in China and Korea and has history of 2000 years of being used for treatment of haemorrhage, haemostasis and menstruation in China (*Chen et al*., 2016).

Immunostimulants are those compound that stimulate the body's own response systems and mechanisms to fight disease but they do not harm the body or and increase the body's own resistance to stress and trauma. The cellular compounds and secondary metabolites derived from edible mushroom have immunomodulatory activity (Shavit, 2009). *Lentinula edodes, Trametes versicolar, Grifola frondosa* and *Ganoderma* species seems to be most promising stimulator of interleukin, interferon and T-cells proliferation. All these show opposite effect Cyclophosphamide, boosting immune system, mushroom

improve humoral immune response and cellular immune response. Mushrooms have immunopotentiation which balance immune suppression that induced by chemotherapy, for cancer treatment. Influence of mushroom metabolites on immune function and hormonal system seems to underlay some of the major beneficial effects of higher fungi (Wasser, 2002; Poucheret *et al.*, 2006).

The study area come under Western Ghats, is rich in plant diversity, it is one of the 34 global hotspots of biodiversity (Prasad and Al-Sagheer, 2012). Western Ghats has many endemic plant and animal species. Shivamogga district in Karnataka has semi-evergreen and moist deciduous forest, these forest has rich diversity of mushrooms (Sentilarasu, 2010; Mohanan, 2014; Krishnappa, 2014; Karun, 2015, 2016; Swapna *et al.*, 2008) surveyed for macrofungal diversity from 2005 to 2007.

The present study is concentrated on the bioprospecting aspects of *E. speculum, Phellinus linteus* and *P. tremulae*. There is no information on the bioprospecting of selected wild macrofungi. Hence, the study undertaken with the following objectives.

Objectives

1. To collect and characterize the macrofungi in the parts of Western Ghats.

2. To extract the secondary metabolites from sporocarps of macrofungi.

3. To culture the selected macrofungi and extract secondary metabolites from culture.

4. To characterize the secondary metabolites.

5. To study the pharmacological properties of secondary metabolites from sporocarp.

Chapter - 2

Review of Literature

Balauiri *et al*. (2016) reviewed the methods of *in vitro* antimicrobial activity. Authors stated that agar well diffusion method is widely used and one of the good method to evaluated antimicrobial activity.

Owaid *et al*. (2015) investigated antimicrobial activity of *Pleurotus ostreatus, P. cosnucopiae* and *P. salmoneostramineus,* fruiting body mycelia as well as culture filtrate. All three extracts were effective against pathogens. But liquid filtrate of *P. salmoneostramineus* was best against *P. aeruginosa* and *Candida paraprilosis*, compared to other filtrate extracts. Mycelia of *P. cosnucopiae* inhibited bacteria effectively.

Dasgupta *et al*. (2015) screened the phytochemistry of *Entoloma lividoalbum* on edible mushroom from Darjeeling. The dried mushrooms were extracted with ethanol. The phytochemical test showed the presence of phenols, β-carotene, lycopene and flavonoids.

Pei *et al*. (2015) cultured the *Phellinus linteus* mycelia and isolated polysaccharide (Pl-NI) from alkaline extract. Pl-NI in antitumour assay *in vitro* showed inhibition of growth of HepG2 cells to certain extent. So polysaccharide from *P. linteus* is a potent antitumour in agent.

Pranitha *et al.* (2014) evaluated the antibacterial activity of *Trametes versicolar* by extracting from methanol against bacteria *P. aeruginosa, Bacillus subitlis, S. aureus* and fungal pathogens *Penicillium* spp., *A. flavus, A. niger, A. fumigatus* using agar well

diffusion method. Methanol extract inhibited *P. aeruginosa* and *S. aureus* maximum in fungi pathogens *A. fumigatus* and *A. niger* were maximum inhibited.

Lin (2014) evaluated the anti-inflammatory activity of *Phellinus linteus* fermented broths, which suppressed macrophages LPS- mediated nuclear factor (NK-kB activity and tumour necrosis factor (TNF)-d *P. linteus* fermented broths contained vast amount of histopolon which showed the greatest anti-inflammatory activity in RAW264.7 cells and murine primary peritoneal exudates macrophages (PEMs).

Kim and Lee (2014) screened the antimicrobial activity of 1,2-Benzendiol which was extracted by roots of *Diospyros kaki*. 1,2-Benzendiol showed antimicrobial activity against *Bacillus cerenus, Listeria monocytogenes, Salmonella enterica, S. typhimurium, Shigella sonnei, Staphylococcus aureus, S. epidermidis* and *S. intermedius.* 1,2-Benzendiol isolated from Persimmo roots and its structural analogues show antimicrobial activities against food-borne bacteria.

Song (2014) proved a sesquiterpenoids from *Phellinus ignarius* was potent enough to inhibit H5N1 influenza, A virus by inhibiting the enzymes Neuraminidase (NA).

Rai *et al.* (2013) tested antimicrobial activity of four wild mushrooms *Meripilus giganteus, Entoloma lividoalbum, Ramaria aurea* and *Pleurotus flabellatus.* Ethanolic and ethyl acetate fraction of wild mushrooms against *P. aeruginosa, E. coli* and *Candida albicans* by disk diffusion method. The ethanolic extraction of *P. flabellatus* and *M. giganteus* showed greater activity than *E. lividoalbum* and *R. aurea*. Both the extracts were effective against bacteria and fungi.

Praveen *et al.* (2013) evaluated *Stereum ostrea,* culture filtrate and methanol extract for antibacterial activity against *Klebsiella pneumonia, P. aureginosa, B. subtilis, S. aureus* and *Micrococcus* species. Crude culture filtrate was high in pathogen inhibition compare to methanol extract of fruiting body.

Ma *et al*. (2013) investigated the anti-inflammatory and anti-cancerous activity from the petroleum ether and ethyl acetate extract of *Inonotus obliguns.* Six main compounds were isolated from two extracts 3β-hydroxy-8, 24-dien-21-al, ergostrerol inotodial ergosterol peroxide, trametenolic acid and lanosterol. Compounds like ergosterol, ergosterol peroxide and trametenolic acid showed anti-inflammatory activity by inhibiting nitric oxide *in vitro*. In *in vitro* anticancer activity ergosterol peroxide and trametenolic acid showed good cytotoxic activity against cell line of prostatic cancer and breast carcinoma cell line.

Wang *et al*. (2013) reviewed analgesic activity of mushroom and their bioactive components and extracts of several mushroom. Crude saponin extract of *Termitomyces albuminosus,* methanol extract of *Inonotus obliquus,* EtOH extract of *Phellinus linteus,* β-glucans of *Pleurotus pulmonarius,* Hydroethanolic extract of *P. florida,* Fucogalactans of *Agaricus brasiliensis* and *A. bisporus,* cordymin of cordyceps and polysaccharopeptides of *Coriolus versicolor* proved potent analgesic effect.

Gonzaga *et al*. (2013) studied the analgesic activity of a polysaccharide from *Agaricus blazei* Murill. by abdominal writhings induced by acetic acid on mice. *A. blazai* showed promising results by showing writhing inhibition value between 85.95 and 91.26%. Isolated polysaccharide is β-(1→6) and α-(1-4).

Ganeshpurkar and Rai (2012) investigated analgesic and anti-inflammatory of *Pleurotus florida* by carrageenan induced anti-inflammatory and analgesis activity was investigated using hot plate method, tail flick method, acetic-acid induced writhing and formalin induced pain in rats and extract showed significant activity in all the test models.

Sonawane *et al*. (2012) revealed that acetone, methanol and ethyl acetate extracts of *Ganoderma* and *Phellinus* showed a broad spectrum antimicrobial activity when tested by different methods. Acetone and methanol being of some polarity showed different activity when tested against seven pathogens.

Nakalembe and Kabasa (2012) identified antimicrobial potential of bioactive compounds of two medicinal mushrooms *Termitomyces* sp. and *Termitomyces microcarpus*. Mushrooms were extracted by petroleum ether and methanol. The extracts were subjected to GCMS analysis the results showed. Palmitic acid, 2-pentanone, 1,2-benzenedicarboxylic acid, 8-11 actodeconoic acid were major compounds. The extracts also showed noticeable antimicrobial activity against *P. aeruginosa* and other test organisms.

Giri *et al*. (2012) determined the antimicrobial activity of 33 different mushroom by extracting all of them by methanol by stirring method, against *Proteus vulgaris, Bacillus cereus, S. aureus, C. albicans, B. subtillis, Lentinus squarrosulu, Russula albonigra* and *Trichoderma giganteum* showed inhibition against all the test microorganisms.

Vamanu (2012) cultured mycelia of *Pleurotus ostreatus* by supplementing different organic and inorganic nitrogen source. The extract was lyophilized and tested antimicrobial activity. The corn extract supplemented mycelia showed notable activity against all test pathogens like *E. coli, B. aureus, L. innocua, Candida* spp., *P. aeruginosa* and *S. aureus*.

Huang *et al*. (2012), isolated Inotilone from *Phellinus linteus* and tested anti-inflammatory activity by Carrageenan induced hind mouse pan edema model. Pure compound from *P. linteus* decreased the inflammation at good levels by suppression of tumour necrosis factor and nitric oxide.

Moro *et al*. (2012) investigated *in vitro* anti-inflammatory activity of methanol extract of wild mushrooms *Agaricus bisporus, Boletus edulis, Cantherellus cibarius, Cantherellus cornucopiodes, Lactarius deliciosus, Pleurotus ostreatus* and proved three mushrooms are potential anti-inflammatory source.

Ede *et al*. (2012) investigated the anti-inflammatory and anti-analgesic activity of *Ganoderma applanatum.* Ethanol extract of *G. applanatum* showed a significant anti-inflammation in albino mice in paw edema method. Analgesic activity was tested by hot plate method.

Suseem *et al.* (2011) worked on the analgesic activity of ethyl acetate, methanol and aqueous extracts of *Pleurotus eous* by using acetic acid induced writhing, hot-plate, tail immersin and tail-clip tests. Three extracts showed the raise in the pain threshold at different time. Authors suggested *P. eous* have potent analgesic property and could serve as a base for future drugs.

Balakumar *et al*. (2011) carried out the study on antibacterial and antifungal activity of *Phellinus.* Methanol and aqueous extracts of fruiting bodies of *Phellinus* spp. were screened against different pathogens like *Streptococcus mutans, P. aeruginosa, S. typi, S. aureus* and *Penicillum* spp., *Aspergillus* spp. and *Mucor indicus* by agar well diffusion method. *Phellinus* spp. showed remarkable inhibition against all test pathogens except *A. fumigatus.*

Jedinak *et al*. (2011) evaluated anti-inflammatory activity of edible oyster mushroom *in vivo* and *in vitro*. Anti-inflammatory activity of *Pleurotus ostreatus* was conformed by the inhibition of proliferation and secretion of interferons from concanavalm A- stimulated mouse splenocytes.

Mourao *et al*. (2011) screened anti-inflammatory activity of *Agaricus blazei* in different stages by carrageanan induced air-pond in animal subcutaneous tissue. Closed basidiocarp extract was effective in 55 and 110 mg/kg dose whereas open basidiocarp extract in 110 mg/kg dose and showed anti-inflammatory response.

Ayodele and Idoko (2011) cultured the mycelial of *Lentinus squarrosulus, Psathysella atroumbonata, Volvariella volvacea* and *Coprinellus mecaceus* in potato dextrose Broth. Culture filtrates were analyzed for antimicrobial activity by bacterial growth by optical density methanol and filter paper disc method. Both the method gave good inhibition against pathogens. The culture filtrate of *Coprinellus micaceus* failed to inhibit *Penicillium notatum*.

Sridhar *et al.* (2011) screened *Ganoderma lucidum* for antibacterial and antifungal activity against *E. coli, P. aureginosa, Salmonella typi, S. aureus* and *Streptococcus*

mutans and five fungal *Penicillium* spp., *A. fumigatus,, Aniger, A. flavus* and *Mucor indicus,* by agar well diffusion method. Among aqueous extract and methanol extract, methanol extract showed grater activity/inhibition against pathogens.

Belsare *et al*. (2011) tested the antibacterial of *Phillinus swieteniae* and *Phillinus merrilli*. Ethyl acetate extracts of *P. swieteniae* inhibited all the strains of *Acinetobacter baumannii*. *P. merrilli* failed to inhibit to strains of *A. baumannii*.

Manjunathan and Kaviyarasan (2011) investigated the growth requirements of mycelia of *Lentinus tuberregium* and confirmed the effective mycelia formation 1:3 ratio of dextrose and yeast. The culture filtrate was extracted by four different solvents. Among them, ethyl acetate extract showed more effective inhibition on human pathogens.

Hassan Iftekhar *et al*. (2011) screened the antibacterial activity of *G. lucidum, A. auricula* and *P. florida*. The ethanol extract of these mushrooms were tested against *S. aureus* and *E. coli. S. aureus* was inhibited by all three mushrooms extracted but *E. coli* was resistant to ethanol extract of the above mentioned mushroom.

Nwachukwu and Uzoeto (2010) studied the antimicrobial activity of *Russula vesca, Auricularia auricular, Pleurotus squarrosulus* and *Volvariaella vulvae* by extracting in three different methods. Hot cold and ethanol against wide range of gram positive and gram negative bacteria. Ethanol and hot water extract were more effective against test pathogens than cold extract. Bioactive compound were found less in cold extract.

Vaz *et al*. (2010) investigated the inhibition of human tumour cell lines *in vitro* by extracts of *Clitocybe alexandri* and *Lepista inverse*. The methanolic ethanolic and water extracts of mushroom inhibited lung, breast, gastric cancer and colon cancer in varied manner but *C. alexandri* was the most potent as growth inhibitor of studied cell lines.

Shekhar *et al*. (2010) screened three species of *Ganoderma* viz., *G. lucidium, G. chalceum* and *G. stipitatum* from Western Ghats of Maharashtra. Mushrooms were extracted by ethyl acetate and extracts were tested against gram positive, gram negative and *Candida albicans.* Sesquiterpene inhibited all the test pathogens.

Akyuz *et al*. (2010) investigated *Pleurotus eryngii, P. astreatus, P. sajor-caju, Terfezia baudieri* and *Agaricus bisporus*. The methyl alcohol extract of mushrooms examined for antimicrobial activity by disk diffusion method against *Candida albicans, C. glabrata, Trichophyton* spp. and *Epidermophyton* species. All the extract showed different degrees of inhibition.

Synytsya *et al*. (2009) proved *Pleurotus* species like *Pleurotus ostreatus* and *Pleurotus eryngii*, boiling water and hot alkali extraction of *Pleurotus* species. The extracts promoted the growth of nine different strains of probiotic *Lactobacillus, Bifidobacterium* and *Enterococcus*. The test organisms showed different growth characteristics, these by proved potential prebiotic in nature.

Joseph *et al*. (2009) investigated anti-inflammatory activity of *Ganoderma lucidum* by Carrageenan induced paw edema in *Swiss albino* mice. *Ganoderma* showed significant inhibitory effect against acute and chronic inflammation by chloroform extract.

Adams *et al.* (2008) studied the anti-cancer activity of *Agaricus bisporus* hot water extract on prostrate cancer cell line, extract inhibited cancer cells *in vitro* in a dose dependent manner and within 72 hrs of treatment apoptosis was induced. Authors also recommended white button mushroom as a dietary component for prevention of prostate cancer in patients.

Smiderle *et al.* (2008) extracted glucan from hot water from *Pleurotus pulmonarius* and investigated anti-inflammatory and analgesic activity, where glucan proved potent anti-inflammatory and analgesic activity which was dose dependent on activity.

Jonathan Gbolagade and Ishola (2007) studied the effect of antimicrobial activity of *A. polytricha, C. occidentalis, D. elegans, D. concentrica* and *T. lobayemsis*. These mushrooms were extracted by methanol and tested against *B. cereus, E. coli, K. pneumoniae, P. vulgaris* and *P. aeruginosa* and *S. aureus* by agar well diffusion method. Mushroom extract inhibited bacteria stronger than fungi.

Iwalokun *et al*. (2007) analyzed the phytochemical present in pet ether and acetone extracts of *Pleurotus ostreatus.* PE extracts showed the presence of lower phenolic content than acetone. PE extract inhibited the gram negative bacteria more effectively than acetone. Acetone extracts elicited higher antioxidant activity *in vitro*.

Ajith and Janardhanan (2007) screened the antitumour activity of *G. lucidum, Phellinus rimosus, Pleurotus florida* and *Pleurotus pulmonaris* and *Phellinus rimosus* extracts found to inhibit Dalton's Lymphoma Ascites (DLA) cell induced tumour in mice

and Ehrlich's Ascites Carcinoma (EAC). *Plurotus florida* and *Pleurotus pulmonaris* showed profound activity against the EAC cell line induced tumour in mice.

Cho *et al*. (2007) screened hypoglycemic effect of exopolyssacharides from a mycelia of *Tremella fuciformis* and *Phellinus beumii* in mice. The mycelial extract of both mushrooms significantly reduced blood sugar levels and improved glucose tolerance with systematic insulin sensitivity without any increase in body weight in mice.

Barros *et al*. (2007) found antimicrobial activity of *Lactarius deliciasus*, *Sarvodon imbricatus* and *Trichoderma portentosum* against *B. cereus, B. subtilis, E. coli, P. aeruginosa, C. albicans* and *C. neoformans. E. coli* was resistant to all the mushroom extracts whereas gram positive bacteria was inhibited by mushroom extracts.

Zheng *et al*. (2006) culture mycelia of *Cordyceps sinensis* and purified an antibacterial protein by culture. Authors purified *C. sinensis* antibacterial protein (CSAP) and test antimicrobial activity against bacteria fungi. Protein showed a significant activity against bacteria but fungi were resistant to protein.

Kim *et al*. (2006) screened the anti-inflammatory effect of *Phellinus linteus* by Heme oxygenase-1 macrophages. *Phellinus* suppressed nitric oxide synthase and increased the production of nitric oxide, *P. linteus* showed anti-inflammator activity in an *in vitro* inflammation model.

Yamac and Bilgili (2006), culture mycelia of eleven macrofungi and extracted culture fluids from chloroform and methanol (9:1) solution. The extracts were screened antimicrobial activity by disk diffusion method. All mycelial extract of all mushroom were effective against more than one of the test microorganisms.

Zaidman *et al*. (2005) reviewed the antitumour properties of mushroom. Calvacin from *Calvatia gigantea,* Lentinan from *Lentinus edodes, D. fraction* and β-glucon from *Grifola frondosa, Schizophyllan* (SPG, *Soni filan, Sizo filan*) from *Schizophyllan commune,* polysaccharide peptide from *Trametes versicolor* and *Ganoderma lucidum* polysaccharide (GLPs) from *Ganoderma lucidum* are the antitumour bioactive compound from mushrooms.

Shittu *et al*. (2005) investigated the antibacterial metabolite from mycelial culture of *Russula* sp. and *Pycnoporus cinnbarinus.* Authors cultured mycelia and extracted mycelia by methanol and culture filtrate by ethyl acetate. Antibacterial activity was tested by agar well diffusion method.

Jeong *et al*. (2004) cultured *Phellinus pini* mycelia and extracted exopolymer to evaluated immunomodulating activity. Mycelia was cultured in potato dextrose broth and extracted by ethanol and dialyzed against distilled water and lyophilized to obtain an exopolymer. Immunomodulating activity was tested by using mice.

Daba *et al*. (2003) and Ren *et al*. (2012) reviewed the antitumour/anticancer activity by the polysaccharides from mushroom, polysaccharides like *Lentinan* from *Lentinus edodes* and *Schizophyllan* from *Schizophyllum commune* are anti-tumour bioactive compounds which suppress the proliferation of cancer cells. Two species viz., *Phellinus gilvus* and *Phellinus lintenus* inhibit lung cancer and melanoma cancer respectively, whereas *Cordyceps militaris* inhibit both polysaccharides from *Ganoderma lucidum* can and inhibit breast cancer, Melanoma lung cancer and cervical cancer. Polysaccharides from fruiting bodies of *Agaricus blazei, Grifola frondosa* and *Trametes vessicolar* inhibit prostate cancer.

Grube *et al.* (2001) investigated *Agaricus bisporus* breast cancer cell proliferation. Chemicals in *A. bisporus* water extract inhibited aromatase activity and proliferation of breast cancer cell in post menopausal women dose dependently.

Hatvani (2001) screened the antibacterial activity of culture fluid of *Lentinus edodes*. Mycelia was cultured in liquid medium containing glucose peptone and yeast, at certain percentage. The culture fluid showed poor inhibition against *C. albicans* and had no effect on *P. fluorescens, M. luteus, E. coli* and *C. jejuni*. Lenthionine was detected in fruiting body of *L. edodes* and also mycelium biomass.

Han *et al*. (1999) and Kozarski *et al*. (2011) screened the antitumour and immunomodulatory activity of polysaccharides. The polysaccharides proved potent against B16F10 melanoma and also act as immunopotent by inhibit growth of tumour in cancer patients without toxicity. The α and β glucans from *Phellinus linteus* showed immunostimulating effect against tumour cells.

Chapter - 3

Materials and Methods

3.1. Study Area

Shivamogga district is the part of Malnad (hilly region) in Karnataka state of India. The district covers 8,465 km² in area more than 50 per cent of land is covered by forest. District is located between 13º 27' and 14º 39' N longitude and 74º 38' and 76º 4' E latitude. Shivamogga district has seven taluks Shivamogga, Bhadravathi, Thirthahalli, Hosanagar, Sagar, Shikaripur and Sorab. The average rainfall is about 140 cm per year. The marofungal survey was conducted in the study area from June 2014 to September 2015.

3.2. Collection

The sporocarps were collected randomly in the forests of Shivamogga district, wherever found. *Entoloma speculum* was collected from the medicinal plant garden (August 2014) of Kuvempu University Campus located in Bhadravathi taluk of Shivamogga district. *Phellinus linteus* collected from Devara Kaadu (July 2015) (sacred groove) located in Sagar taluk. *Phellinus tremulae* was collected from the dead wood in the Banajale estate near Sagar, Sagar taluk.

Specimen collected from Sagar was difficult to identify it by morphologically so far molecular identification sample was sent to chromos biotic for sequencing, sequence of internal transcribed spacers (ITS) with ITS1 and 4 markers. The obtained DNA sequence was blasted in National Center for Biotechnological Information (NCBI). According to NCBI data base results specimen was identified as *P. tremulae* with maximum number of hits (16).

Classification

***Phellinus tremulae* (Bondartsev) Bondartsev & P.N. Borisov**

Kingdom	:	Fungi
Phylum	:	Basidiomycota
Class	:	Agaricomycetes
Order	:	Hymenochaetales
Family	:	Hymenochaetaceae
Genus	:	*Phellinus*
Species	:	*P. tremulae* (Bondartsev) Bondartsev & P.N. Borisov, 1953

Mycobank number : **344218**

Fruit body Basidiocarps perennial, pileate, developing around branch scars, firmly attached on substrate, with a slight sweet odour and hard corky when fresh, woody hard and heavy in weight when dry. Pilei ungulate, projecting up to 7 cm, 12 cm wide, and 4 cm wide. Pileal surface greyish black to black, obscurely concentrically sulcate with narrow zones, velutinate, becoming glabrous with age, eventually forming a crust, margin obtuse. Pore surface deep brown to cocoa-brown; sterile margin usually wide, rust-brown, matted, upto 3 mm wide; pores circular, 5–6 per mm; dissepiments thick, entire. Context umber to dark brown, contrasting with tubes by a darker hues, woody corky, up to 2 cm thick, white mycelial strands present, a granular core present at the place of attachment. Tubes umber-brown to dull brown, woody hard, up to 2 cm long, usually filled with white mycelial strands in old tubes, tube layers usually distinct, separated by a thin layer of context-like mycelium (Plate-1 A & B and Plate-3).

Distribution : Banajale estate, Sagar taluk

Habitat : Dead wood log

***Phellinus linteus* (Berk. & M.A. Curtis) Teng**

Kingdom : Fungi

Phylum : Basidiomycota

Class : Agaricomycetes

Order : Hymenochaetales

Family : Hymenochaetaceae

Genus : *Phellinus*

Species : *P. linteus* (Berk. & M.A. Curtis) Teng

Mycobank number : **319769**

Basidiocarps perennial, dimidiate, woody hard; upper surface dark brown, matted tomentose, concentrically zonate, shallowly sulcate; edge more or less acute; poroid surface brown to dull brown, sterile margin very narrow to indistinct, yellowish brown, pores circular, 6-8(-9) per mm (n=120/4), dissepiments thin to fairly thick, entire. Context brown to dull brown, woody hard, up to 1 cm thick, somewhat radially fibrous and lustrous, a thin black line present between upper matted tomentum and context in the type, the black line becoming thick and forming a black crust in old basidiocarps. Tubes dull brown, up to 1.5 cm long, tube layers indistinct. Spores, Basidiospores basically subglobose, mature spores thick-walled, yellowish brown (Plate-1 C & D and Plate-2A).

Distribution : Devarakaadu, Sagar taluk

Habit : *Autocarpus heterophyllus*

***Entoloma speculum* (Fr.) Quel**

Kingdom : Fungi

Phylum : Basidiomycota

Sub-Phylum : Agaricomycotina

Class : Agaricomycetes

Order : Agaricales

Family : Entolomataceae

Genus : *Entoloma*

Species : *E. speculum* (Fr.) Quel

Mycobank number : **221523**

Pileus 20.0–80.0 mm dia, white (7A1) to pinkish white (7A2), conical or bell–shaped, sometimes flattening with age, smooth, margin involute, glabrous at the centre. Lamellae white (7A1) to pinkish white (7A2), attached, almost distant, emarginated, ventricose. Stipe 25.0–60.0×2.0–10.0 mm, white (1A1), cylindrical, fistulose, striate of silver lining along the length, fragile and easily splitting. Basidia 25.0–35.5×7.4–15.7 µm, clavate, 4–sterigmate. Cystidia absent, clamp connections present. Spore–print Pink. Basidiospores 8.5–12.5×7.2–11.5 µm (Plate-1 E & F and Plate-2B).

Distribution : Shankaraghatta

Habitat : Soil, litter

PLATE-1

A – *Phellinus tremulae* on host

B – Collection of *Phellinus tremulae*

C – *Phellinus linteus* on host

D – Collection of *Phellinus linteus*

E – *Entoloma speculum*

F – Drying of samples

PLATE-1

PLATE-2

A – Spores of *Phellinus linteus*

B – Spores of *Entoloma speculum*

PLATE-2

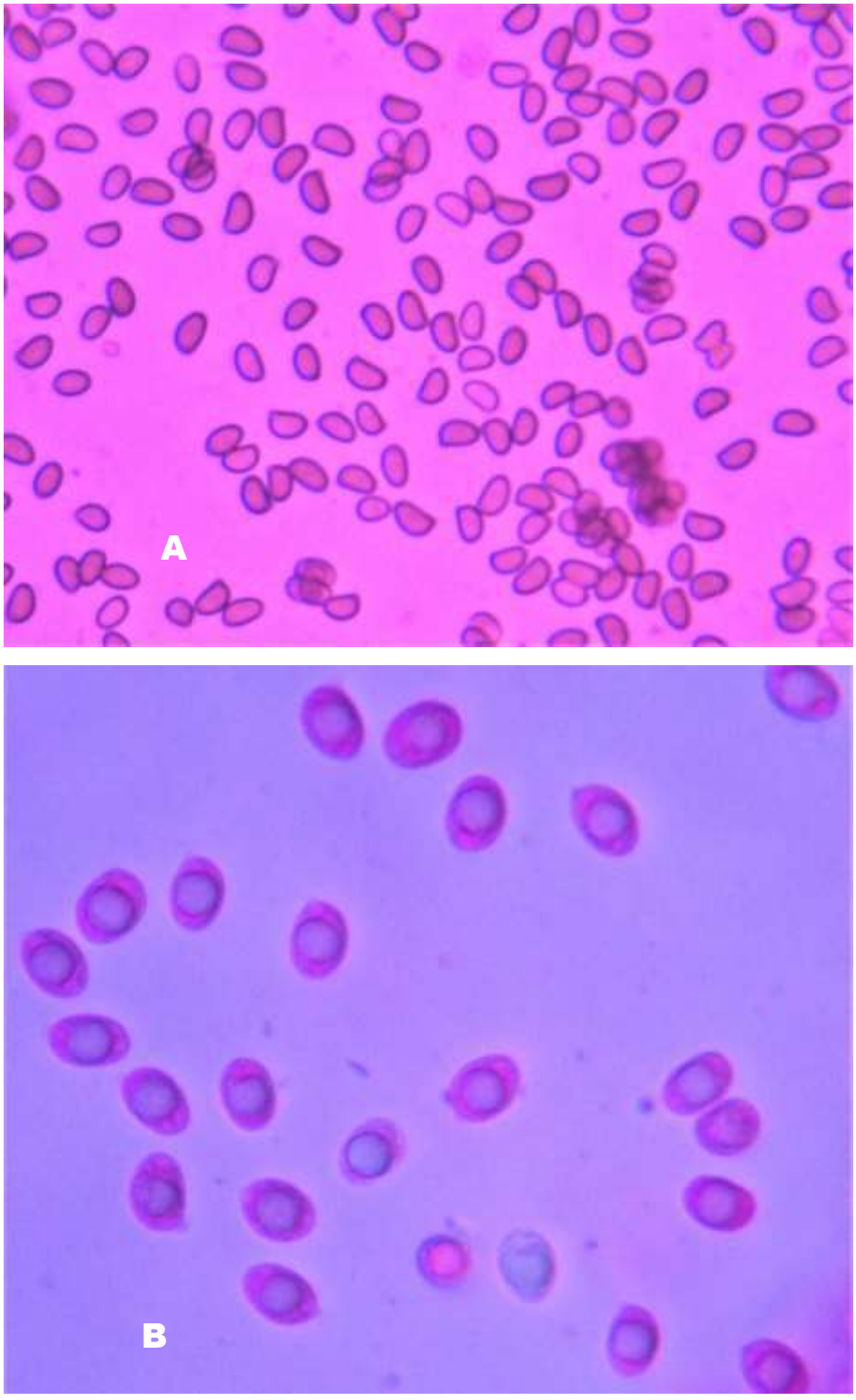


PLATE-3

A & B - Spores of *Phellinus tremulae*

PLATE-3

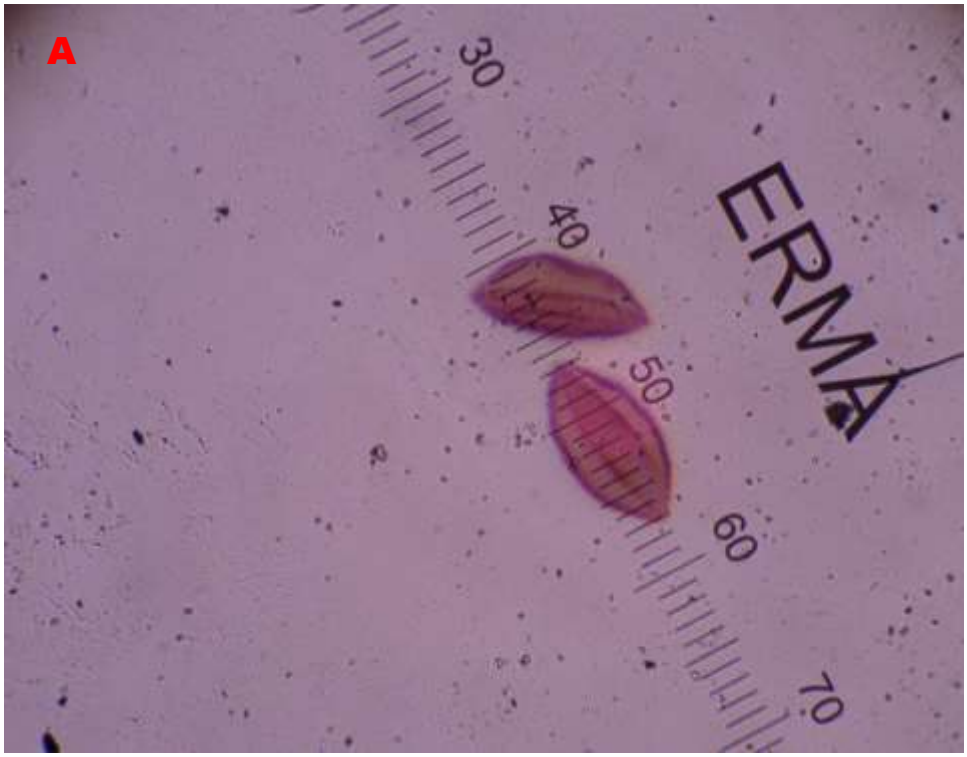


PLATE-4

A & B – Mycelial culture on PDA media of *Phellinus linteus*

C & D – Broth culture of mycelia of *Entoloma speculum*

PLATE-4

PLATE-5

Antibacterial activity of methanol extract of *Entoloma speculum* against

A – *Salmonella typhi*

B – *Staphylococcus aureus*

C – *Pseudomonas aeruginosa*

D – *Escheritia coli*

Antibacterial activity of chloroform extract of *Phellinus linteus* against

E – *Pseudomonas aeruginosa*

F – *Agrobacterium tumefaciens*

PLATE-5

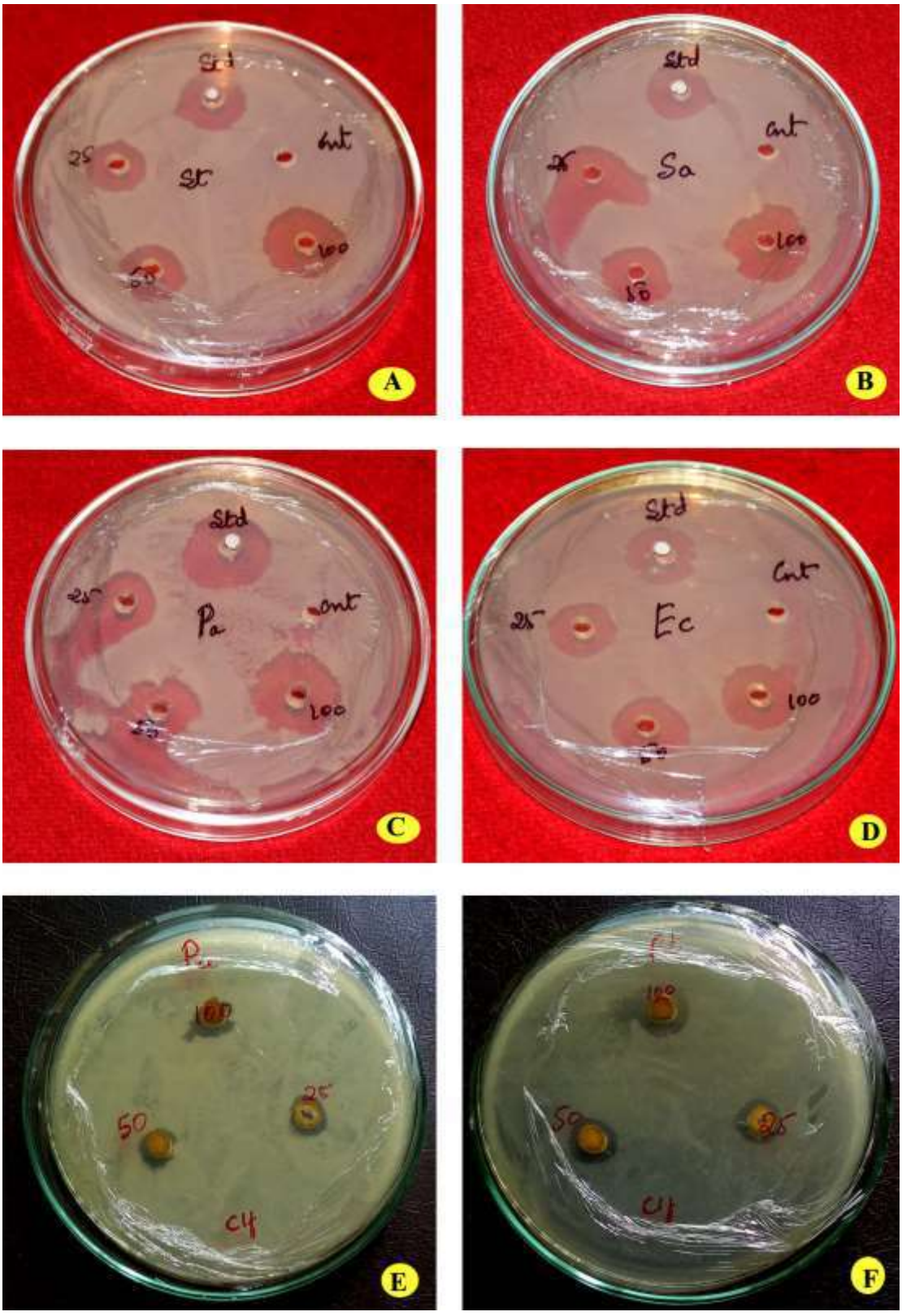

Std
Cnt
25
St
100
50
A
Std
Cnt
25
Sa
100
50
B
Std
25
Pa
Cnt
100
C
Std
Cnt
25
Ec
100
D
100
50
25
E
100
50
25
F

PLATE-6

A – Antibacterial activity of chloroform extract of *Phellinus tremulae* against *Pseudomonas syringe*

Antibacterial activity of pet ether extract of *Phellinus tremulae* against

B – *Staphylococcus aureus*

C – *Pseudomonas syringe*

D – *Pseudomonas aeruginosa*

E – *Salmonella typhi*

F – Antibacterial activity of methanol extract of *Phellinus tremulae* against *Klebsiella pneumonia*

PLATE-6

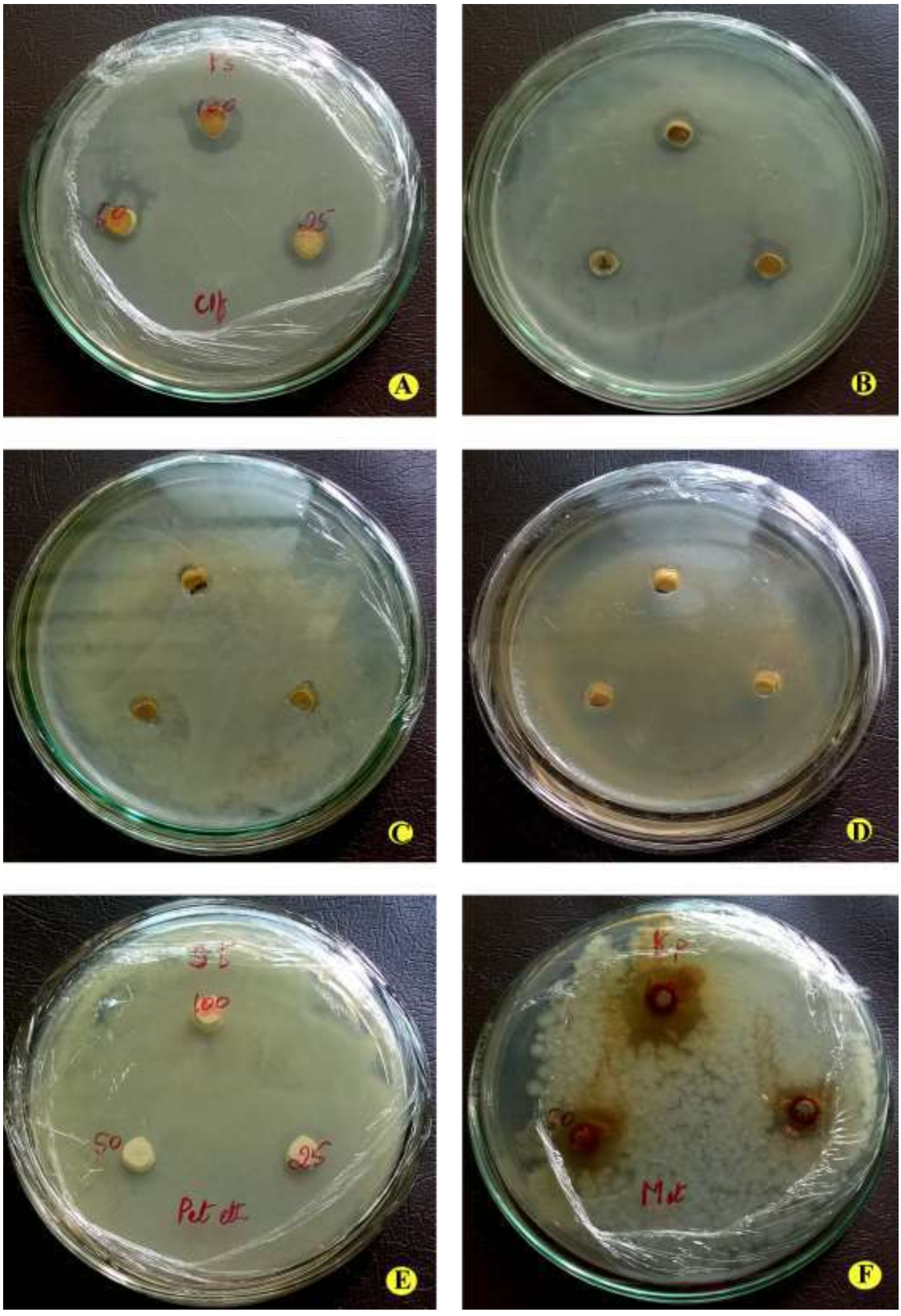


PLATE-7

Antifungal activity of mycelial extract of *Entoloma speculum* against

A – *Candida albicans*

B – *Trichophyton rubrum*

C – *Aspergillus* sp.

PLATE-7

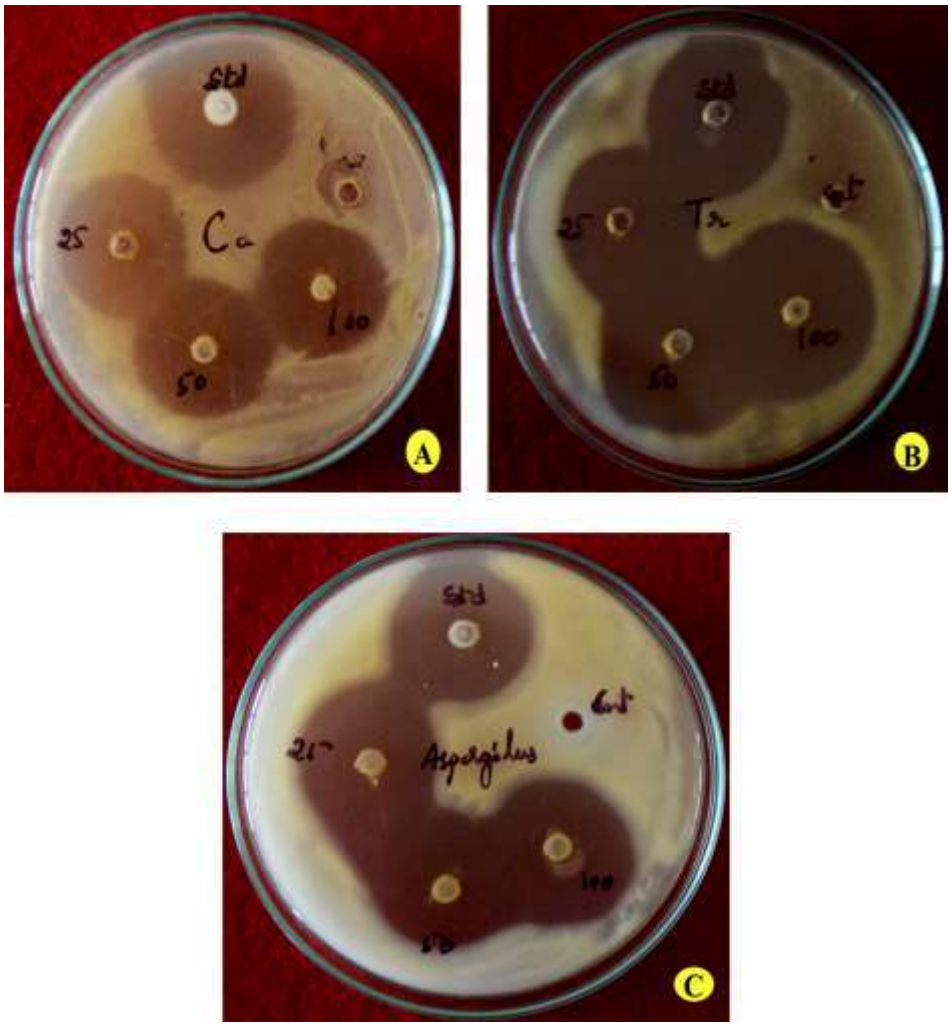


PLATE-8

A – Rat from the standard group not showing any writhings

B, C and D – Rats showing writhings at different intervals

PLATE-8

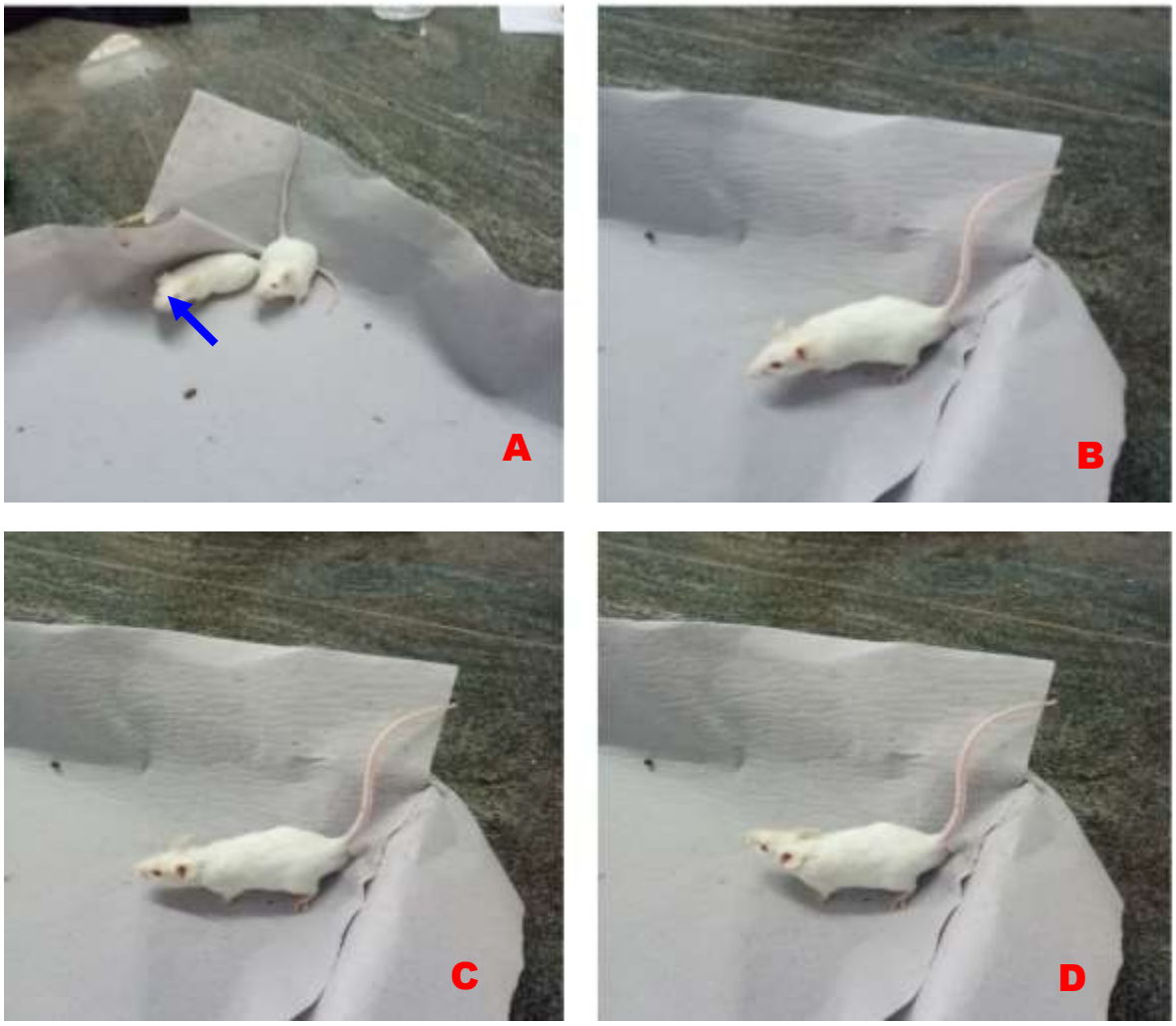


PLATE-9

A – Injecting carragenan to right hind paw of rat

B & C – Measurement of inflammation

D - Plethysmometer

PLATE-9

3.3. Proximate analysis

Determination of foreign matter

One gram of sample was weighed and foreign matter was carefully separated. The matter differing in colour and texture were considered as foreign. The separated matter was weighed and subtracted from one gram and percentage was calculated (Gupta, 1984).

Determination of moisture content

One gram of powder was weighed and dried at 80°C for 24 h in hot air oven. After 24 h, the powder was weighed again and the difference in the weight was determined. The percentage of moisture was calculated (Gupta, 1984).

Determination of pH

The 5% (w/v) (5 g in 100 ml of water) powder was kept on shaker for 5 h with 140 rpm and filtered. The filtrate was analysed for the pH using pH meter (Elico, India) (Iqbal *et al.,* 2010).

Determination of water soluble extractive

Five Grams of powder was weighed and added into a 100 ml conical flask, 25 ml of distilled water is added into to it and kept on a rotator shaker (140 rpm) for 24 hour. After 24 h it was filtered and dried in hot air oven at 80ºC for 24 h and weighed again. The difference in weight was determined and percentage of water soluble extractive were calculated (Gupta, 1984).

Determination of alcohol soluble extractive

Five grams of powdered material is taken in a 100 ml conical flask, 25 ml of absolute alcohol is added to it and kept on rotator shaker at 140 rpm for 24 h. After 24 h

it was filtered and dried in hot air oven set at 80ºC for 24 h and weighed again. The difference in weight was determined and percentage of Alcohol soluble extractive were calculated (Gupta, 1984).

Determination of total ash content

The clean and dried silica crucible was weighed, 10 g of powder was taken and kept in muffle furnace and heated up to 300ºC for 3-4 h until the whole powder turns into ash. The crucible was cooled and weighed again. The difference in the weight was calculated (Gupta, 2003; Indrayan *et al.*, 2005).

Determination of Water Soluble Ash

One g of powder was weighed and 10 ml of distilled water is added into it. The mixture was kept on a shaker with 140 rpm for 8 h and filtered through ash less filter paper. The ash remained in the paper was kept in a crucible (silica) and burnt ash again in a muffle furnace for 3-4 h. The weight of ash obtained was noted and percent of water soluble ash was determined (Ahmad and Sharma, 2001).

Determination of acid insoluble ash

1 g of ash was weighed and 10 ml of distilled water is added into it. The mixture was kept on a shaker with 140 rpm for 8 h and faltered through ash less filter paper. The ash remained in the paper was kept in a crucible (Silica) and burnt to ash again in a muffle furnace for 3-4 h. The weight of ash obtained was noted and percentage of acid insoluble ash was determined (Ahmad and Sharma, 2001).

3.4. Preparation of extraction

The collected sporocarps were brought to lab in sterile polythene bags for further work. sporocarps were shade dried for 30 days. The dried mushrooms were ground to course powder by grinder and stored in air tight polythene bag at room temperature (27ºC) for further use. The powder was weighed accurately and subjected for extraction in a Soxhlet apparatus, 300 gm of powdered sporocarps was loaded in Soxhlet with petroleum ether, chloroform and methanol successively. Extract was evaporated to dryness and stored at 4ºC until the further use.

Soxhlet Apparatus

3.5. Qualitative tests for secondary metabolites

Biochemical screening for secondary metabolites for all the three solvents (petroleum ether, chloroform and methanol) extract of *Entoloma speculum*, *Phellinus linteus* and *P. tremulae* were carried out by the standard methods of Visweswari *et al.* (2013) and Kokate (2000).

Test for Alkaloids

a. **Mayer's test:** To the 2 ml of sample, 4 to 5 ml dil. HCl was added and shaken well, then Mayer's reagent was added and appearance of white or pale yellow precipitate indicates the presence of alkaloids.

b. **Wagner's test:** To the 2 ml of sample, 4 to 5 ml dil. HCl was added and shaken well. Later Wagner's reagent was added. Appearance of white precipitate indicates the presence of alkaloids.

c. **Dragendroff's test:** To the 2 ml of sample, 2 ml Dragendroff's reagent and 1ml dil. HCl were added. Appearance of orange precipitate indicates the presence of alkaloids.

Test for Flavonoids

a. **Flavonoid test:** To the 2 ml of sample, few drops of Conc. H_2SO_4 was added through the sides of the tube. Appearance of magenta red or deep cherry indicates flavonoids and scarlet colour indicates the presence of flavones.

b. **Zinc-hydrochloric acid reduction test:** To the 2 ml of sample, zinc dust and few drops of dilute HCl were added. Appearance of magenta red colour indicates the presence of flavonoids.

c. **Ferric chloride test:** To the 2 ml of sample, few drops of neutral ferric chloride solution was added. Appearance of blackish green colour indicates the presence of flavonoids.

d. **Alkaline reagent test:** To the 2 ml of sample, few drops of NaOH solution was added. Increase in intensity of yellow colour which becomes colourless on addition of dilute acid indicates the presence of flavonoids.

e. **Lead acetate test:** To the 2 ml of sample, few drops of 10% (v/v) lead acetate solution was added. Appearance of yellow precipitate indicates the presence of flavonoids.

f. **Pew's Shinoda and NaOH test or Shinoda's test:** To the 0.5 ml of sample, 10 drops dilute HCl and a piece of magnesium were added. Appearance of pink, reddish or brown colour indicates the presence of flavonoids.

Test for Glycosides

a. **Kellar Kiliani test:** To the 2 ml of sample, few drops glacial acetic acid was added and then boiled for a minute and cooled. Then 2 drops of ferric chloride which was transferred to another tube containing Conc. Sulphuric acid was added. Appearance of reddish brown ring at junction of two layers indicates the presence of glycosides.

b. **Molisch's test:** To the 2ml of sample, 1ml Molisch's reagent was added. When 1 ml Conc. H_2SO_4 poured through sides of the tube, a reddish violet ring formation at the junction of two layers indicates the presence of glycosides.

c. **Raymond's test:** To the 2 ml of sample, dinitrobenzene in hot methanolic alkali was added. Appearance of violet colour indicates the presence of glycosides.

d. Bromine water test: To the 2 ml of sample, few drops of bromine water was added. Appearance of yellow precipitate indicates the presence of glycosides.

e. Legal's test: To the 2 ml of sample, pyridine (made alkaline by adding sodium nitroprusside solution) was added. Appearance of pink to red colour indicates the presence of glycosides.

Test for Lignin

a. Furfuraldehyde test: To the 2 ml of sample, few drops of Furfuraldehyde solution (2% v/v) was added. Appearance of red colour indicates the presence of lignins.

Test for Phenolics

a. Phenol test: To the 2 ml of sample, 0.5 ml Ferric chloride solution was added. Formation of intensive colour indicates the presence of phenolics.

b. Ellagic acid test: To the 2 ml of sample, few drops of 5% mixture containing glacial acetic acid and 5% (w/v) sodium nitrate solution were added. Formation of muddy yellow or olive brown or niger brown or deep chocolate colour indicates the presence of phenolics.

c. Hot water test: To the 2 ml of sample, few drops of hot water was added. Formation of intensive colour at the junction indicates the presence of phenolics.

Test for Steroids

a. Salkowski's test: To the 2 ml of sample, few drops of conc. H_2SO_4 was added. Formation of wine red colour indicates the presence of steroids.

Test for Tannins

a. **Sodium chloride test:** To the 2 ml of sample, few drops NaCl solution was added. Formation of precipitate indicates the presence of tannins.

b. **Gelatin test:** To the 2 ml of sample, gelatin solution was added. Formation of white precipitate indicates the presence of tannins.

c. **Ferric chloride test:** To the 2 ml of sample, few drops of 5% (w/v) ferric chloride solution was added. Green colour indicates the gallotannins; brown colour indicates the presence of tannins.

Test for Triterpenoids

a. **Salkowski's test:** To the 2 ml of sample, few drops of conc. H_2SO_4 was added. Production of golden yellow colour at the lower layer indicates the presence of triterpenoids.

9. Test for Saponins

a. **Foam test:** To the 5 ml of sample, a drop of sodium bicarbonate solution was added, shaken well and left for 3 minutes. Formation of honey comb like froth indicates the presence of saponins.

3.5. Antibacterial activity

Antibacterial activity of collected mushroom extracts were determined by the Agar well diffusion method. Pathogenic bacteria were from MTCC Chandighar, India. Bacteria were maintained in nutrient broth. 6 mm wells were made in NA plates and inoculated with test organisms. Other wells were loaded with extracts in dimethyl sulfoxide (DMSO) in terms of weight is to volume into different concentration 100, 50

and 25% using micro pipette. Amoxycillin is used as standard and DMSO used as control for pathogenic bacteria. The inoculated plates were incubated for 27±2ºC for 24 hours. The zone formation was observed in plates. The experiment is repeated in 4 replicates and results are tabulated in terms of mean of 4 replicates.

Test organisms

- *Xanthomonas campestris* MTCC-2286
- *Pseudomonas synringae* MTCC-1604
- *Agrobaterium tumefaciens* MTCC-431
- *Klebsiella pneumonia* MTCC-7028
- *Escheritia coli* MTCC-1559
- *Salmonella typhi* MTCC-734
- *Pseudomonas aeruginosa* MTCC-1934
- *Staphylococcus aureus* MTCC-902
- *Stretptomyces pneumoneae* MTCC-47

3.6. Antifungal activity

Antifungal activity of selected sporocarps extracts were determined by agar well diffusion method. Pathogenic fungi were from MTCC Chandighar, India. Fungi were maintained in potato dextrose agar media. The spores of pathogens were dissolved in tween 20, so that they spread uniformly on media. Wells were made with the help of 6 mm cork borer and loaded with different concentration extracts dissolved in DMSO in terms of weight is to volume using micropipette. Terbinafine is used as standard and DMSO used as control for antifungal activity. The inoculated plates were incubated for 24±2ºC for 5 days. The zone formation was observed n plates and measured in mm. the experiment is repeated in 4 replicates the results are tabulated in terms of mean of 4 replicates.

Test organisms

- *Candida albicans* MTCC-1637
- *Chrysosporium merdarium* MTCC-4608
- *Trichophyton rubrum* MTCC-3272
- *Chrysosporium keratinophilum* MTCC-1367
- *Fusarium solani* MTCC-1040
- *Penicillium chrysogenum* MTCC-947
- *Aspergillus flavus* MTCC-1783
- *Aspergillus niger* MTCC-514

3.7. Mycelial culture and extraction

The fresh sporocarp of *E. speculum* and *Phellinus linteus* were collected and washed with distilled water, kept for spore print under aseptic condition. Petri plates containing subourdous dextrose agar medium and inoculated with spore from spore print and inoculated for 7 days at ambient temperature. From the 7^{th} day old pure culture, 6 mm diameter discs were inoculated to 250 ml conical flask containing 100ml potato dextrose broth of pH 5 and kept in rotatory shaker at 120 rpm, at 25ºC for 10 days. After 10 days culture filtrate was filtered by Whatmann paper No. 1 and dried at 80ºC for 30 min. Dried mycelia was powered and weighed and subjected for extraction with 150 ml of methanol. The residue was then extracted twice with another 150 ml methanol (Hwang, 2004). The total extract was then evaporated to dryness. These extracts were screened against pathogenic fungi and bacterial species.

Culturing of *Phellinus tremulae* and also by bits of sporocarp on media was failed and no method was successful in culturing. So only *Entoloma speculum* and *Phellinus linteus* was able to culture.

3.8. Anticancer test

The test compounds studied for short term *in vitro* cytotoxicty using or Ehrlich Ascites Carcinoma (EAC) cells and Dalton's lymphoma ascites (DLA). The tumour cells aspirated from the peritoneal cavity of tumour bearing mice were washed thrice with PBS or normal saline. Cell viability was determined by trypan blue exclusion method. Viable cell suspension ($1x10^6$ cells in 0.1 ml) was added to tubes containing various concentrations of the test compounds and the volume was made upto 1 ml using phosphate buffered saline (PBS). Control tube contained only cell suspension. These assay mixture were incubated for 3 hours at 37ºC. Further, cell suspension was mixed with 0.1 ml of 1% trypan blue and kept for 2-3 minutes and loaded on a haemocytometer. Dead cells take up the blue colour of trypan blue white live cells do not take up the dye. The number of stained and sustained cells were counted separately.

$$\% \text{ cytotoxicity} = \frac{\text{No. of dead cells}}{\text{No. of live cells + No. of dead cells}} \times 100$$

Control tube contains only one dead cells DMSO dissolved.

3.9. Amylase inhibition assay

The inhibition assay was performed using the chromogenic DNSA method (Miller, 1959; Sudha *et al*., 2011). The total assay mixture composed of 1400 ml of 0.05 μ sodium phosphate buffer (pH 6.9), 50 μl of amylase and samples of concentration 100, 250 and 500 μg were incubated at 37ºC for 10 minutes. After pre-incubation, 500 μl of 1% (w/v) starch solution in the above buffer was added to each tube and incubated at 37ºC for 15 minutes. The reaction was terminated with 1.0 ml DNSA reagent, placed in boiling water bath for 5 min, cooled to room temperature and the absorbance measured at

540 nm. The control amylase represented 100% enzyme activity and did not contain any sample of analysis.

To eliminate the absorbance produced by sample, appropriate extract controls with the extract in the reaction mixture in which the enzyme was added after adding DNS. The maltose liberated was determined by the help of standard maltose curve and activities were calculated according to the following formula:

$$\text{Activity} = \frac{\text{Conc. of Maltose liberated x ml of enzyme used}}{\text{Mol. Wt of maltose x incubation time (min)}} \text{ x Dilution factor}$$

One unit of enzyme activity is defined as the amount of enzyme required to release one micro mole of maltose from starch per min under the assay condition.

The inhibitory/induction property shown by the sample was compared with that of control and expressed as per cent induction/inhibition. This was calculated according to the following formula:

$$\%\ \text{inhibition / induction} = \frac{\text{Activity in presence of compound}}{\text{Control activity}} \text{ x } 100$$

DNS Solution: 1 g of DNS dissolved in 2N NaOH, 30 g of potassium sodium tartarate was added and whole volume was made up to 100 ml.

3.10. Anti-inflammatory activity (*in vivo* model)

Anti-inflammatory activity by carrageen in induced rat hind paw edema method

The experiment was conducted in pharmacy college of Harapanahalli. *Wister albino* rats were used for experiment and they were purchased by Sri Venkateshwara Enterprises, Bengaluru. Animals were divided into control, standard, different test groups

comprising of five animals in each group. They were fasted overnight with free access to water before experiment. In all groups, acute inflammation was produced by subplanter injection of 0.1 ml of freshly prepared 1% suspension of carrageenin in the right hind paw of the rats and paw of the rats and paw volume was measured plethysmometrically at 0 hr and 3 hrs after carrageenin injection. The test compounds (50 mg/kg) was administered orally, standard group was treated with diclofenac (50 mg/kg) orally 1 hr. before by injection and control group received only vehicle. Mean difference in paw volume was measured and percentage inhibition was calculated by following formula:

$$\% \text{ inhibition of edema} = \frac{Vc - Vt}{Vc} \times 100$$

where, Vt = mean paw volume of test group

Vc = mean paw volume of control group

3.11. Analgesic activity

The experiment was conducted in pharmacy college of Harapanahalli. SAM mice were used for experiment. This study was carried out using acetic acid induced abdominal writhing reflex pain model. Mature mice were randomly divided into 8 groups (1-4) of 6 mice per group, fasted for 12 hours and treated as follows, Group 1 (control group) received normal saline, group 2 Standard received Ibuprofen 100 mg/kg of Acetyl salicyclic acid; groups 3, 5 and 7 received LD 250 mg/kg of test mushroom extracts, group 4, 6 and 8 received HD 500 mg/kg body weight of test mushroom extracts. One hour after drug and extract administration, 0.6% glacial acetic acid (10 ml/kg) was administered intra-peritoneally (I.P) to all the mice to induce abdominal contortions or writhings. The analgesic effect was assessed in each mouse for 30 minutes and recorded.

The degree of analgesia was calculated using the following formula:

$$\% \text{ of inhibition} = \frac{A - B}{A} \times 100$$

where, A is the mean number of writhing produced by the control group

B is the mean number of writhing produced by the test group.

This represents the percentage of inhibition of writhing.

Note: 1. LD_{250} (LD means Low dose 250 mg/kg body weight of the animal)

2. HD_{500} (LD means High dose 500 mg/kg body weight of the animal)

Statistical analysis

Statistical analysis of results was done by using MS-Excel, One-Way ANOVA followed by Dunnett's test and the results were compared with control.

Chapter - 4

Results

4.1. Study area

Study area receives 140 cm rainfall per year. Shivamogga district is hilly region located in between 13° 27' and 14° 39' N longitude and 74° 38' and 76° 4' E latitude. 50 per cent of the land (8465 km^2 total area) is covered by forest.

4.2. Collection

Entoloma speculum was collected from medicinal plants garden of Kuvempu University campus between July and August of 2014 in Bhadravathi taluk. *Phellinus linteus* was collected from Devara Kaadu (sacred groove) near Jambekoppa village of Sagar taluk. *Entoloma speculum* and *Phellinus linteus* were identified by morphological characters (Krick *et al.*, 2008).

Phellinus tremulae was collected from Banajaale estate of Sagar taluk was difficult to identify by morphology. The fresh material was molecularly identified by DNA sequencing at Chromos Biotec. (Annexure-1). The obtained DNA was blasted in NCBI database. Based on results, the specimen was identified as *Phellinus tremulae* (16) with maximum number of hits.

4.3. Mycelia culture

Culturing of *Phellinus tremulae* was not successful like other two species. *Entoloma speculum* and *Phellinus linteus* was successfully cultured by spore print (Plate-3) on potato dextrose agar media and then transferred to potato dextrose broth for mass culture. 7^{th} day the characters were noticed.

Entoloma speculum and *Phellinus linteus* was cultured by spore print. Mycelia of *Entoloma* was Ambonate, medium growth irregular margin yellowish to pale yellow in colour, hypae was septate 2-7 μm wide and clamp connections were abundant in mycelia when observed under microscope. Mycelia of *Phellinus linteus* was white, thick walled, little branched 2-3.5 μm wide, skeletal hyphae, clamp connection was not observed in mycelia.

4.4. Physico-chemical analysis

The dried sample of selected macro fungi were subjected to physico-chemical analysis. The results were varied with all the three macro fungi as they were collected from different places and from totally different environment. *Entoloma speculum* showed more foreign matter (0.9%) than other two macro fungi (0.8%). Moisture content was more in *Phellinus tremulae* 16.6% followed by *P. linteus* 7.3% and *E. speculum* 5.3%, water soluble extraction was 38.16% in *P. tremulae* and 8.88% in *P. linteus* and only 6.64% in *E. speculum*. Alcohol soluble extraction was almost equal in both *E. speculum* and *P. tremulae* 12.8 and 12.5% respectively. pH was neutral in *E. speculum* and *P. tremulae* is acidic (5.9%) and pH of *P. linteus* is slightly acidic by 6.64% (Table 1).

Table 1. Percentage of physico-chemical analysis

Physico-chemical parameters	***Entoloma speculum***	***Phellinus tremulae***	***Phellinus linteus***
Foreign matters	0.9	0.8	0.8
Moisture content	5.3	16.6	7.3
Water soluble extractive	6.64	38.16	8.88
Alcohol soluble extractive	12.8	12.5	3.2
pH	7	5.9	6.64
Determination of ash	10.4	12.4	21.6
Water soluble ash	83	95	85
Acid insoluble ash	12	16	21

Ash was 21.6% in *P. linteus*, 10.4% and 12.4% in *E. speculum* and *P. tremulae* respectively. Water soluble ash was 95% in *P. tremulae* followed by 85% in *P. linteus* and 83% in *E. speculum*. Acid insoluble ash was 21 in *P. linteus* and 16% in *P. tremulae* and only 12% in *E. speculum*.

4.5. Biochemical analysis

The selected macrofungi were extracted with pet ether, chloroform and methanol respectively. All the three extracts of *E. speculum* show positive results for alkaloids, tannins, flavonoids, glycosides and phenols. Only pet ether extract showed positive result for steroids. Alkaloids, tannins and flavonoids were present in all the extract of *P. tremulae*. Steroids, glycosides and phenols were present only in chloroform extract of *P. tremulae*. Methanol extracts showed positive result for glycosides, triterpenoids and phenols. Pet ether extracts showed positive to only alkaloids and tannins but chloroform and methanol extracts of *P. linteus* showed positive to all test except saponins (Table 2).

Table 2. Biochemical test results

Tests	*Entoloma speulum*			*Phellinus tremulae*			*Phellinus linteus*		
	Pet ether	**Chloroform**	**Methanol**	**Pet ether**	**Chloroform**	**Methanol**	**Pet ether**	**Chloroform**	**Methanol**
Alkoloids	+	+	+	+	+	+	+	+	+
Saponins	-	-	-	-	-	-	-	-	-
Tannins	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	-	+	+
Steroids	+	-	-	-	+	_	-	+	+
Glycosides	+	+	+	-	+	+	-	+	+
Triterpenoids	-	-	-	-	-	+	-	+	+
Phenols	+	+	+	-	+	+	-	+	+

4.6. Characterization (HPLC and GCMS)

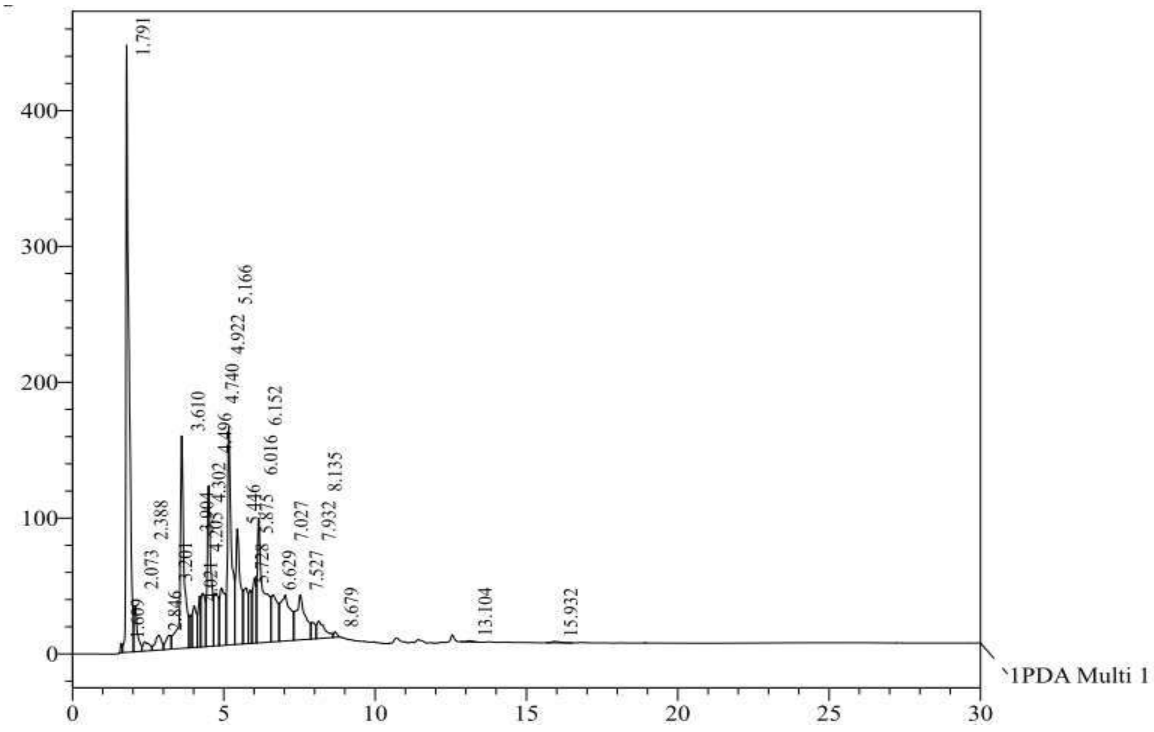


Fig. 1. HPLC chromatogram of methanol extract of *Entoloma speculum*.

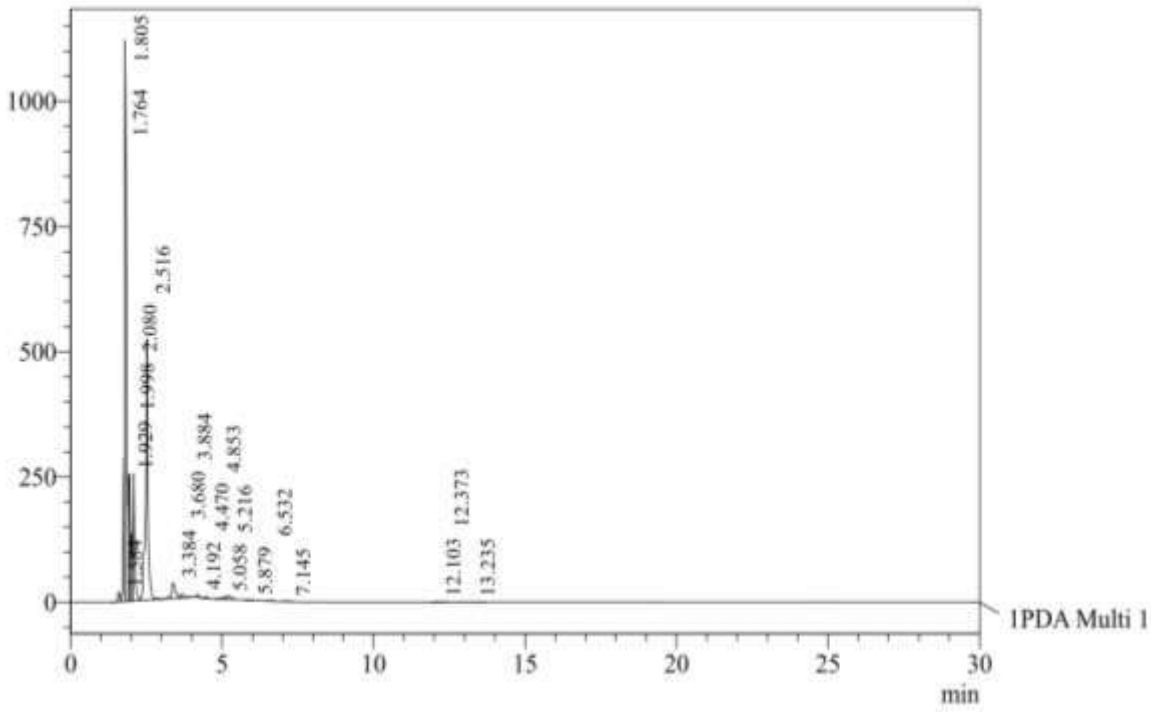


Fig. 2. HPLC chromatogram of methanol extract of *Phellinus tremulae*.

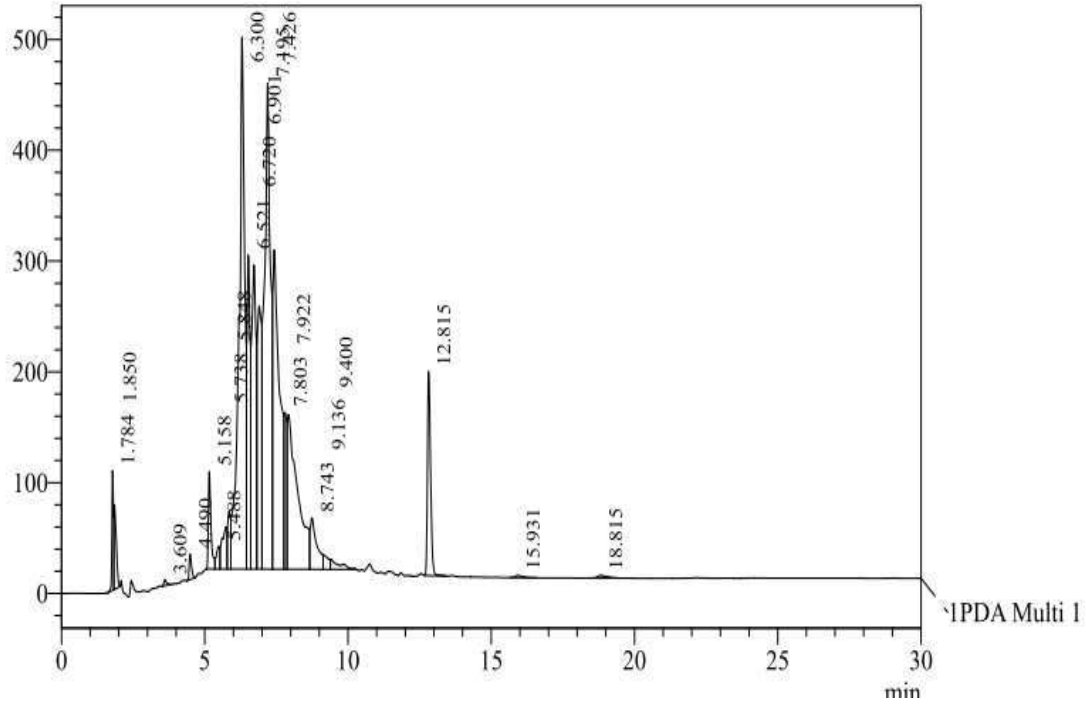


Fig. 3. HPLC chromatogram of methanol extract of *Phellinus linteus.*

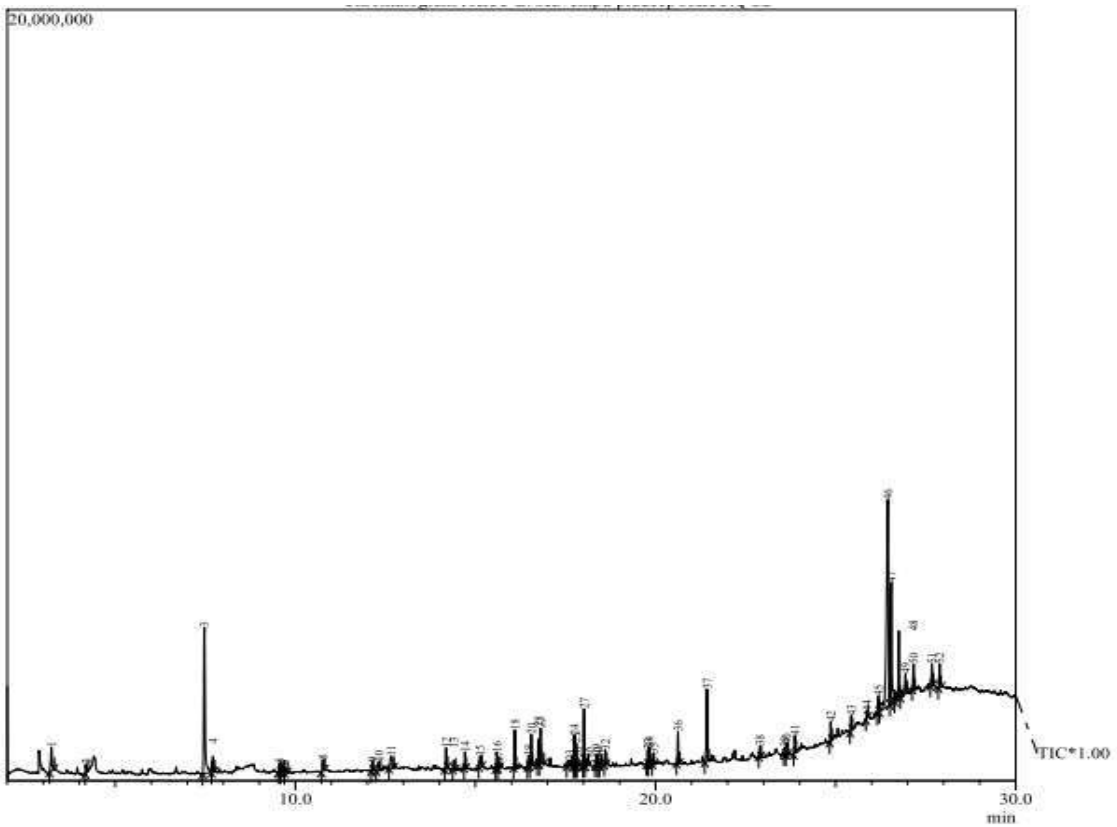


Fig. 4. GCMS chromatogram of methanol extract of *Entoloma speculum.*

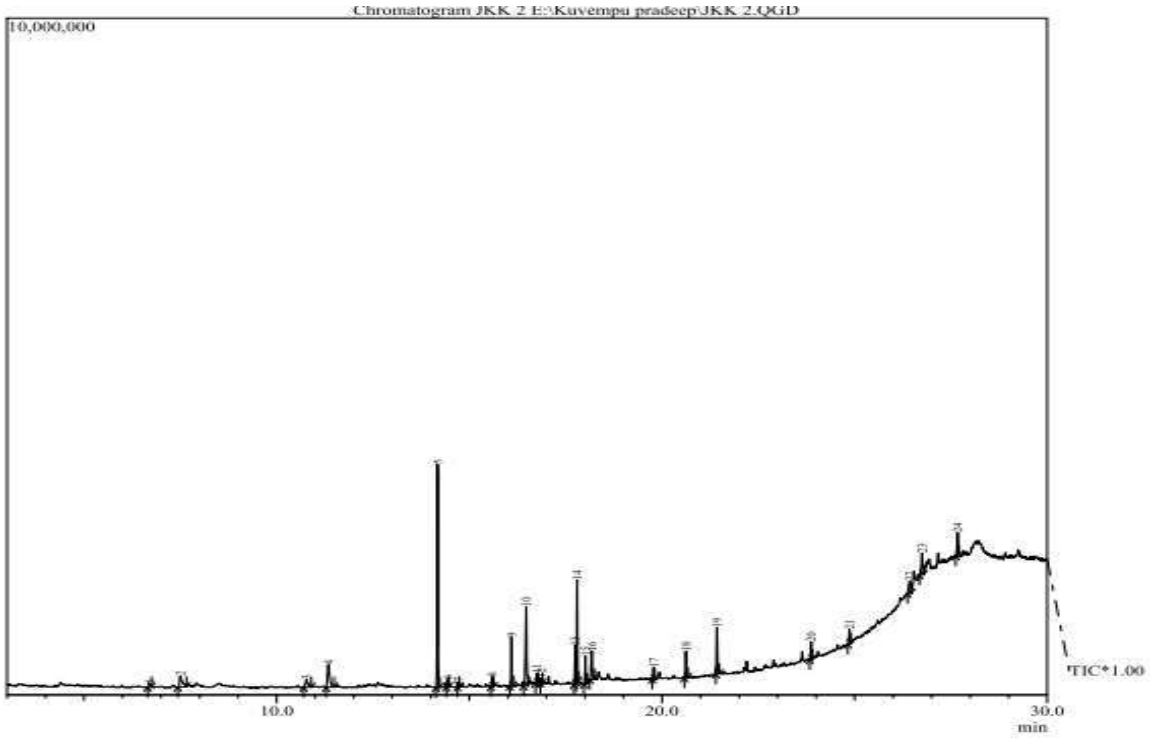


Fig. 5. GCMS chromatogram of methanol extract of *Phellinus tremulae.*

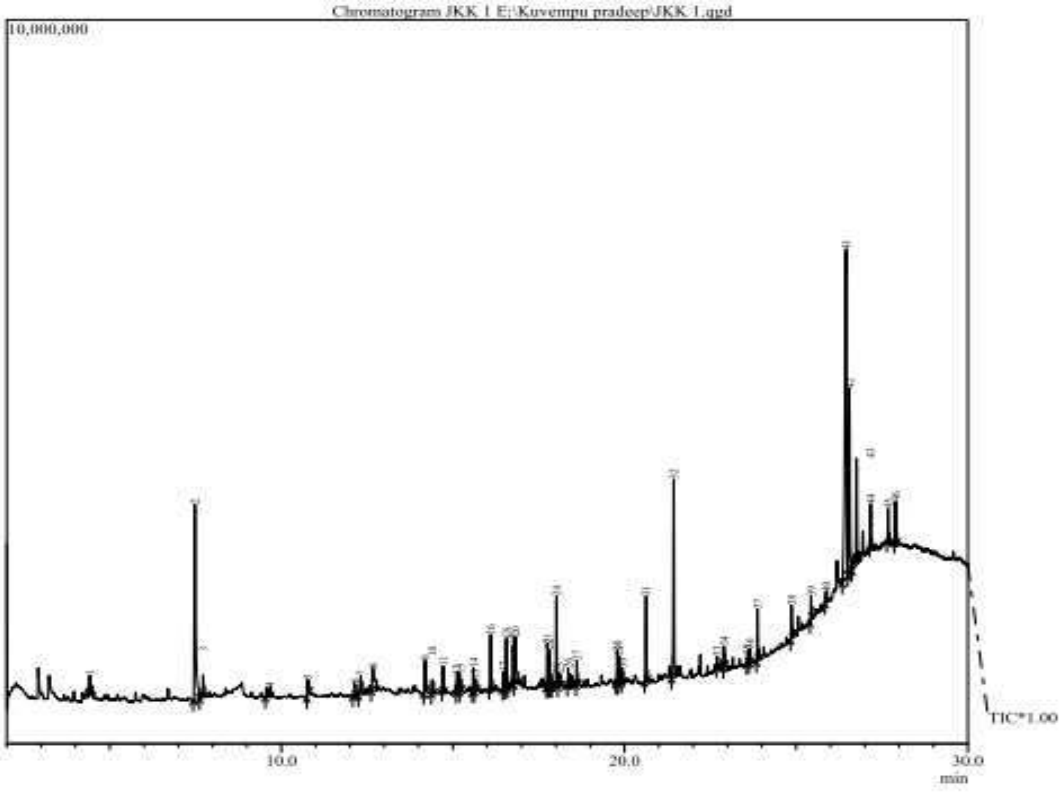


Fig. 6. GCMS chromatogram of methanol extract of *Phellinus linteus.*

4.7. Characterization (HPLC and GCMS)

Table 3. Chemicals obtained in GCMS analysis of methanol extract of *E. speculum*

Peak No	Retention Time	Area %	Compound	Activity
1	3.226	2.09	(S)-2-Hydroxypropanoic acid	Antiseptics and antiviral, Anticancer (Stomach)* Anticancer (Skin)* Analgesic-Synergist* Antidiabetic (Insulin-Sparing)* Antidote (Snake Venom)CNS-Sedative* DNA-Synthesis-Inhibitor*Ovulation-Stimulant* Erik (1985)
2	4.187	0.52	Ethanol, 2,2'-oxybis- (CAS) Diethylene glycol	Food Additives PubChem CID: 8117
3	7.473	10.99	1,2-Benzenediol	Anti-inflammatory Zheng (2008)
4	7.714	0.86	2,3-Dihydro-benzofuran	Antihyperexcitability Yong-JinWu *et al.,* 2004 PubChem CID: 10329
5	9.558	0.51	Phenol, 2,6-dimethoxy- (CAS) 2,6-Dimethoxyphenol	flavouring ingredient. PubChem CID: 7041
6	9.655	0.41	Decanoic acid (CAS) Capric acid	intermediate for food-grade additives and perfumes PubChem CID: 2969
7	9.723	0.37	Benzaldehyde, 4-hydroxy- (CAS) p-Hydroxybenzaldehyde	Flavouring Agent: FLAVOURING_AGENT PubChem CID: 126
8	10.763	0.79	Benzaldehyde, 2-hydroxy-6-methyl-	Alpha-Amylase-Inhibitor* Analgesic* Anthelmintic* Antiacne* Anti-Itching* Anti-HIV-Integrase*
9	12.133	0.42	Dodecanoic acid (CAS) Lauric acid	Insecticide, Acaricide, Herbicide, Plant growth regulator, antimicrobial PubChem CID: 3893
10	12.320	0.99	Benzoic acid, 4-hydroxy-3-methoxy- (CAS) Vanillic acid	Testosterone-Hydroxylase-Inducer* Anti-analgesic (Yoa *et al*., 2016)
11	12.659	1.76	2-Methoxy-5-formyl-1,3(2H)-benzoxodiole	Pesticide, Anti-HIV-Integrase* Hallucinogenic* Hormone Balancing* https://www.pharmacompass.com/chemistry-chemical-name/bendiocarb
12	14.186	1.25	1-(4-Hydroxybenzylidene) acetone	------
13	14.395	0.55	Tetradecanoic acid	Arachidonic acid-Inhibitor*
14	14.710	0.68	1-Heptadecene (CAS) Hexahydroaplotaxene	-------

Contd….

15	15.125	0.84	3,5-Heptanedione, 2,2,6,6-tetramethyl	Casein-Kinase-II-Inhibitor*
16	15.593	0.80	8-Octadecanone	----
17	15.650	0.23	Hexadecen-1-ol, trans-9-	Reverse-Transcriptase-Inhibitor*
18	16.094	1.87	Eicosanoic acid, methyl ester (CAS) Arachidic acid methyl ester	Surfactants, Urine-Acidifier* https://pubchem.ncbi.nlm.nih.gov/compound/Arachidic_acid#section=Associated-Disorders-and-Diseases
19	16.474	0.63	Hexadecanoic acid (CAS) Palmitic acid	Anti-inflammatory [28], Anti-androgenic*, Anti-fibrinolytic*, Hypercholesterolemic*, Nematicide Verma 2015
20	16.553	1.62	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	-------
21	16.751	1.56	1-Hexadecanol	----
22	16.810	3.19	4-Hydroxy-2-methoxycinnamaldehyde	Testosterone-Hydroxylase-Inducer*
23	17.631	0.55	1-Docosene	--------
24	17.749	1.41	9,12-Octadecadienoic acid, methyl ester, (E,E)-	Acidulant*
25	17.795	1.06	9-Octadecenoic acid, methyl ester, (E)- (CAS) Methyl elaidate	Antitumor (Esophagus)*
26	17.842	0.61	Tetradecanoic acid, 12-methyl-, methyl ester (CAS) Methyl 12-methyltetradecanoate	----------
27	18.015	2.49	Octadecanoic acid, methyl ester (CAS) Methyl stearate	----------
28	18.105	0.38	5,6,7,8-Tetrahydro-4H-[1,2,4]triazolo[5,1-b]quinazolin-9-one	---------
29	18.357	0.50	9,12-Octadecadien-1-ol (CAS) OCTADECA-9,12-DIEN-1-OL	-----
30	18.400	0.29	(E)-9-Octadecenoic acid ethyl ester	-------
31	18.472	0.33	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl	Antitumor (Thyroid)* Blood-Thinning* Thyrostimulant*
32	18.608	0.73	Docosanoic acid, ethyl ester	----
33	19.783	0.77	Hexadecanal (CAS) Palmitic Aldehyde	------

Contd….

34	19.824	0.88	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	------
35	19.934	0.48	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	-----
36	20.623	1.59	Hexadecanal (CAS) PALMITIC ALDEHYD	----
37	21.429	4.27	Octadecanal (CAS) Stearaldehyde	Used to test Sjogren-Larsson syndrome. PubChemCID:12533
38	22.897	0.51	Tetracosanoic acid, methyl ester	-----
39	23.583	0.46	Tetraethylene glycol monododecyl ether	Glycolytic
40	23.639	0.50	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-(CAS) Squalene	-----
41	23.870	0.93	Octadecanoic acid, 2-hydroxy-, methyl ester	-----
42	24.864	1.08	9(11)-Dehydroergosteryl benzoate	-----
43	25.432	0.76	1-Eicosanol	-----
44	25.868	0.37	Cholest-5-en-3-ol (3.beta.)-(CAS) Lanol	Fertility-Enhancing* Memory-Enhancer*
45	26.186	0.98	Dehydroergosterol 3,5-dinitrobenzoate	------
46	26.449	25.71	Ergosterol	Helps in LACTATION PubChem CID 444679
47	26.548	8.24	Ergosta-7,22-dien-3-ol, (3.beta.,22E)-	----
48	26.756	4.65	7,22-Ergostadienone	------
49	26.941	1.92	7-Ergostenol	------
50	27.165	1.67	Cholest-8-en-3.beta.-ol, acetate	Fertility-Enhancing*
51	27.677	1.45	10,13-dimethyl-17-(1,4,5-trimethyl-Hex-2-Enyl)-1,2,9,10,11,12,13,15,16	-----
52	27.890	1.47	16-Hentriacontanone (CAS) Palmitone	------

"*" represents the compound having known biological activity cited in Dr. Duke's phytochemical and ethnobotanical database

Table 4. Chemicals obtained in GCMS analysis of methanol extract of *P. tremulae*

Peak No	Retention time	Area %	Compound	Activity
1	6.699	0.44	2,3-Dihydro-3,5-dihydroxy-6-methyl-4H-pyran-4-one	Anti-HIV-Integrase* Antidote (Heavy Metals)* Hallucinogenic* Hirudicide* Hormone Balancing* Improve Cerebral Hypoxia*
2	7.500	3.41	1,2-Benzenediol (CAS) Pyrocatechol	Anti-inflammatory (Zheng, 2008)
3	10.771	2.06	3,4-Altrosan	-------
4	11.342	5.84	D-Allose	Anticancer (Duodenum)* CNS-Depressant* Coronary-Dilator* DNA-Topoisomerase-Inhibitor* Food Dye* Provide Vit D* Urine-Deodorant*
5	14.178	23.94	Benzene, 1,2,4,5-tetrachloro-3,6-dimethoxy	-------
6	14.439	0.75	2-methoxytetrachlorophenol	--------
7	14.712	0.40	1-Tetradecene (CAS) n-Tetradec-1-ene	Antitumor (Nasopharynx)* Nematistat* Nephroprotective* Neuroexcitant* Nitric-Oxide-Synthase-Inhibitor*(anti-inflammatory)
8	15.595	1.60	3-Octanol (CAS) n-Octan-3-ol	Increase Natural Killer (NK) Cell Activity* Antitumor (Nasopharynx)* Neurosedative*
9	16.094	4.73	Eicosanoic acid, methyl ester (CAS) Arachidic acid methyl ester	------
10	16.476	11.23	n-Hexadecanoic acid	Myo-neuro-stimulant* Neurodepressant* Neuroprotective* NO-Scavenger*

Contd….

11	16.749	1.58	1-Octadecanol	-------
12	16.869	0.57	13-Docosenoic acid, methyl ester, (Z)- (CAS) Methyl erucate	-------
13	17.750	3.95	9,12-Octadecadienoic acid, methyl ester	------
14	17.798	9.07	10-Octadecenoic acid, methyl ester	------
15	18.014	2.66	Octadecanoic acid, methyl ester	-------
16	18.178	4.87	9-Hexadecenoic acid (CAS)	------
17	19.784	1.34	Hexadecanal	------
18	20.624	2.87	Hexadecanal (CAS) PALMITIC ALDEHYDE	-----
19	21.430	5.08	Octadecanal (CAS) Stearaldehyde	-------
20	23.874	1.99	99 Octadecanoic acid, 2-hydroxy-, methyl ester	-----
21	24.867	1.70	9(11)-Dehydroergosteryl benzoate	
22	26.438	2.97	Ergosta-5,7,22-trien-3-ol, (3.beta.,22E)- (CAS) Ergosterol	Anticancer (Esophagus)* Ectoparasiticide* Memory-Enhancer* Fertility-Enhancing*
23	26.751	3.95	7,22-Ergostadienol acetate	-----
24	27.676	2.99	10,13-dimethyl-17-(1,4,5-trimethyl-Hex-2-Enyl)-1,2,9,10,11,12	------

"*" represents the compound having known biological activity cited in Dr. Duke's phytochemical and ethnobotanical database

Table 5. Chemicals obtained in GCMS analysis of methanol extract of *P. linteus*

Peak No.	Retention time	Area %	Compound	Activity
1	4.418	0.90	1,2,3-Propanetriol (CAS) Glycerol	----
2	7.484	10.72	1,2-Benzenediol	-----
3	7.723	1.09	2,3-dihydro-benzofuran	------
4	9.657	0.67	Decanoic acid (CAS) Capric acid	Urinary-Acidulant* Inhibit Production of Uric Acid*
5	10.774	0.64	Benzaldehyde, 2-hydroxy-6-methyl	Testosterone-Hydroxylase-Inducer*
6	12.135	0.47	Dodecanoic acid (CAS) Lauric acid	------
7	12.318	1.10	Benzoic acid, 4-hydroxy-3-methoxy- (CAS) Vanillic acid	Urine-Acidifier* Anti-analgesic (Yoa *et al*., 2016)
8	12.675	2.35	2-Methoxy-5-formyl-1,3(2H)-benzoxodiole	Anti-HIV-Integrase* Hepatoprotective* Herbicide* Hormone-Balancing* Hydroperoxidase-Inhibitor* Improve Cerebral Hypoxia*
9	14.191	1.25	1-(4-Hydroxybenzylidene)acetone	-------
10	14.398	0.53	Tetradecanoic acid	Inhibit Production of Uric Acid*
11	14.715	0.68	E-15-Heptadecenal	
12	15.117	1.30	Phosphonofluoridic acid, (1-methylethyl)-, hexyl ester	------
13	15.210	0.55	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	Oligosaccharide Provider*
14	15.597	0.79	8-Octadecanone	------
15	15.654	0.40	1-Eicosanol	------
16	16.098	1.78	Eicosanoic acid, methyl ester (CAS) Arachidic acid methyl ester	Inhibit Production of Uric Acid*
17	16.475	0.79	9-Octadecenoic acid (Z)- (CAS) Oleic acid	Provide Zinc*
18	16.557	1.61	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	Acidifier*
19	16.753	1.59	1-Nonadecene	------
20	16.813	3.01	3-(p-hydroxy-m-methoxyphenyl)-2-propenal	Anticancer (Mammary)* Anticancer (Mouth)* Antidote (Mushroom)* Contraceptive (Male)*Memory-Enhancer* Molluscicide* Mosquitocide* Mutagenic* Anticancer (Pancreas)* Anticancer (Prostate)* Asthma-preventive* Balance Prolactin Production* Fistula-Preventive* Parasiticide* Purgative*

Contd….

21	17.752	1.32	9,12-Octadecadienoic acid (Z,Z)-, methyl ester (CAS) Methyl linoleate	-------
22	17.799	1.07	9-Octadecenoic acid, methyl ester, (E)- (CAS) Methyl elaidate	Anticancer (Esophagus)*
23	17.848	0.45	Cyclopentaneundecanoic acid, methyl ester (CAS) methyl 11-cyclopentylun	Fertility-Enhancing* Memory-Enhancer*
24	18.017	2.64	Octadecanoic acid, methyl ester (CAS) Methyl stearate	------
25	18.107	0.42	Dihydropyranno(3,2-G) chromanne	Antidote (Poison Gas)* Anticancer (Gastric)*
26	18.360	0.50	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]	------
27	18.611	0.83	Ethyl ester of docosanoic acid	-------
28	19.788	1.20	Octadecanal (CAS) Stearaldehyde	----
29	19.829	0.90	2-Propenoic acid, 3-(4-methoxyphenyl)-, 2-ethylhexyl ester	-----
30	19.939	0.43	Cyclohexane, eicosyl	----
31	20.627	2.75	Hexadecanal	----
32	21.434	7.01	Octadecanal (CAS) Stearaldehyde	-----
33	22.700	0.58	9-Octadecenoic acid (Z)-, 2,3-dihydroxypropyl ester	Increase Zinc Bioavailibility*
34	22.902	0.57	Tetracosanoic acid, methyl ester	-------
35	23.583	0.49	Tetraethylene glycol monododecyl ether	Glycolytic*
36	23.643	0.42	2,6,10,14,18,22-Tetracosahexaene, 2,6,10,15,19,23-hexamethyl-, (all-E)-	-------
37	23.876	1.72	Octadecanoic acid, 2-hydroxy-, methyl ester	Testosterone-Hydroxylase-Inducer*
38	24.89	1.12	9(11)Dehydroerosteryl benzoate	------
39	25.436	0.86	1-Eicosanol	-----
40	25.871	0.38	CHOLESTERINPALMITAT	-----
41	26.454	24.11	Ergosterol	-----
42	26.551	8.04	Ergost-5,8(14)-dien-3-ol	----
43	27.171	5.05	7,22-Ergostadienone	----
44	27.171	1.83	beta.-Sitosterol	-----
45	27.684	1.41	0,13-Dimethyl-17-(1,4,5-Trimethyl-Hex-2-Enyl)-1,2,9,10,11,12,13,15,16	-----
46	27.897	1.68	16-Hentriacontanone (CAS) Palmitone	-----

"*" represents the compound having known biological activity cited in Dr. Duke's phytochemical and ethnobotanical database

4.8. Antimicrobial activity

The study of antimicrobial activity of crude extracts from mushrooms against test microorganisms were compared with the control DMSO and standard Amoxicillin for bacteria and Terbinafine for fungi. The antimicrobial activity of mushroom samples varied according to the solvents and also concentration of extract. Mushrooms used for study were found effective against the tested microorganisms. The clear zone of inhibition produced by the bacteria and fungi around the tested mushroom extracts was evidence of potency of mushroom. Methanol yield was maximum followed by chloroform and petroleum ether.

Physico-chemical analysis of *Phellinus linteus* showed 0.8 per cent of foreign matter, 7.3 per cent of moisture content 8.88 per cent of water soluble extractive, 3.2 per cent of alcohol soluble extractive; pH is 6.4, 21.6 per cent of determination of ash, 85 per cent of water soluble ash and 21 per cent of acid insoluble ash (Table 1). The extracts were tested for presence of secondary metabolite. Petroleum ether extract showed the presence of alkaloids and tannins. Chloroform extracts showed positive to alkaloids, tannins, steroids, glycosides and phenols. Methanol extract showed the presence of flavonoids, glycosides triterpenoids and phenols.

The extracts showed varied antimicrobial result when tested against pathogens. The petroleum ether extract of *Phellinus linteus* inhibited *Salmonella typhi* and *Staphylococcus aureus* (12 mm) at maximum. *Agrobacterium tumefaciens, Klebsiella pneumonia, Pseudomonas aeruginosa* were (10 mm) moderately inhibited. *Xanthomonas campestris, Pseudomonas syringae, Escherichia coli* and *Streptomyces pneumoneae* were

(8–0) least inhibited by petroleum ether extracts (Table 7). The fungal pathogens like *Trichophyton rubrum, Fusarium solani, Aspergillus flavus* and *Aspergillus niger* were completely resistant showing no inhibition whereas *Chrysosporium keratinophilum* was inhibited maximum that of other test organism. *Candida albicans* and *Chrysosporium merdarium* were inhibited moderately (Table 6).

Chloroform extracts of *Phellinus linteus* inhibited test organisms better than petroleum ether extract. *K. pneumonia* and *S. aureus* were inhibited maximum by showing 16 and 17 mm zone. The chloroform extract inhibited *P. syringae, E. coli, P. aeruginosa, S. pneumoneae, A. tumefaciens* and *S. typhi* moderately by showing 13–14 mm inhibition zone. Whereas *X. campestris* were least inhibited with 8 mm. *F. solani* (22 mm) had the maximum effect of chloroform extract followed by *A. niger* and *C. keratinophilum* (20 mm). *C. merdarium, T. rubrum* and *P. chrysogenum* were moderately affected with 18–19 mm inhibition zone (Table 6 and Plate-5 E&F). *A. flavus* and *C. albicans* were least inhibited by chloroform extract. The GCMS analysis of methanol extract showed the presence of ergosterol (Table 5).

A. tumefaciens S. aureus and *P. aureginosa* showed more susceptibility to methanol extract followed by *K. pneumonia, S. typhi, P. syringae*, whereas *X. campestris* was resistant to methanol extract of *Phellinus linteus*. *C. keratinophilum* and *C. albicans* were maximum inhibited by methanol extracts, almost equal to inhibition zone of standard drug. *C. merdarium, F. solani, A. flavus* showed less susceptibility than the *C. keratinophilum* and *C. albicans* but *T. rubrum, P. chrysogenum* and *A. niger* were least inhibited by methanol extract compared to another test organism (Table 6 and Fig. 7).

Table 6. Antifungal activity of *Phellinus linteus* at different concentration and different solvent

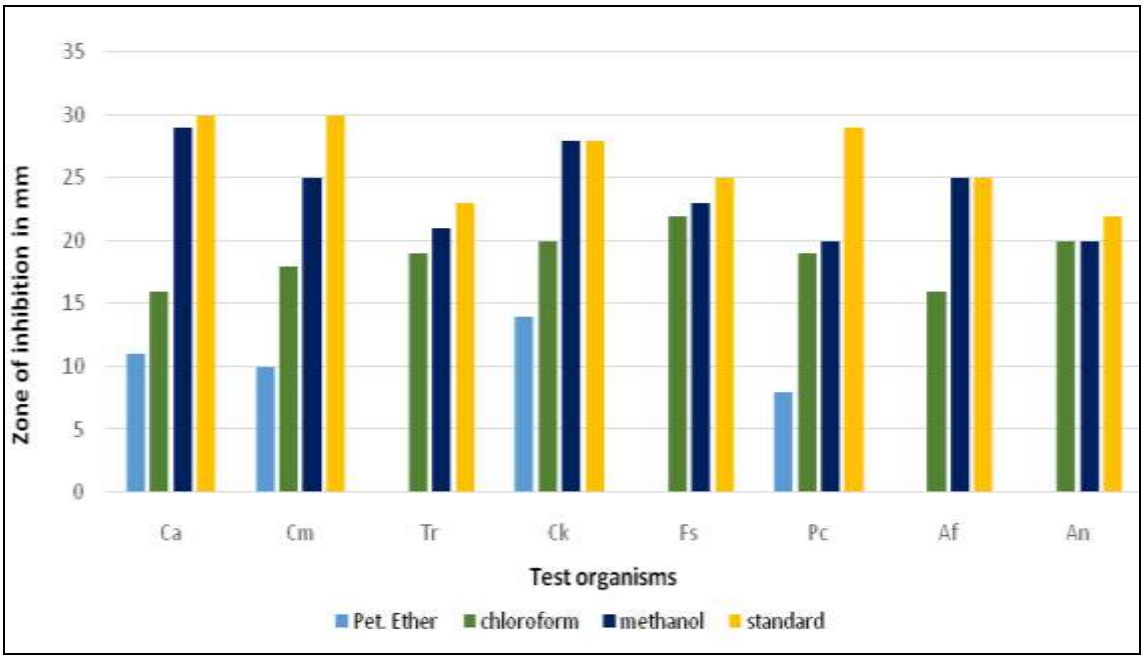
Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Ca	11±1.6	10±2	8±1	16±0	12±0.5	10±0.6	29±1.5	22±2	19±1.5	30±2.3
Cm	10±0	8±1	0	18±1	10±0	8±0	25±0	19±0	15±0	30±1
Tr	0	0	0	19±1.2	16±0	14±0	21±0	19±0	14±0	23±1.2
Ck	14±1	8±0.5	0	20±1,3	13±0	6±0	28±0	25±0	20±0	28±0.5
Fs	0	0	0	22±1.8	19±0	15±0	23±0	20±0	15±0	25±0
Pc	8±2	6±0.5	0	19±0.4	14±0	9±0	20±0	15±0	11±0	29±0
Af	0	0	0	16±1	14±1.5	12±0.6	25±0.5	20±0	15±0	25±0
An	0	0	0	20±2.2	14±0.5	8±0.5	20±0.5	15±0	10±0	22±0

Ca = Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and *An = Aspergillus niger*

Table 7. Antibacterial activity of *Phellinus linteus* at different concentration and different solvent

Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Xc	8±0	0	0	8±0.5	6±1	0	20±1	15±2	9±1.5	19±1
Ps	8±0	0	0	14±0.6	10±2	7±1	24±1.5	16±1	10±1.5	25±1
At	10±0	0	0	13±0.5	10±1.6	8±1	30±0.5	24±1	15±1	36±1
Kp	10±0	0	0	16±1	10±0.7	6±0.8	25±2	20±1.5	15±1	28±2
Ec	9±o	0	0	15±1	8±2	0	24±1.5	20±1.5	18±1	24±2
St	12±	8±	0	13±0.5	10±1.4	6±1	24±2.3	20±1.3	18±2	26±1.5
Pa	10±0	0	0	14±1	10±1	8±1	28±1.2	20±1.5	10±2	28±1
Sa	12±0	8±1	0	17±0.8	13±0.7	9±1.5	28±2	19±1.5	10±2	30±1.2

Xc = Xanthomonas campestris, Ps = Pseudomonas syringae, At = Agrobacterium tumefaciens, Kp = Klebsiella pneumonia, Ec = Escheritia coli, St = Salmonella typhi, Pa = Pseudomonas aeruginosa and *Sa = Staphylococcus aureus*

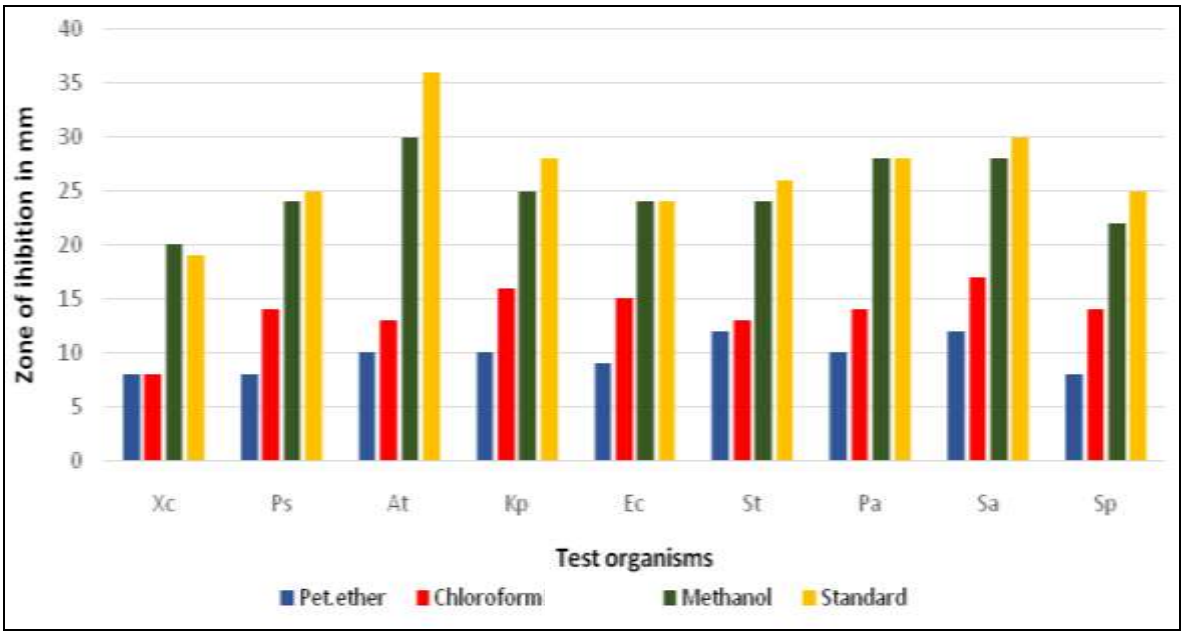


Ca=*Candida albicans*, Cm = *Chrysosporium merdarium*, Tr = *Trichophyton rubrum*, *Ck = Chrysosporium keratinophilum*, Fs = *Fusarium solani*, Pc = *Penicillium chrysogenum*, Af = *Aspergillus flavus* and An =*Aspergillus niger*.

Fig. 7. Antifungal activity of *Phellinus linteus* at 100% concentration of different solvents.

Mycelia of *Phellinus linteus* inhibited *K. pneumonia, X. campestris* and *A. tumefaciens* maximum almost equal to standard with 27 mm and *E. coli* was least inhibited with 18 mm. Sporocarp inhibited *K. pneumonia* maximum with 29 mm greater standard and *E. coli* was inhibited with 20 mm zone as least (Fig. 8 and Table 8).

In antifungal activity mycelia suppressed the growth of *C. merdarium* 28 mm to its maximum and *Fusarium solani* was inhibited with only 20 mm zone reporting least zone of inhibition, but mushroom inhibited *Fusarium solani* with maximum zone 27 mm and least reported was 12 mm against *T. rubrum* (Fig. 9, 10; Table 9 and Plate-4).



Xc = *Xanthomonas campestris*, Ps = *Pseudomonas syringae*, At = *Agrobacterium tumefaciens*, Kp = *Klebsiella pneumonia*, Ec = *Escherihia coli*, St = *Salmonella typhi*, Pa = *Pseudomonas aeruginosa*, Sa = *Staphylococcus aureus* and Sp = *Streptomyces pneumoneae*

Fig. 8. Antibacterial activity of *Phellinus linteus* at 100% concentration of different solvents.

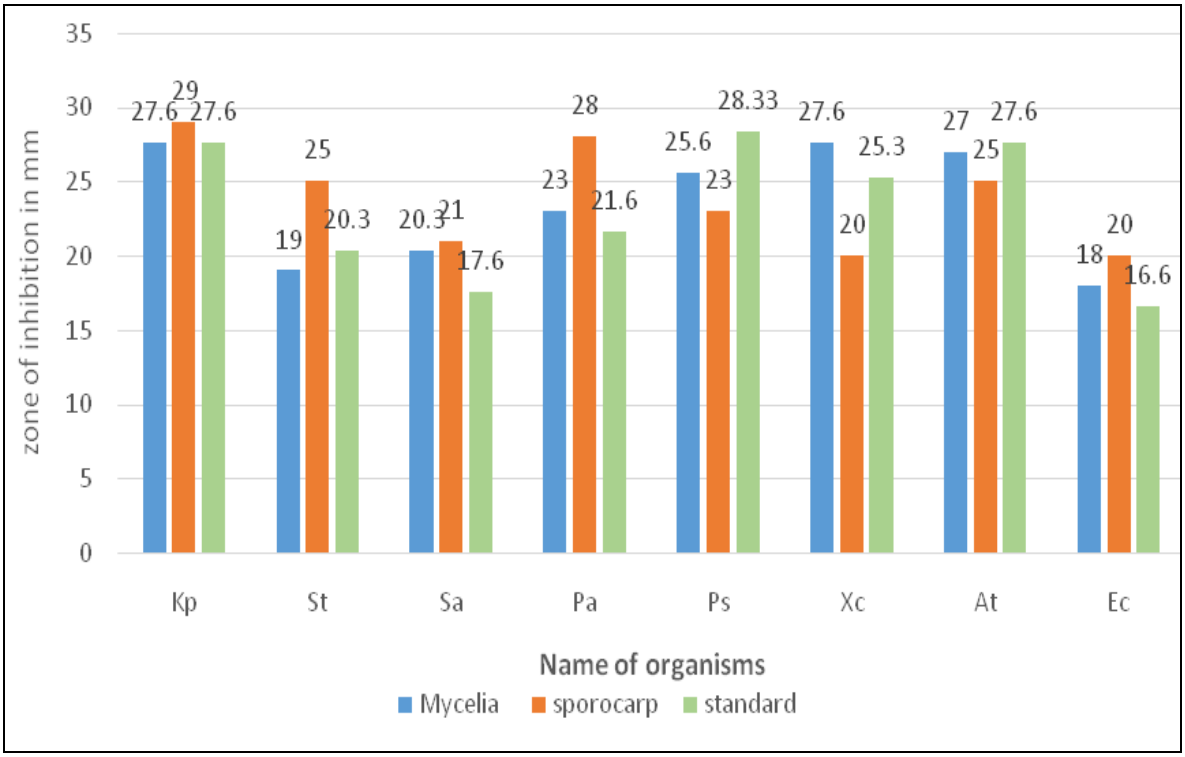


Fig. 9. Antibacterial activity of mycelia and sporocarp of *Phellinus linteus.*

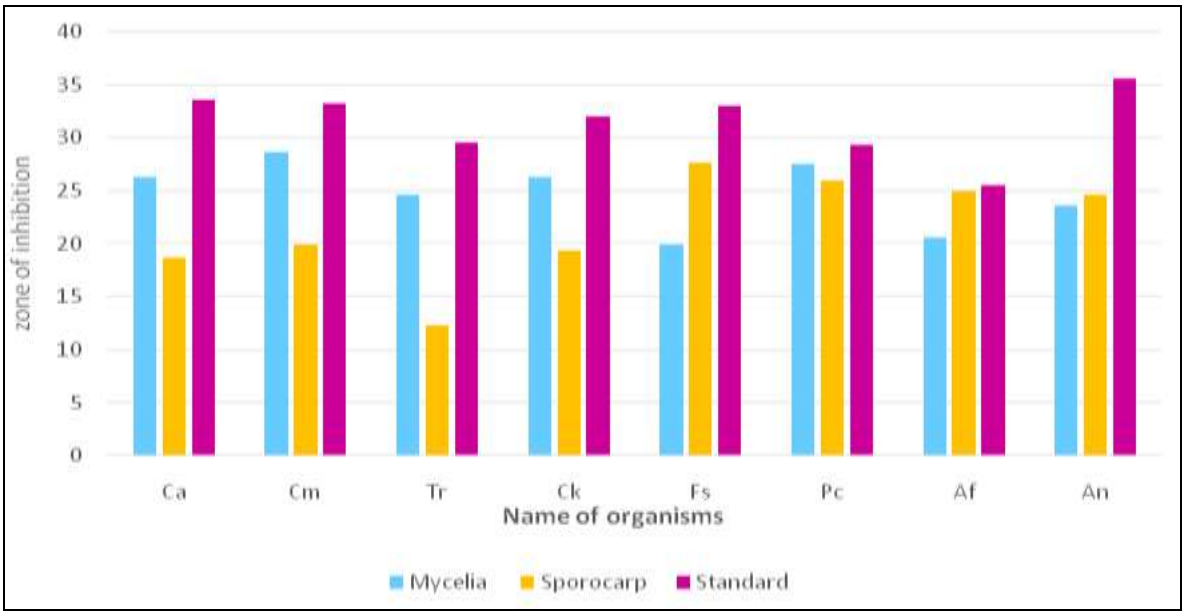


Fig. 10. Antifungal activity of mycelia and sporocarp of *Phellinus linteus.*

Petroleum ether extract of *Phellinus tremulae* exhibited very poor antibacterial activity inhibiting only *X. campestris* at 6.33 mm among all the test bacteria (Fig. 11), but *C. albicans* and *Fusarium solani* were only fungi inhibited by pet ether extract 8 and 9 mm respectively (Plate-6 C, D & E).

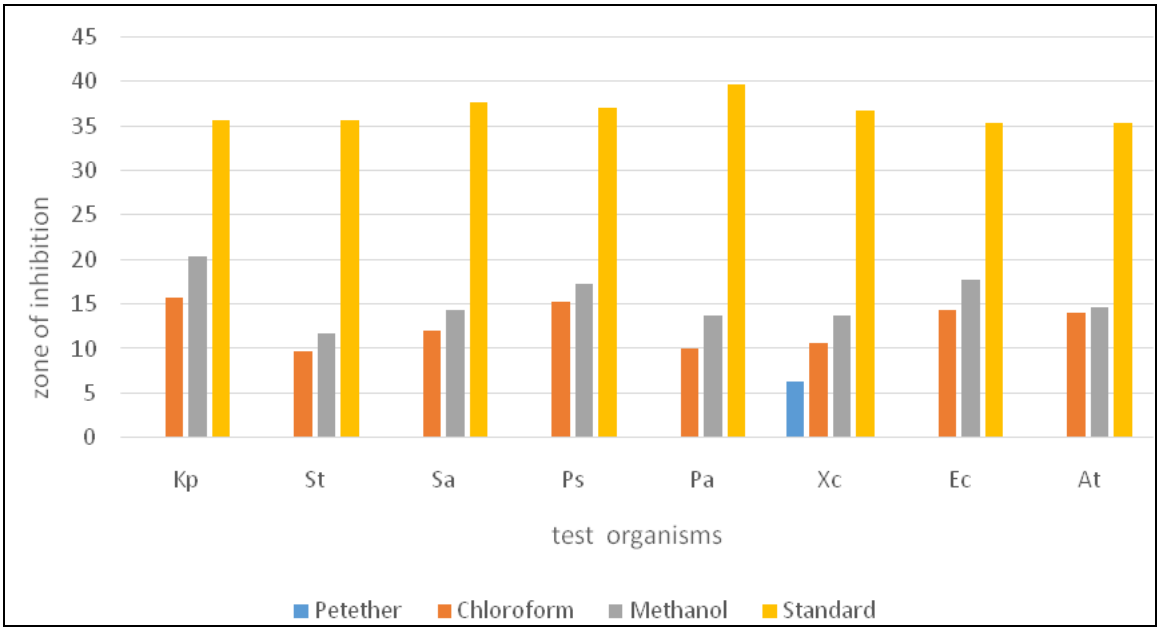


Fig. 11. Antibacterial activity of *Phellinus tremulae* at 100% concentration of different solvents.

Table 8. Antibacterial activity of mycelia and sporocarp of *Phellinus linteus* at different concentrations

Organisms	Mycelia			Standard	Mushroom		
	100	**50**	**25**		**100**	**50**	**25**
Kp	27.6±0.4	25.3±0.57	20.3±0.47	27.6±0.47	29±1.5	22±2	19±1.5
St	19±0.8	16.3±1.15	15±0	20.3±0.47	25±0.5	19±1.52	15±0
Sa	20.3±0.4	18.6±0.57	14.6±0.94	17.6±0.47	21±1.5	19±2	14±0
Pa	23±0.81	15±0	12.3±0.47	21.6±1.24	28±0.57	25±0.47	20±0.47
Ps	25.6±1.88	21.6±1.5	17.6±0.47	28.33±1.24	23±1	20±1.52	15±1.52
Xc	27.6±0.47	24±1	19.3±0.47	25.3±0.4	20±1.57	15±0.47	11±0.47
At	27±0.81	21.3±1.52	18.3±1.24	27.6±0.4	25±0.5	20±1.52	15±0.47
Ec	18±0.81	15.3±0.57	12.3±0.47	16.6±1.24	20±0.5	15±1.52	10±1.52

Kp = Klebsiella pneumonia, St = Salmonella typhi, Sa = Staphylococcus aureus, Pa = Pseudomonas aeruginosa, At = Agrobacterium tumefaciens, Ps = Pseudomonas syringae, Xc = Xanthomonas campestris and *Ec = Escheritia coli*

Table 9. Antifungal activity of mycelia and sporocarp of *Phellinus linteus* at different concentrations

Organisms	Mycelia			Standard	Mushroom		
	100	50	25		100	50	25
Ca	26.33 ±1.52	23.6 ±2.08	21.33±2.3	33.6±1.5	18.66±0.57	14.6±2.51	11.66±0.57
Cm	28.66 ±1.15	24.33 ±1.15	21±1	33.3±1.15	20±1	17.66±0.577	15±1
Tr	24.6 ±2.51	22.33 ±2.08	19.33±1.15	29.6±1.52	12.33±0.577	9.666±0.577	8.333±0.57
Ck	26.3 ±1.52	23.6 ±1.15	20.6±1.15	32±2	19.33±0.577	10.33±0.57	0±0
Fs	20 ±2	18 ±2	15.6±2.08	33±2	27.66±0.577	18±1	11.66±0.577
Pc	27.6 ±2.51	24.3 ±4.04	18.66±5.5	29.3±1.15	26±1	19.66±0.577	17.666±0.57
Af	20.6 ±1.15	16.6 ±2.3	13.66±1.52	25.6±1.52	25±1	19.66±0.577	14.333±0.577
An	23.6 ±2.08	26.6 ±2.88	22±1.73	35.6±1.15	24.66±0.577	19±1	13.6±0.577

Ca = Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and *An = Aspergillus niger*

Chloroform extract of *Phellinus tremulae* inhibited *K. pneumonia* and *P. syringae* maximum by exhibiting 15 mm and least inhibition was observed against *P. aeruginosa* 10 mm (Table 10).

Fusarium solani, *A. flavus* and *A. niger* were inhibited equally by 19 mm. *T. rubrum* and *P. chrysogenum* were least inhibited with 10 mm (Table 10 and Plate-6 A & B). Methanol extract inhibited *K. pneumonia* maximum 20 mm and *S. typhi* is least inhibited by 11 mm zone but against *Fusarium solani* methanol showed 27 mm considerable zone, but *T. rubrum* were was least inhibited 12 mm by methanol extract (Fig. 12 and Table 11).

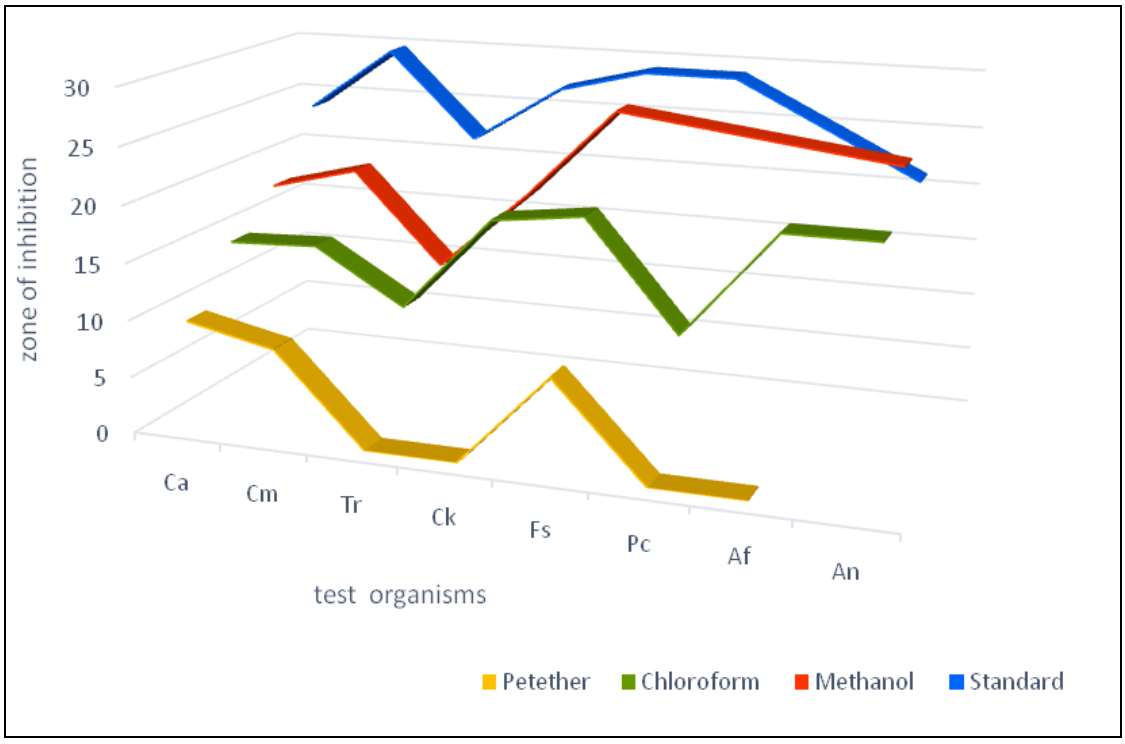


Fig. 12. Antifungal activity of *Phellinus tremulae* at 100% concentration of different solvents.

Table 10. Antifungal activity of *Phellinus tremulae* at different concentration and different solvent

Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Ca	9.66±0.57	6.33±0.57	0	14.66±0.577	13.33±0.57	9.66±0.57	18.66±0.57	14.6±2.51	11.66±0.57	24±1
Cm	8±0	0	0	15±1	11.66±0.57	8.6±0.57	20±1	17.66±0.57	15±1	29.66±0.5
Tr	0	0	0	10.33±0.57	8.33±0.57	6.33±0.57	12.33±0.57	9.666±0.57	8.333±0.5	22.66±1.5
Ck	0	0	0	18.66±0.57	14.66±0.57	11.66±0.57	19.33±0.57	10.33±0.57	0	27±1
Fs	8.33±0.57	0	0	19.66±0.57	14.66±0.57	11±1	27.66±0.57	18±1	11.66±0.5	29.33±0.5
Pc	0	0	0	10.33±0.57	8.66±0.57	6.333±0.57	26±1	19.66±0.57	17.666±0.57	29±1
Af	0	0	0	19.66±0.57	14.33±0.57	10.33±0.57	25±1	19.66±0.57	14.333±0.57	25±1
An	0	0	0	19.66±0.57	14.33±0.57	8.66±0.57	24.66±0.57	19±1	13.6±0.57	21.33±0.5

Ca = Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and *An = Aspergillus niger*

Table 11. Antibacterial activity of *Phellinus tremulae* at different concentration and different solvent

Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Kp	0±0	0±0	0±0	15.66±0.57	12±1	11±1	20.33±0.57	14.33±0.57	11.6±0.57	35.66±1.52
St	0±0	0±0	0±0	9.66±0.57	8±0	6.66±0.57	11.66±0.57	8±1	7±1	35.66±0.57
Sa	0±0	0±0	0±0	12±1	9.66±0.57	7.66±0.57	14.333±0.57	9.66±0.57	9±0	37.66±1.15
Ps	0±0	0±0	0±0	15.33±0.57	11.33±0.57	8.33±0.57	17.33±0.57	12±1	9.66±0.57	37±1
Pa	0±0	0±0	0±0	10±0	8.333±0.57	0±0	13.66±0.57	10.66±0.57	9.66±0.57	39.66±1.52
Xc	6.33±0.57	0±0	0±0	10.66±0.57	0±0	0±0	13.66±0.57	11.66±0.57	9.33±0.57	36.66±0.57
Ec	0±0	0±0	0±0	14.33±0.57	9.66±0.57	8.33±0.57	17.66±0.57	9.66±0.57	8.33±0.57	35.33±0.57
At	0±0	0±0	0±0	14±0	9.66±0.57	8.33±0.57	14.66±0.57	9.66±0.57	8.66±0.57	35.33±0.57

Kp = Klebsiella pneumonia, St = Salmonella typhi, Sa = Staphylococcus aureus, Ps = Pseudomonas syringae, Pa = Pseudomonas aeruginosa, Xc = Xanthomonas campestris, Ec = Escheritia coli and *At = Agrobacterium tumefaciens*

Methanol extract of *Entoloma speculum* showed positive result to all tests except Saponins and Triterpinoids hence it was subjected to GCMS analysis to know/find out bioactive compounds (Table 3). The methanol fraction of *E speculum* showed different bioactive compounds. 1,2-Benzenediol, Octadecanoic acid methyl ester (CAS) methyl stearate, Ergosterol and Ergosta-7, 22-dien-3-ol,are the major compounds. Similar compounds are found from methanol extract of *Termetomyes* species from GCMS analysis (Osman, 2015).

The minimum inhibitory concentration values of pet ether chloroform and methanol extract varied from 6-15, 7-29 and 11-37 respectively. Petroleum ether extract showed maximum inhibition against *Staphylococcus aureus* and *Pseudomonas aeruginosa* showed minimum inhibition against *Chrysosporium merdarium* and *Fusarium solani* (Plate-5 A to D). Whereas petroleum ether extract of *E. speculum* did not show any inhibition against *Chrysosporium keratinophilum* (Table 12) and *Penicillium crysoginum*. But *Aspergillus flavous, A. niger, Candida albicans, Salmonella typhi* and *Escherichia coli* were moderately inhibited (Fig. 13).

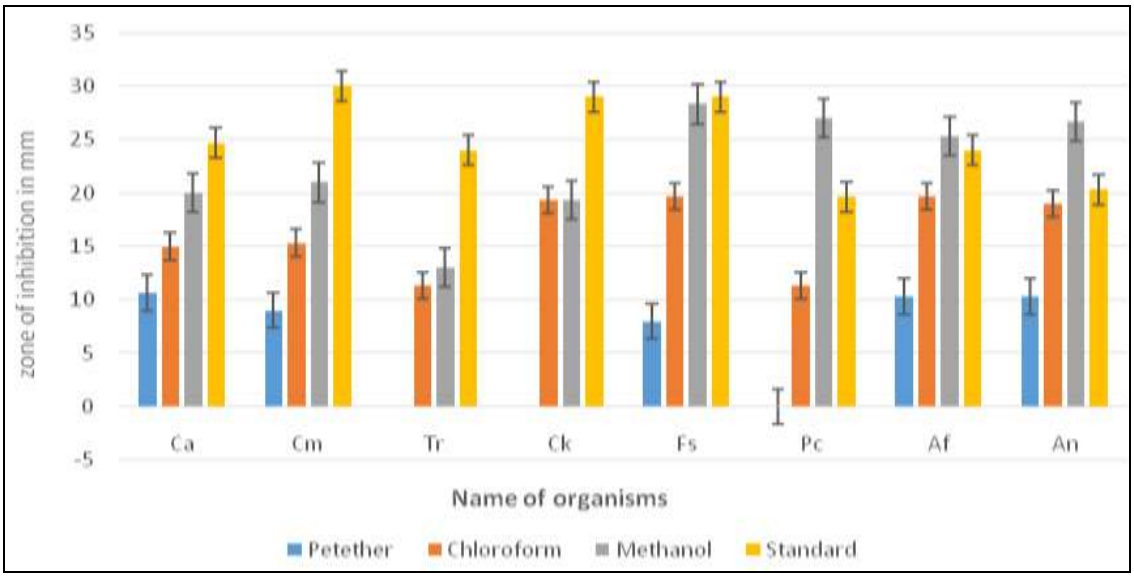


Fig. 13. Antifungal activity of *Entoloma speculum* at 100% concentration of different solvents.

Chloroform extract showed maximum inhibition against *Klebsiella pneumonia*, *Staphylococcus aureus* and *Pseudomonas aeruginosa* higher than the standard drug (Fig. 14). Chloroform extract of *E. speculum* showed minimum effect against *Candida albicans* and *Trichophyton rubrum* and other test organisms were moderately affected.

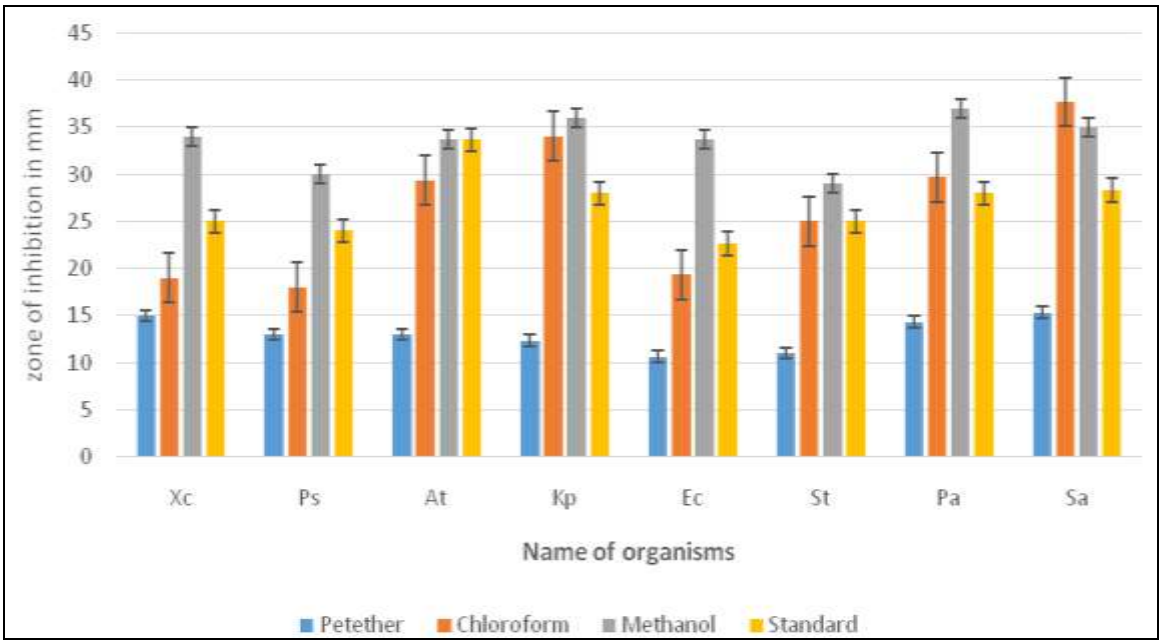


Fig. 14. Antibacterial activity of *Entoloma speculum* at 100% concentration of different solvents.

Methanolic extract exhibited a maximum activity against all the test organisms. The best was 37 mm against *Pseudomonas aeruginosa*, followed by *Klebsiella pneumonia* (36 mm) (Table 13), *Staphylococcus aureus* (35 mm), *Fusarium solani* (28 mm) (Fig. 15), *Penicillium crysoginum* (27 mm) and *Apergillus niger* (26 mm) and the least inhibition was observed against *Trichophyton rubrum* (13 mm) and all other organisms were inhibited moderately with 13-21 mm. Comparatively methanol showed good result this may be due to the presence of 1,2-Benzenediol.

Table 12. Antifungal activity of *Entoloma speculum* at different concentration and different solvent

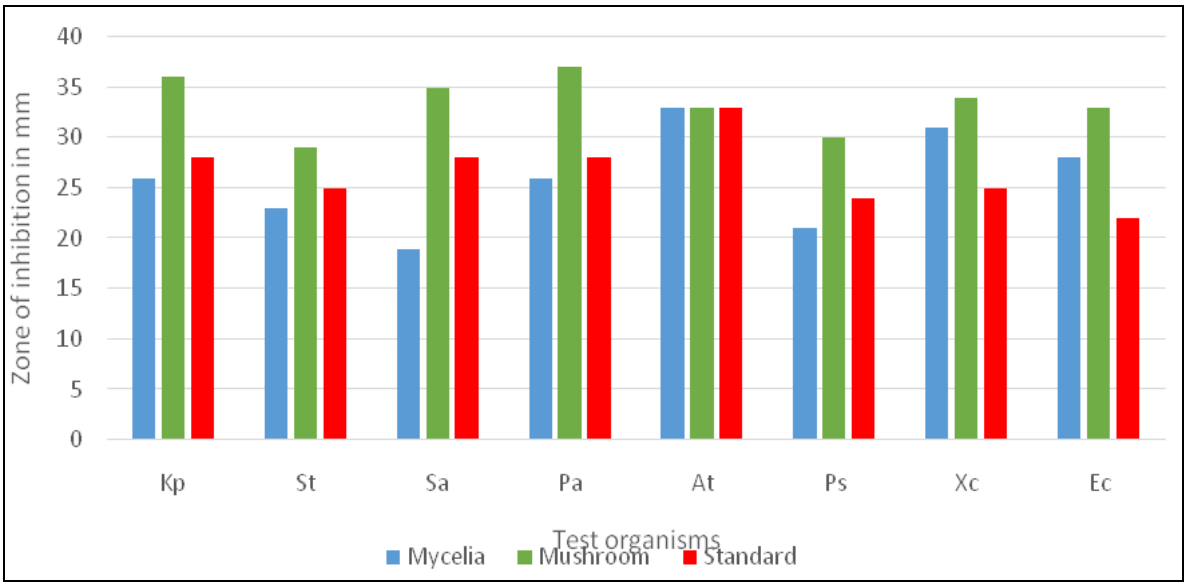
Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Ca	10.66±0.57	6.33±1.52	0±0	15±1	14±1	11±1	20±1	15±1	12±1	24.66±0.57
Cm	9±1	0±0	0±0	15.33±2.51	13±1	9.33±0.57	21±1	17.33±1.15	14±1	30±1
Tr	0±0	0±0	0±0	11.33±1.52	9±1	7±1	13±1	11±1	8.33±0.57	24±1
Ck	0±0	0±0	0±0	19.33±0.57	15.66±1.15	12.33±0.57	19.33±1.52	10.66±1.15	0±0	29±1
Fs	8±0	0±0	0±0	19.66±1.52	14.66±0.57	11±1	28.33±1.52	18±1	13±1	29±1
Pc	0±0	0±0	0±0	11.33±1.52	10±1	7.33±1.52	27±1	21±1.73	18.33±0.57	19.66±0.57
Af	10.33±0.57	9±1	7±1	19.66±1.52	15±1	11.33±1.52	25.33±1.52	22.33±2.08	15.33±1.52	24±1
An	10.33±1.15	10±1	6.66±1.15	19±1	14±1	10±1	26.66±1.52	21.66±2.08	14±1	20.33±1.52

Ca = Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and *An = Aspergillus niger*

Table 13. Antibacterial activity of *Entoloma speculum* at different concentration and different solvent

Organisms	Zone of inhibition in mm (Mean± SD)									
	Petroleum ether			Chloroform			Methanol			Standard
	100%	50%	25%	100%	50%	25%	100%	50%	25%	
Xc	15±1	8.66±1.15	7±1	19±1	15±1	12±1	34±1	28±1	26±1	25±1
Ps	13±2	10±2	7.33±2.08	18±0	14.66±0.57	12.33±1.52	30±1	24.66±0.57	19±1	24±1
At	13±1.73	12±0	9±1	29.33±0.57	24±1	13.66±1.52	33.66±1.52	28.66±1.52	25.33±1.52	33.66±2.51
Kp	12.33±1.15	10.33±0.57	10±0	34±1	24±1	20±1	36±1	28.33±1.52	26±1	28±1
Ec	10.66±1.15	8.66±1.15	7.33±1.15	19.33±0.57	16±1	12.66±0.57	33.66±1.52	28.66±1.52	25.33±1.52	22.66±1.52
St	11±1	11±1	8±0	25±6.08	9.66±0.7	12.66±1.15	29±1	17±0.7	21.33±1.52	25±1
Pa	14.33±1.52	12±1	12±2	29.66±0.57	24.33±0.57	19±1	37±1	29.66±1.52	24.66±0.57	28±1
Sa	15.33±0.57	13±2	7.66±0.57	37.66±1.52	30±1	21±2	35±1	30±1	23.33±2.08	28.33±1.52

Xc = Xanthomonas campestris, Ps = Pseudomonas syringae, At = Agrobacterium tumefaciens, Kp = Klebsiella pneumonia, Ec = Escheritia coli, St = Salmonella typhi, Pa = Pseudomonas aeruginosa and *Sa = Staphylococcus aureus*

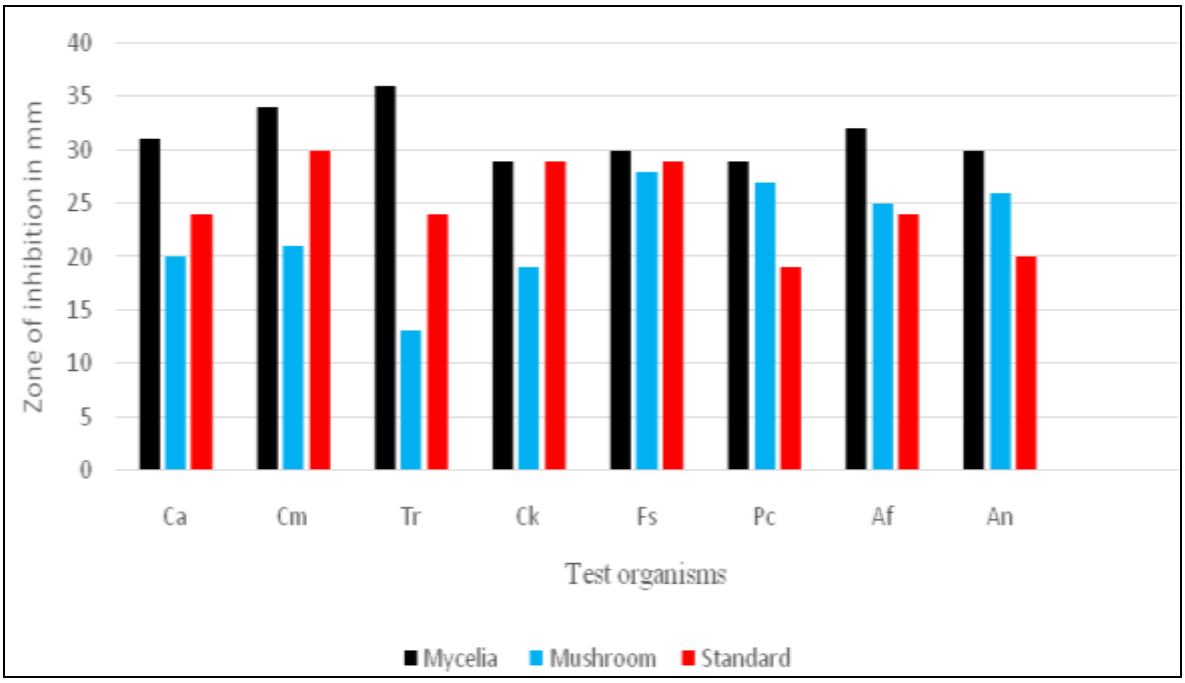


Kp = Klebsiella pneumonia, St = *Salmonella typhi*, *Sa = Staphylococcus aureus*, *Pa = Pseudomonas aeruginosa*, *At = Agrobacterium tumefaciens*, *Ps = Pseudomonas syringae*, *Xc = Xanthomonas campestris* and *Ec = Escherihia coli*

Fig. 15. Antibacterial activity of mycelial and sporocarp extract of *E. speculum*.

In the present study the antimicrobial activity of mycelia and sporocarps of *E. speculum* is studied, fruiting bodies and mycelia showed significant activity against test organisms. Mycelia inhibited *T. rubrum* maximum (36 mm), mushroom inhibited *F. solani* (28 mm) maximum, whereas mushroom extract least inhibited *T. rubrum* (13 mm). *C. keratinophilum* and *P. chrysogenum* were inhibited by mycelial extract of *Entoloma speculum* equally with (29 mm) but *P. chrysogenum* was inhibited with 27 mm by mushroom extract. *F. solani* and *A. niger* was inhibited with 30 mm inhibition zone by mycelial extract (Fig. 16, Table 14 and Plate-7).

A. tumefaciens inhibited by mycelia extract with 32 mm zone. Mushroom extract of *Entoloma speculum* showed 26 mm inhibition zone against *A. niger*. 25 per cent concentration of mushroom extract did not show any activity on *C. keratinophilum*, but all the three concentration (100, 50 and 25 per cent) of mycelia showed good inhibition on *C. keratinophilum* range from 29-24 mm (Plate-4).



Ca=Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and *An = Aspergillus niger*

Fig. 16. Antifungal activity of mycelial and sporocarp extract of *E. speculum*.

Whereas *P. chrysogenum* was inhibited by mycelia and mushroom extract both. *C. albicans* was significantly inhibited by mycelium but moderately inhibited by mushroom. Pathogenic bacteria were inhibited by Sporocarp extract than the mycelia. Sporocarp suppressed the growth of all bacterial pathogens. *X. campestris* was susceptible to mycelial extract greater than the standard Amoxycilin. *E. coli* inhibited with 28 mm zone while *K. pneumonia* and *P. aeruginosa* inhibited with 26 mm zone. *S. aureus* was least inhibited with 19 mm. *S. typhi* showed 23 mm inhibition by mycelia extract but mushroom extracts inhibited *S. typhi* by 29 mm greater than standard (25 mm). *P. aeruginosa* showed 37 mm zone highest of all the other test organisms (Fig. 16).

Table 14. Antifungal activity of mycelial and sporocarp extract of *Entoloma speculum* in different concentrations

Organisms	Mycelial extract in different concentration (%)			Standard Terbinafine	Mushroom extract in different concentration (%)			Standard Terbinafine
	100	50	25		100	50	25	
Ca	31±1	28.33±1.52	25.66±1.15	31±3.6	20±1	15±1	12±1	24.66±0.57
Cm	34±2	29±1	25±1	37.33±1.15	21±1	17.33±1.15	14±1	30±1
Tr	36.66±1.52	31±3.6	26.33±1.52	32.66±2.3	13±1	11±1	8.33±0.57	24±1
Ck	29.33±1.15	27±1	24.66±1.52	34.66±1.52	19.33±1.52	10.66±1.15	0±0	29±1
Fs	30.66±1.15	27±1.73	21.33±1.52	28.33±4.16	28.33±1.52	18±1	13±1	29±1
Pc	29.33±1.52	24.66±0.57	21.33±1.52	31.33±1.15	27±1	21±1.73	18.33±0.57	19.66±0.57
Af	32±2	27.66±0.57	25.33±1.52	32±3	25.33±1.52	22.33±2.08	15.33±1.52	24±1
An	30.66±1.15	27.33±1.15	23.66±1.52	31.66±1.52	26.66±1.52	21.66±2.08	14±1	20.33±1.52

Ca = Candida albicans, Cm = Chrysosporium merdarium, Tr = Trichophyton rubrum, Ck = Chrysosporium keratinophilum, Fs = Fusarium solani, Pc = Penicillium chrysogenum, Af = Aspergillus flavus and An = Aspergillus niger

Table 15. Antibacterial activity of mycelial and mushroom extract of *Entoloma speculum* in different concentrations

Organisms	Mycelial extract in different concentration (%)			Standard Amoxycilin	Mushroom extract in different concentration (%)			Standard Amoxycilin
	100	50	25		100	50	25	
Kp	26.66±1.52	20.66±5.13	17.66±6.8	30.66±1.15	36±1	28.33±1.52	26±1	28±1
St	23.33±2.88	21.66±3.21	19.33±3.05	25.33±0.57	29±1	17±0.7	21.33±1.52	25±1
Sa	19.66±1.52	16.33±1.52	10.66±1.15	20.66±1.15	35±1	30±1	23.33±2.08	28.33±1.52
Pa	26.33±1.15	15±2	10.33±0.57	25.33±0.57	37±1	29.66±1.52	24.66±0.57	28±1
At	33.33±1.15	30.33±0.57	26±1	30.66±1.15	33.66±1.52	28.66±1.52	25.33±1.52	33.66±2.51
Ps	21±3.6	18.66±4.04	16.33±4.5	23.33±0.57	30±1	24.66±0.57	19±1	24±1
Xc	31.33±1.15	28.33±2.88	24.66±4.16	30.66±1.15	34±1	28±1	26±1	25±1
Ec	28.66±1.52	21.66±2.08	13.66±1.15	32.66±1.15	33.66±1.52	28.66±1.52	25.33±1.52	22.66±1.52

Kp = Klebsiella pneumonia, St = Salmonella typhi, Sa = Staphylococcus aureus, Pa = Pseudomonas aeruginosa, At = Agrobacterium tumefaciens, Ps = Pseudomonas syringae, Xc = Xanthomonas campestris and *Ec = Escheritia coli*

4.9. Analgesic activity

Pain is one of the unpleasant sensation. Pain may be beneficial to man and animal as it is protective mechanism to body, not only triggers reaction and also induces learned avoidance behaviours. Pain may be predisposing, persistent or due to consequence of brain or nerve injury. Pathological condition and tissue injury is immediate cause of pain, which result in release of number of chemicals than act of nerve terminal and increase sensitivity for (Suseem *et al.*, 2011) stimulation. Anti-analgesic drugs in market are with more side effects, so attention is focused in the natural remedies with are cheaper in cost and have no side effects.

Table 16. Analgesic activity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum*

Treatment	Dose mg/kg	Number of writhings (Average) 30 min	AVG±STD	% inhibition
Control	Saline	74.33	74.33±1.52	0
Standard (Ibuprofen)	100	17.83	17.83±1.25	76.0123***
Phellinus linteus	250	45.66	45.66±2.08	38.57***
Phellinus linteus	500	27	27±1.73	63.67***
Phellinus tremulae	250	54	54±1	27.35***
Phellinus tremulae	500	21.33	21.33±1.52	71.30***
Entoloma speculum	250	30	30±1	59.63***
Entoloma speculum	500	22	22±1	70.40***

Results are expressed as Mean ± SEM. The difference between experimental groups was compared by ANOVA followed by Dunnett's test. The results were considered statistically significant when $P < 0.0001$

Analgesic activity was tested in SCS Pharmacy College, Harapanahalli (Annexure-2). Analgesic activity was carried by acetic acid induced writhing method according to Collier *et al.* (1968). The animals of control group were received only saline, standard group received Ibuprofen as standard drug, rest groups are served with different (250 &500mg/kg) dose of all the test extracts. One hour after administration of extracts glacial acetic acid 10ml/kg b.w was administered intra peritoneally (I.P) to all the mice to induce abdominal contortion nor writhings. The maximum number of writhings were observed in control group (74.3±1.52) with no inhibition (Fig. 17 and Table 16), and the least number of inhibition were noted in standard group (17.83±1.25). *Phellinus tremulae* showed considerable activity followed by *Entoloma speculum* and *Phellinus linteus. P. linteus* inhibited writhings by 38.57% in low dosage and 63.67%, while *P. tremulae* inhibited writhings by 27.35% in low dose and 71.30% inhibition in high dose, whereas *E. speculum* inhibited upto 59.63% in low dose and in high dose 70.40% writhings were inhibited (Plate-8).

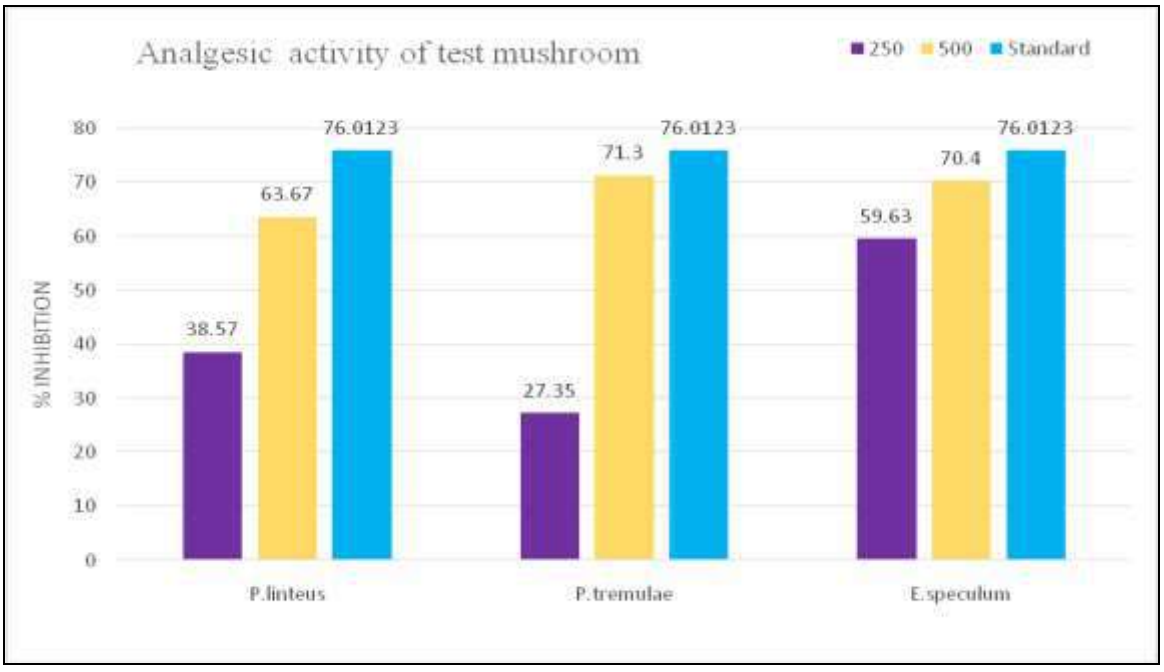


Fig. 17. Analgesic activity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum.*

4.10. Amylase inhibition

Diabetes mellitus (DM) is a chronic metabolic disorder caused by insulin dysfunction, results in rise of blood sugar. Diabetes also produce oxidative stress. Recent discoveries have opened up an exciting opportunity for developing new types of therapeutics from medicinal mushrooms to control DM and its complications. To date, more and more active components including polysaccharides and their protein complexes, dietary fibres, and other compounds extracted from fruiting bodies, cultured mycelium, or cultured broth of medicinal mushrooms have been reported as to having anti-hyperglycaemic activity. These compounds exhibit their antidiabetic activity via different mechanisms (Wasser, 2011; Prabhu, 2014).

Table 17. Amylase inhibition of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum*

Sl. No.	Sample	OD	Conc. of Maltose	Activity (µg/ml/min)	Percentage inhibition
1	Control	1.63	133.25	0.04	0
3	*Phellinus linteus* 100 µg/ml	0.84	67.42	0.02	49.43
4	*Phellinus linteus* 250 µg/ml	0.58	45.75	0.01	65.68
5	*Phellinus linteus* 500 µg/ml	0.18	12.42	0	90.69
6	*Phellinus tremulae* 100 µg/ml	0.93	74.92	0.02	43.8
7	*Phellinus tremulae* 250 µg/ml	0.32	24.08	0.01	81.93
8	*Phellinus tremulae* 500 µg/ml	0.2	14.08	0	89.44
9	*Entoloma speculum* 100 µg/ml	1.43	116.58	0.03	12.55
10	*Entoloma speculum* 250 µg/ml	0.9	72.42	0.02	45.68
11	*Entoloma speculum* 500 µg/ml	0.35	26.58	0.01	80.06

The difference between experimental groups was compared by ANOVA followed by Dunnett's test. The results were considered statistically significant when $P < 0.0001$

Amylase inhibition activity was carried out in Biogenics, Hubballi (Annexure-3). *Phellinus linteus* inhibited α-amylase by 90.69% at 500 µg/ml concentration being highest in test mushrooms followed by *P. tremulae* 89.44% and the least was *E. speculum* 80.06%. *P. linteus* 100 µg inhibited 49.43% which is greater than *E. speculum*'s 250 µg inhibition (45.68%). *E. speculum* 100 µg is the least inhibition (12.55%) observed in the present investigation. Both *Phellinus* species inhibited α-amylase significantly (Fig. 18 and Table 17).

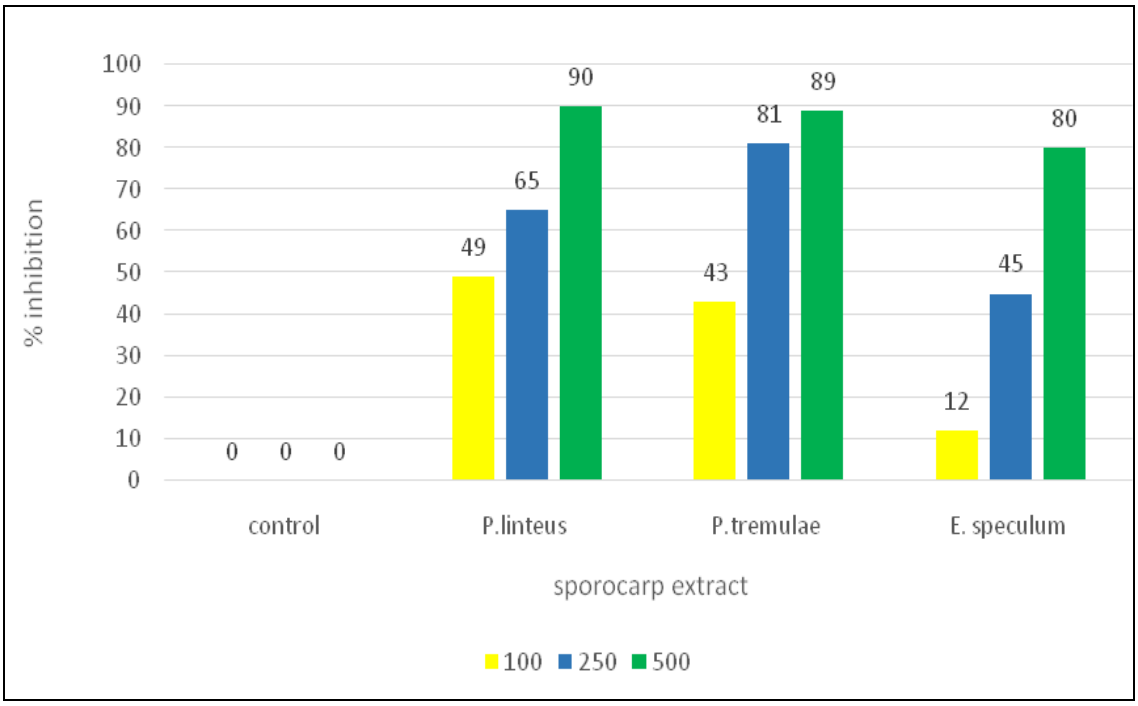


Fig. 18. Amylase inhibition of *P. linteus, P. tremulae* and *E. speculum*.

4.11. Cytotoxic activity

Cancer is abnormal growth of cells. Cancer may be due to three causes, pollution, infection, and poor nutrition. Pollutant-caused cancers are may be due to the use of tobacco products, involving the lung, oropharynx, kidney larynx, and bladder. Infectious

causes affect the stomach; cervix; liver; nasopharynx; neural, hematological and lymphatic systems; and bladder. Cancer due to poor nutrition involve the stomach, colon, esophagus, breast, liver, oropharynx, and prostate cancers. As treatments for cancer surgery, chemotherapy and radiotherapy are there but there is need of combination of these three with immunotherapy also without any side effects.

Cytotoxic activity was carried out in Amala Cancer Research Center, Thrissur, Kerala (Annexure-4). The present investigation is to study cytotoxicity of *P. linteus*, *P. tremulae* and *E. speculum* against DLA and EAC cells which were taken from the infected mice. *P. linteus* showed greater inhibition against DLA cells followed by *E. speculum* and *P. tremulae* (Fig. 19).

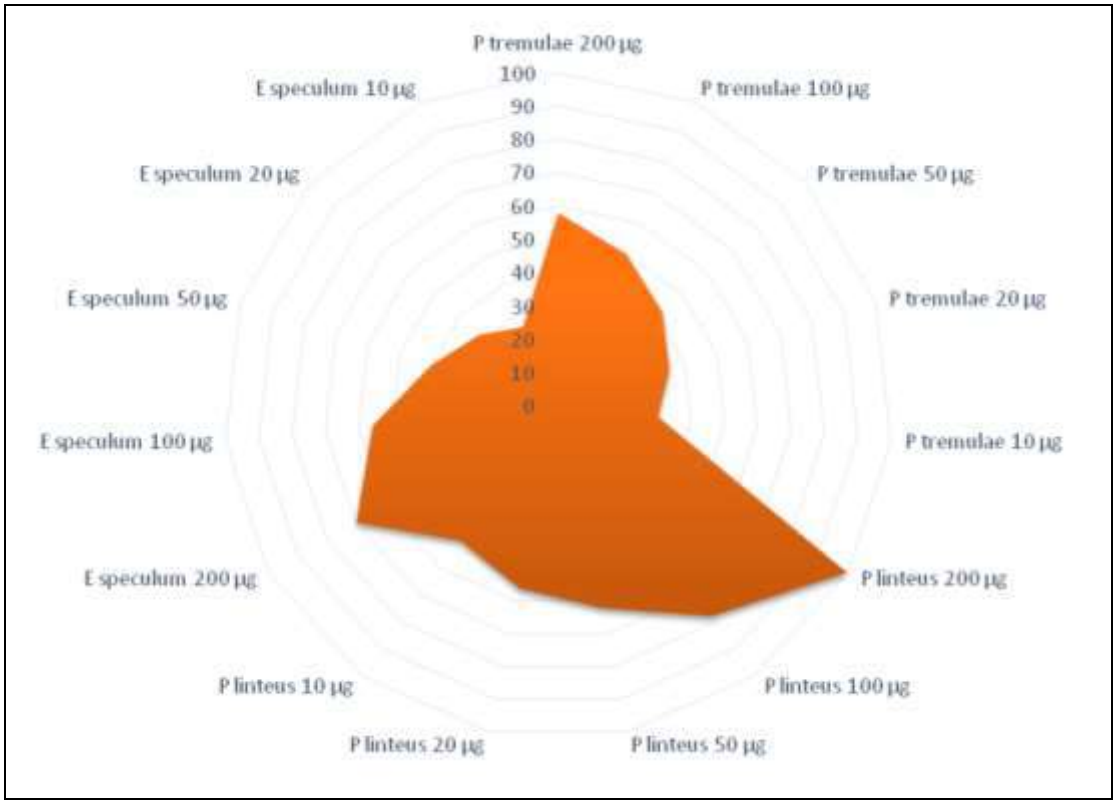


Fig. 19. Cytotoxicity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum* against DLA cells.

In cytotoxicity against EAC cells again *P. linteus* inhibited 100 per cent of cancer cells followed by *P. tremulae* (68%) and *E. speculum* (62%) at 200 µg concentration. *P. tremulae* and *E. speculum* were poor in inhibiting both DLA and EAC cells respectively. *P. linteus* inhibited DLA cells by 78 per cent at 100 µg which is more than *E. speculum* 70 per cent at 200 µg concentration (Table 18 and Fig. 19).

Table 18. Cytotoxicity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum* against DLA cells

Sl. No.	Drug conc. µg/ml	Percentage cytotoxicity
1	*P tremulae* 200 µg	58±1
	P tremulae 100 µg	50±0.5
	P tremulae 50 µg	42±0.27
	P tremulae 20 µg	35±0.3
	P tremulae 10 µg	30±0.5
2	*P linteus* 200 µg	100±0.5
	P linteus 100 µg	78±1.5
	P linteus 50 µg	62±2.5
	P linteus 20 µg	56±1.5
	P linteus 10 µg	50±0.5
3	*E speculum* 200 µg	70±0.3
	E speculum 100 µg	56±0.5
	E speculum 50 µg	40±0.5
	E speculum 20 µg	32±0.5
	E speculum 10 µg	26±0.32
ANOVA	**P value**	**< 0.0001**

Results are expressed as Mean ± SEM. The difference between experimental groups was compared by ANOVA followed by Dunnett's test. The results were considered statistically significant when $P < 0.0001$

Table 19. Cytotoxicity of *Phellinus linteus*, *Phellinus tremulae* and *Entoloma speculum* against EAC cells

Sl. No.	Drug conc. µg/ml	Percentage cytotoxicity
1	*P tremulae* 200 µg	68±1
	P tremulae 100 µg	52±0.5
	P tremulae 50 µg	43±0.27
	P tremulae 20 µg	35±0.3
	P tremulae 10 µg	20±0.5
2	*P linteus* 200 µg	100±0.5
	P linteus 100 µg	76±1.5
	P linteus 50 µg	60±2.5
	P linteus 20 µg	58±1.5
	P linteus 10 µg	50±0.5
3	*E speculum* 200 µg	62±0.3
	E speculum 100 µg	52±0.5
	E speculum 50 µg	38±0.5
	E speculum 20 µg	22±0.5
	E speculum 10 µg	12±0.32
ANOVA	**P value**	**< 0.0001**

Results are expressed as Mean ± SEM. The difference between experimental groups was compared by ANOVA followed by Dunnett's test. The results were considered statistically significant when $P < 0.0001$

In EAC cell inhibition, *P. tremulae* ranged from 20±0.5% to 68±1%, and by *E. speculum's* toxicity ranged from 50±0.5% to 100±0.5%, inhibition of EAC cells of 10 µg concentration of *P. linteus* (50±0.5%) is equal to 100 µg of *P. tremulae* (50±0.5%) and *E. speculum* (52±0.5%) (Fig. 20 and Table 19).

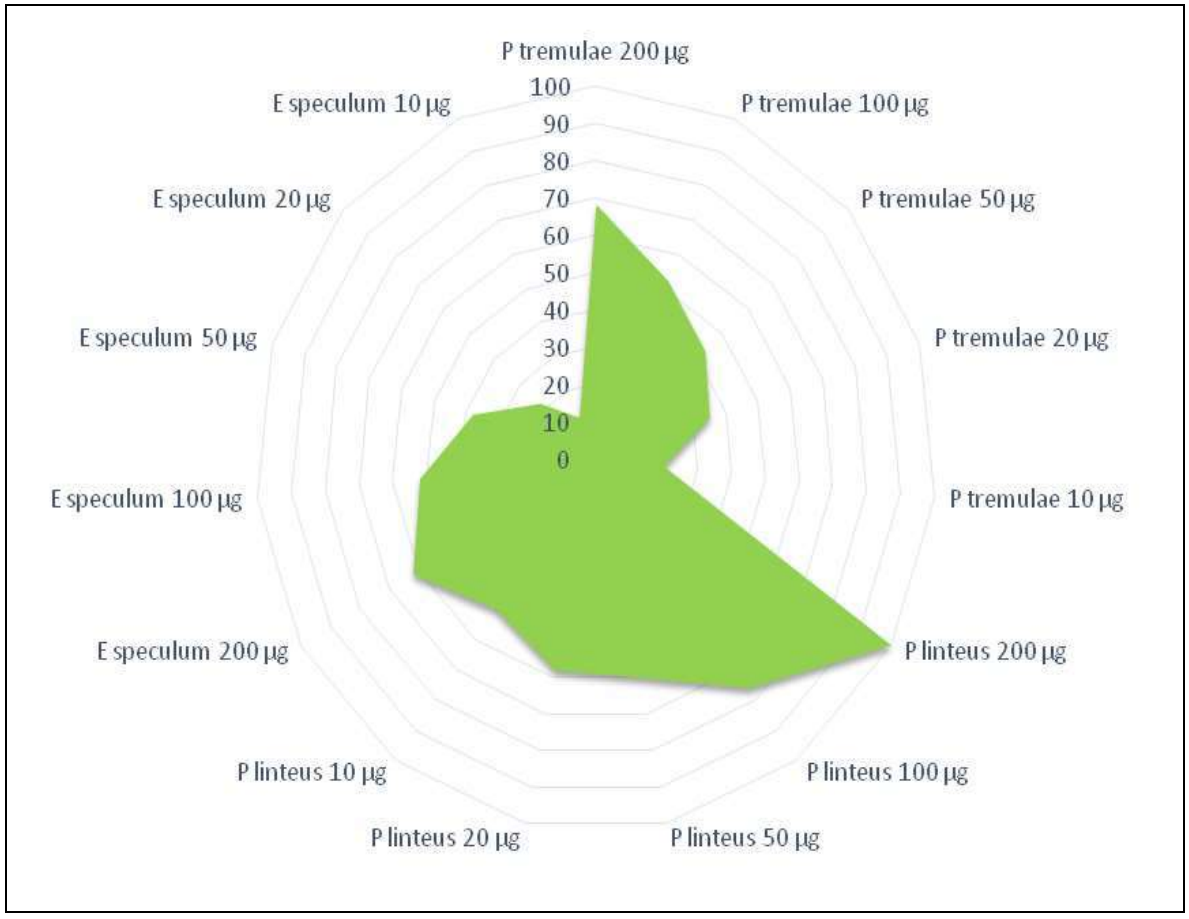
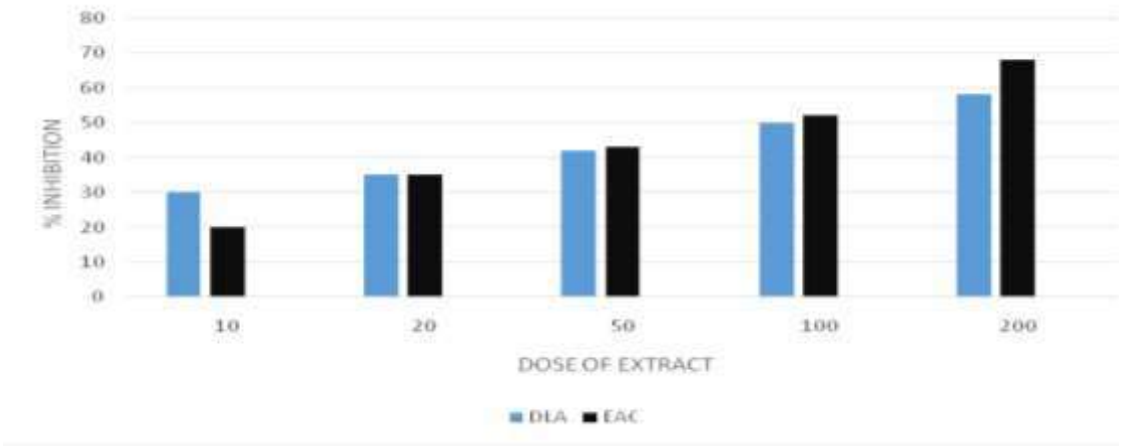
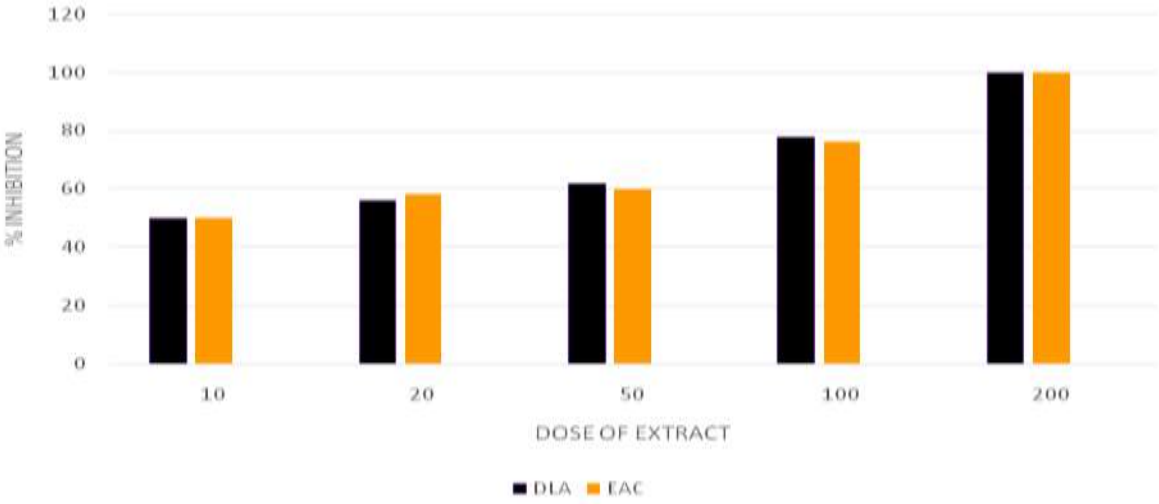


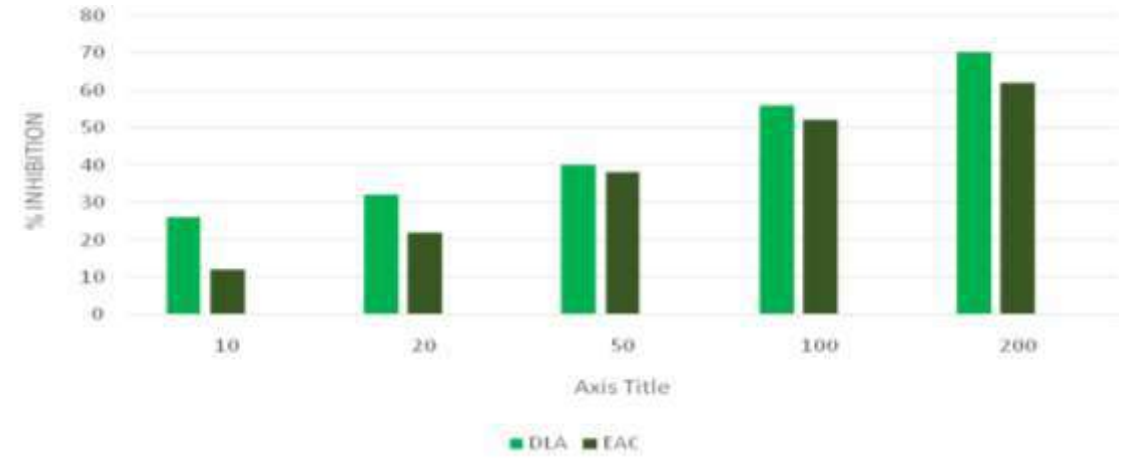
Fig. 20. Cytotoxicity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum* against EAC cells.



21a. Cytotoxic activity of *Phellinus tremulae* on DLA and EAC cells.



21b. Cytotoxic activity of *Phellinus linteus* on DLA and EAC cells.



21c. Cytotoxic activity of *Entoloma speculum* on DLA and EAC cells.

Fig. 21. Cytotoxicity of selected mushrooms.

4.12. Anti-inflammatory

Inflammation is biological response of vascular tissue to stimuli like damaged cell, irritants and pathogens in the process of healing inflammation is necessary step (Denko, 1992). Carrageenan induced hind paw edema is the standard method to evaluate the acute inflammation in rats. In present study anti-inflammatory activity the animals were injected carrageenan (1% w/v suspension) in the sub-planter region of right hind paw. The test groups of rats were given orally 250 and 500 mg/kg of methanolic extract of mushroom one hour before the carrageenan injection. The controls were given the same volume of saline as in test group. The inflammation was quantitated in terms of ml i.e. replacement of water by oedema using a plethysmometer immediately before carrageenan injection and then 1, 2, 4 and 6 hours after carrageenan injection. The per cent inhibition of oedema as calculated for each group with respect to its vehicle-treated control group. The anti-inflammatory activity was calculated. The activity of methanol extracts of test mushrooms was dose dependent.

Anti-inflammation of test mushrooms were tested at two different dose 250 mg/kg and 500 mg/kg. Standard drug (Diclofenac sodium) was administered orally (10 mg/kg). Only standard drug showed reduction in 0-2 hrs of time, but extracts showed reduction in inflammation at 4^{th} and 6^{th} hr. All the mushroom extracts showed reduction at late hours significantly. Diclofenac sodium showed 10-55 per cent reduction in inflammation from 0-6 hrs after inducing inflammation by carragenan. *Phellinus linteus* showed 34 per cent inhibition at 250 mg/kg dose, and 36 per cent at 500 mg/kg dose. *Phellinus tremulae* inhibited 23 and 25 per cent at 4^{th} and 6^{th} hr respectively at 500 mg/kg. *Entoloma speculum* showed maximum inhibition compared to *Phellinus linteus* and *Phellinus tremulae*. *Entoloma speculum* inhibited 42 and 46 per cent at 500 mg/kg dose in 4^{th} and 6^{th} hr respectively. *Entoloma speculum* is stronger than two *Phellinus* spp. in the present investigation (Table 20, 21; Fig. 22a, b, c and plate-9).

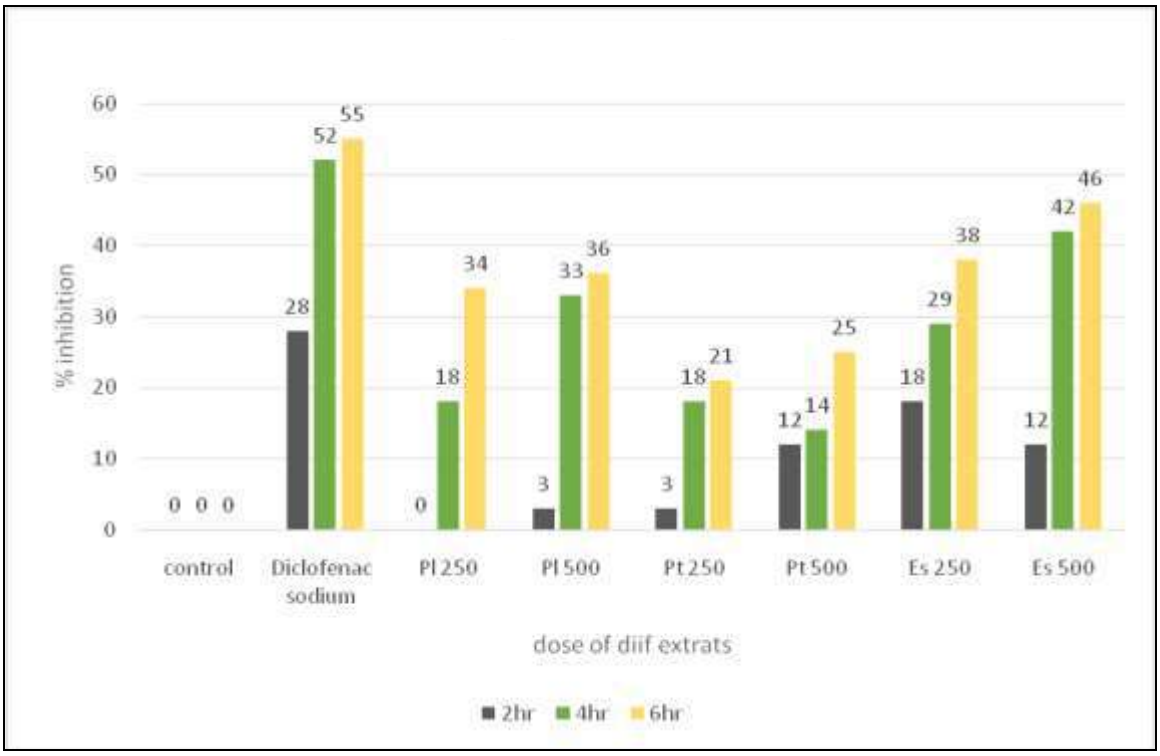


Fig. 22a. Anti-inflammatory activity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum*.

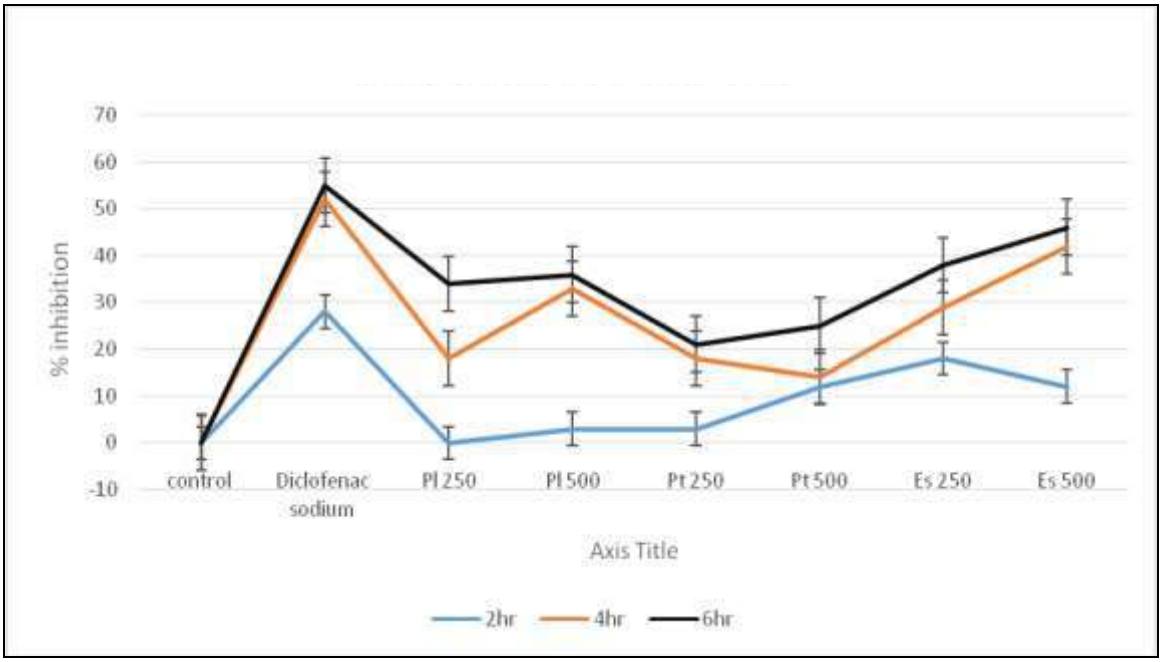


Fig. 22b. Anti-inflammatory activity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum*.

Table 20. Anti-inflammatory activity of *Phellinus linteus, Phellinus tremulae* and *Entoloma speculum*

Groups	Treatment	Dose (mg/kg)	Paw volume (ml) (Hour)				
			0	1	2	4	6
I	Control	0.1ML	0.5±0.05	0.76±0.03	0.81 ±0.04	1.06 ±0.076	1.15±0.056
II	Diclofenac sodium	10mg/kg	0.45±0.02	0.68±0.03	0.58 ±0.03***	0.5 ±0.025***	0.51±0.016***
III	*Phellinus linteus*	250mg/kg	0.533±0.02	0.7±0.025	0.83 ±0.033	0.86 ±0.049**	0.75±0.042***
IV	*Phellinus linteus*	500mg/kg	0.5±0.02	0.75±0.042	0.78 ±0.03	0.71 ±0.03***	0.73±0.033***
V	*Phellinus tremulae*	250mg/kg	0.53±0.02	0.66 ±0.03	0.78 ±0.016	0.86 ±0.021**	0.9±0.025***
VI	*Phellinus tremulae*	500mg/kg	0.5±0.025	0.7±0.02	0.7 ±0*	0.91 ±0.016*	0.86±0.021***
VII	*Entoloma speculum*	250mg/kg	0.5±0.025	0.7±0.036	0.66 ±0.033**	0.75 ±0.022***	0.71±0.047***
VIII	*Entoloma speculum*	500mg/kg	0.65±0.02**	0.75±0.042	0.71 ±0.016	0.61 ±0.016***	0.616±0.03***

Results are expressed as Mean ± SEM. The difference between experimental groups was compared by ANOVA followed by Dunnett's test.

The results were considered statistically significant when $P < 0.0001$

Table 21. Percentage inhibition of anti-inflammation activity of *Phellinus linteus, P. tremulae* and *Entoloma speculum*

Groups	Treatment	Dose (mg/ kg)	% Inhibition				
			0hr	1hr	2hr	4hr	6hr
I	Control	0.1 ML	0	0	0	0	0
II	Diclofenac sodium	10	10	10.5	28	52	55
III	*P. linteus*	250	0	8	2	18	34
IV	*P. linteus*	500	0	0	3	33	36
V	*P. tremulae*	250	0	13	3	18	21
VI	*P. tremulae*	500	0	7	13	23	25
VII	*E. speculum*	250	0	8	18	29	38
VIII	*E. speculum*	500	0	1	12	42	46

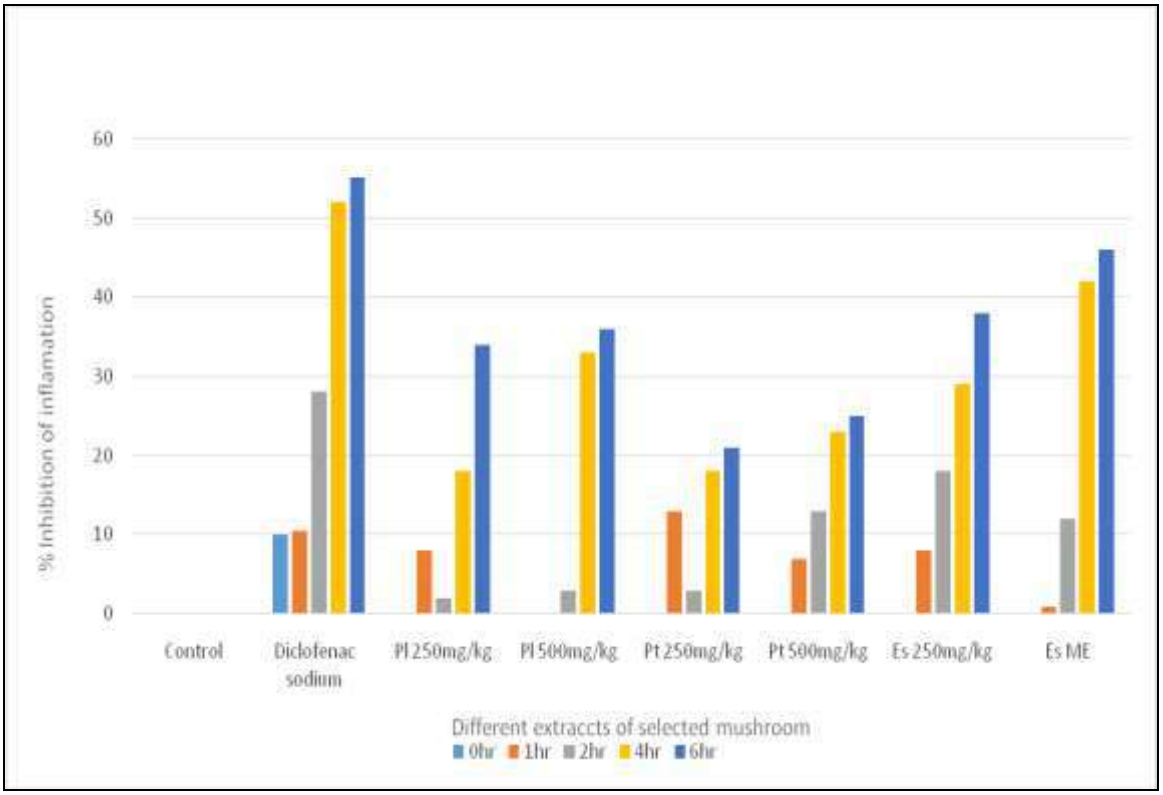


Fig. 22c. Percentage inhibition of anti-inflammation activity of *Phellinus linteus, P. tremulae* and *Entoloma speculum.*

Chapter - 5

Discussion

Study area receives 140 cm rainfall per year. Shivamogga district is hilly region located in between 13° 27' and 14° 39' N longitude and 74° 38' and 76° 4' E latitude. 50 per cent of the land (8465 km^2 total area) is covered by forest. *Entoloma speculum* was collected from medicinal plants garden of Kuvempu University campus between July and August of 2014 in Bhadravathi taluk. *Phellinus linteus* was collected from Devara Kaadu (sacred groove) near Jambekoppa village of Sagar taluk. *Entoloma speculum* and *Phellinus linteus* were identified by morphological characters (Krick *et al.*, 2008).

Phellinus tremulae was collected from Banajaale estate of Sagar taluk. In the field and in the lab it was difficult to identify by morphology. The fresh material was molecularly identified by DNA sequencing at Chromos Biotec. The obtained DNA was blasted in NCBI database. Based on results, the specimen was identified as *Phellinus tremulae* (16) with maximum number of hits. Culturing of *Phellinus tremulae* was not successful like other two species. *Entoloma speculum* and *Phellinus linteus* was successfully cultured by spore print on potato dextrose agar media and then transferred to potato dextrose broth for mass culture. 7^{th} day the characters were noticed.

Entoloma speculum and *Phellinus linteus* was cultured by spore print (Plate-2). Mycelia of *Entoloma* was Ambonate, medium growth irregular margin yellowish to pale yellow in colour, hypae was septate 2-7 μm wide and clamp connections were abundant in mycelia when observed under microscope. Mycelia of *Phellinus linteus* was white, thick walled, little branched 2-3.5 μm wide, skeletal hyphae, clamp connection was not observed in mycelia.

The dried sample of selected macro fungi were subjected to physico-chemical analysis. The results were varied with all the three macro fungi as they were collected from different places and from totally different environment. *Entoloma speculum* showed more foreign matter (0.9%) than other two macro fungi (0.8%). Moisture content was more in *Phellinus tremulae* 16.6% followed by *P. linteus* 7.3% and *E. speculum* 5.3%, water soluble extraction was 38.16% in *P. tremulae* and 8.88% in *P. linteus* and only 6.64% in *E. speculum*. Alcohol soluble extraction was almost equal in both *E. speculum* and *P. tremulae* 12.8 and 12.5% respectively. pH was neutral in *E. speculum* and *P. tremulae* is acidic (5.9%) and pH of *P. linteus* is slightly acidic by 6.64% (Table 1).

Ash was 21.6% in *P. linteus*, 10.4% and 12.4% in *E. speculum* and *P. tremulae* respectively. Water soluble ash was 95% in *P. tremulae* followed by 85% in *P. linteus* and 83% in *E. speculum*. Acid insoluble ash was 21 in *P. linteus* and 16% in *P. tremulae* and only 12% in *E. speculum*.

Biochemical analysis

Pet ether extract showed positive result for steroids. Alkaloids, tannins and flavonoids were present in all the extract of *P. tremulae*. Steroids, glycosides and phenols were present only in chloroform extract of *P. tremulae*. Methanol extracts showed positive result for glycosides, triterpenoids and phenols. Pet ether extracts showed positive to only alkaloids and tannins but chloroform and methanol extracts of *P. linteus* showed positive to all test except saponins (Table 2). Phytochemical tests are in agreement with Islam *et al*. (2015) and Gul *et al*. (2017).

Phytochemical test revealed the presence of tannins, phenols and flavonoids, this is in agreement with Wandati (2013) and Karun *et al*. (2016), worked on *Auricularia auricular*-judae and termitomyces unknown showed the presence of tannins, flavonoids and phenols. Saponins are absent in the present investigation in *P. linteus, P. tremulae* and *E. speculum*, but reported in two *Ganoderma* species (Rajesh and Dhanasekaran, 2014).

Antimicrobial activity

The study of antimicrobial activity of crude extracts from mushrooms against test microorganisms were compared with the control DMSO and standard Amoxicillin for bacteria and Terbinafine for fungi. The antimicrobial activity of mushroom samples varied according to the solvents and also concentration of extract. Mushrooms used for study were found effective against the tested microorganisms. The clear zone of inhibition produced by the bacteria and fungi around the tested mushroom extracts was evidence of potency of mushroom. Methanol yield was maximum followed by chloroform and petroleum ether. As studied by (Tiwari *et al*., 2011) more number of bioactive compounds obtained in methanol, compared to other two solvents. The result of secondary metabolites tests are tabulated in Table 2.

The extracts showed varied antimicrobial result when tested against pathogens. The petroleum ether extract of *Phellinus linteus* inhibited *Salmonella typhi* and *Staphylococcus aureus* (12 mm) at maximum. *Agrobacterium tumefaciens, Klebsiella pneumonia* and *Pseudomonas aeruginosa* were (10 mm) (Plate-5 E&F) moderately inhibited. *Xanthomonas campestris, Pseudomonas syringae, Escherichia coli* and

Streptomyces pneumoneae were (8–0) least inhibited by petroleum ether extracts (Table 7 and Fig. 8). The fungal pathogens like *Trichophyton rubrum, Fusarium solani, Aspergillus flavus* and *Aspergillus niger* were completely resistant showing no inhibition whereas *Chrysosporium keratinophilum* was inhibited maximum that of other test organism. *Candida albicans* and *Chrysosporium merdarium* were inhibited moderately (Table 6 and Fig. 7). Chloroform extracts of *Phellinus linteus* inhibited test organisms better than petroleum ether extract. *K. pneumonia* and *S. aureus* were inhibited maximum by showing 16 and 17 mm zone.

The chloroform extract inhibited *P. syringae, E. coli, P. aeruginosa, S. pneumoneae, A. tumefaciens* and *S. typhi* moderately by showing 13–14 mm inhibition zone. Whereas *X. campestris* were least inhibited with 8 mm. *F. solani* (22 mm) had the maximum effect of chloroform extract followed by *A. niger* and *C. keratinophilum* (20 mm). *C. merdarium, T. rubrum* and *P. chrysogenum* were moderately affected with 18–19 mm inhibition zone (Table 6). *A. flavus* and *C. albicans* were least inhibited by chloroform extract. The GCMS analysis of methanol extract showed the presence of ergosterol (Table 5). This agrees with the result of Reis *et al.* (2014) which is anti-tumor component (Chen *et al.* 2016).

Petroleum ether extract of *Phellinus tremulae* exhibited very poor antibacterial activity inhibiting only *X. campestris* at 6.33 mm among all the test bacteria (Fig. 11), but *C. albicans* and *Fusarium solani* were only fungi inhibited by pet ether extract 8 and 9 mm respectively (Table 10).

Chloroform extract of *Phellinus tremulae* inhibited *K. pneumonia* and *P. syringae* maximum by exhibiting 15 mm and least inhibition was observed against *P. aeruginosa* 10 mm (Table 10 and Plate-6). *Fusarium solani, A. flavus* and *A. niger* were inhibited equally by 19 mm. *T. rubrum* and *P. chrysogenum* were least inhibited with 10 mm (Table 10). Methanol extract inhibited *K. pneumonia* maximum 20 mm and *S. typhi* is least inhibited by 11 mm zone but against *Fusarium solani* methanol showed 27 mm considerable zone, but *T. rubrum* were was least inhibited 12 mm by methanol extract (Table 11 and Fig. 12). The relative decrease in the inhibition with the concentration of the extract shows that amount of bioactive components in extracts play an important role in inhibition of pathogens. The number of organisms inhibited decrease with the decrease in concentration of extracts. The observed result of the effectiveness of extracts, methanol proved best and petroleum ether showed less inhibition effect which is favourable with the findings of (Ehssan and Saadabi, 2012). *Phellinus tremulae* is perennial in nature and may be the effects of environmental factor made *P. tremulae* to have less potency to affect the test microorganisms. The relative decrease in the inhibition with the concentration of the extract shows that amount of bioactive components in extracts play an important role in inhibition of pathogens.

The observed result of the effectiveness of extracts, methanol extract proved best and petroleum ether extract showed less inhibition effect which is favourable with the findings of (Ehssan and Saadabi, 2012). *S. aureus and E. coli* are inhibited maximum by methanol extract this agrees with the result of (Bala *et al.*, 2011). Sonawane *et al.* (2012) reported methanol extract showed better activity than ethyl acetate, in present work methanol extract of *Phellinus linteus* and *Phellinus tremulae* showed good result than

chloroform extract. Antifungal activity of extracts was lower compared to bacteria this agrees with the result of Jonathan and Fasidi (2003, 2005) (Fig. 11 and 12). Oyetayo (2009) studied the effect of ethanol extracts of *Termitomyces* sp. and *Lentinus* sp. against *C. albicans* and *S. aureus,* ethanol extract showed good inhibition against *C. albicans* but *S. aureus* was inhibited hardly by 8 mm inhibition zone. Methanol extract showed good inhibition zone for *E. coli*, *S. typhi*, *P. aeruginosa* and *S. aureus,* this agrees with the result of Balakumar (2010).

When (Iftekhar *et al.,* 2011) worked on the antibacterial activity of *Ganoderma lucidum, Auricularia auricular* and *Pleurotus florida* against *S. aureus* and *E. coli* none of the three mushroom inhibited *E. coli* but in present study *E. coli* is inhibited in all the concentration by all solvent extracts. Methanol extract of *Entoloma speculum* showed positive result to all tests except Saponins and Triterpinoids hence it was subjected to GCMS analysis to know/find out bioactive compounds (Table 3). The methanol fraction of *E speculum* showed different bioactive compounds. 1,2-Benzenediol, Octadecanoic acid methyl ester (CAS) methyl stearate, Ergosterol and Ergosta-7, 22-dien-3-ol,are the major compounds. Similar compounds are found from methanol extract of *Termetomyes* species from GCMS analysis (Osman, 2015).

The best was 37 mm against *Pseudomonas aeruginosa*, followed by *Klebsiella pneumonia* (36 mm) (Table 13 and Fig. 14), *Staphylococcus aureus* (35 mm), *Fusarium solani* (28 mm), *Penicillium crysoginum* (27 mm) and *Apergillus niger* (26 mm) and the least inhibition was observed against *Trichophyton rubrum* (13 mm) and all other organisms were inhibited moderately with 13-21 mm. Comparatively methanol showed good result this may be due to the presence of 1,2-Benzenediol, (Chetaol: commercial

name) being a phenol its antimicrobial in nature (Das, 2010) and used in manufacture of pesticides (Osman, 2015).

Among the solvents used, methanolic extract found to be superior to chloroform and petroleum ether extracts and found to have more potential antimicrobial properties Methyl alcohol extract of *Pleurotus* spp. against *C. albicans* the inhibition zone was 7.5-8.5 mm (Rajoriya, 2015) but in present study the chloroform and methanol extract of *E. speculum* showed higher inhibition zone range from 6-20 mm against *Candida albicans*.

The observed result of effectiveness of extracts, methanol proved best and Petroleum ether showed less inhibition effect. This is favourable with the findings of Ehssan and Saadabi (2012). The antimicrobial activity of ethanol extract of *Rusulla delica* against *E. coli* (9 mm), *S. aureus* (10 mm), *P. aeruginosa* (7 mm) (Pranita, 2014) and but in present study the chloroform extracts of *E. speculum* inhibited *E. coli* (12-19 mm), *S. aureus* (21-35 mm), *P. aeruginosa* (19-29 mm) better than *Rusulla delica*. It was observed that different concentrations of all the three solvents (petroleum ether, chloroform and methanol) extracts of *E. speculum* posed different in inhibition level. This maybe due to the concentration of mushroom extract used also play crucial role in inhibiting potential of macrofungi (Omar, 2011). Generally observed the *E. speculum* activity against pathogenic bacteria is high compared to fungi supporting the result of Jonathan and Awotona from *Lycoperdon* species.

Phytochemical tests of sporocarp and mycelial extracts of *Entoloma speculum* both showed the presence of phenols, steroids, terpenoids and alkaloids in qualitative

chemical screening. Methanol extracts of mushroom and mycelia of *Entoloma speculum* showed maximum inhibition of test organism. In the present study the antimicrobial activity of mycelia and sporocarps of *E. speculum* is studied, fruiting bodies and mycelia showed significant activity against test organisms. Mycelia inhibited *T. rubrum* maximum (36 mm), mushroom inhibited *F. solani* (28 mm) maximum, whereas mushroom extract least inhibited *T. rubrum* (13 mm). *C. keratinophilum* and *P. chrysogenum* were inhibited by mycelial extract of *Entoloma speculum* equally with (29 mm) but *P. chrysogenum* was inhibited with 27 mm by mushroom extract. *F. solani* and *A. niger* was inhibited with 30 mm inhibition zone by mycelial extract (Table 14, Plate-7 and Fig. 16). *A. tumefaciens* inhibited by mycelia extract with 32 mm zone. Mushroom extract of *Entoloma speculum* showed 26 mm inhibition zone against *A. niger*. 25 per cent concentration of mushroom extract did not show any activity on *C. keratinophilum*, but all the three concentration (100, 50 and 25 per cent) of mycelia showed good inhibition on *C. keratinophilum* range from 29-24 mm (Fig. 16 and Plate-4).

Whereas *P. chrysogenum* was inhibited by mycelia and mushroom extract both. *C. albicans* was significantly inhibited by mycelium but moderately inhibited by mushroom. Pathogenic bacteria were inhibited by Sporocarp extract than the mycelia. Sporocarp suppressed the growth of all bacterial pathogens. *X. campestris* was susceptible to mycelial extract greater than the standard Amoxycilin. *E. coli* inhibited with 28 mm zone while *K. pneumonia* and *P. aeruginosa* inhibited with 26 mm zone. *S. aureus* was least inhibited with 19 mm. *S. typhi* showed 23 mm inhibition by mycelia extract but mushroom extracts inhibited *S. typhi* by 29 mm greater than standard

(25 mm). *P. aeruginosa* showed 37 mm zone highest of all the other test organisms (Fig. 15 and Table 15).

Mushroom extract inhibited *X. campestris* equal to standard (33.66), but *A. tumefaciens* inhibition was equal to standard (Table 15). Mushroom inhibited bacteria greater than fungi, this is in agreement with the result of Jonathan (2003). Mycelia inhibited fungi greater than bacteria, this is in favourable with Osman (2015) and Kalyoncu (2010).

Pranita (2015), investigated the antimicrobial activity of *Trametes versicolar*, against *P. aeruginosa is* 20 mm inhibition zone, *S. aureus* 20 mm *Peniillium* sp. 12 mm *A. flavus* 13 mm, *A. niger* 14 mm, *E. coli* 18 mm, in present study the methanol extract of *E. speculum* inhibited *A flavus* at 32 mm, *A. niger* 30 mm, *S. aureus* 19 mm, *P. aeruginosa* 26 mm, higher than *Trametes versiolar*, Omar (2011) revealed that mycelia of *Lentinus squarrosulus* possess the antioxidant activity and antitumor potential.

Cho (2007) investigated hypoglycaemic of exopolysaccharides from mycelial culture of *Tremellafuci formis* and *Phellinus boumni* found excellent activity on mice. Hatvani (2001) investigated the antibacterial effect of culture fluid of *Lentinus edodes* mycelium showed poor activity against *S. aureus* and *S. pyogens* and *Candida albicans E. coli* and *K. pneumonia*, but in present study mycelium extract inhibited *E. coli, K. pneumonia***,** *C. Albicans* and *S. aureus* significantly. Manjunathan (2011) worked on the antimicrobial activity of *Lentinus tuberregium* mycelium extracts on *S. aureus* (9 mm) *P. aeruginosa* (8 mm), *E. coli* (11 mm), *S. typhi* (12 mm) and *C. albicans* (10 mm). Rai (2013) investigated the antimicrobial effect of the extract of *Entoloma*

lividoalbum fruiting body against *C. albicans* which exhibited complete resistant and *E. coli* showed 10 mm and *P. aeruginosa* 8 mm inhibition zone, but *E. speculum* fruiting body and mycelia both inhibited pathogens significantly. Ishikawa (2001) studies on antimicrobial activity of *Lentin ulaedodes* mycelium extract *K. pneumonia* showed 4 mm and *P. aeruginosa* 0 mm and *S. aureus* 13 mm inhibition zone in disc diffusion method. In the present investigation the maximum antifungal activity is showed by mycelia and maximum antibacterial activity shown by sporocarp.

Analgesic activity

Pain is one of the unpleasant sensation. Pain may be beneficial to man and animal as it is protective mechanism to body, not only triggers reaction and also induces learned avoidance behaviours. Pain may be predisposing, persistent or due to consequence of brain or nerve injury. Pathological condition and tissue injury is immediate cause of pain, which result in release of number of chemicals than act of nerve terminal and increase sensitivity for (Suseem *et al.*, 2011) stimulation. Anti-analgesic drugs in market are with more side effects, so attention is focused in the natural remedies with are cheaper in cost and have no side effects (Table 16 and Fig. 17).

Ethyl acetate, methanol and water extracts of *Pleurotus eous* showed significant inhibition of pain (raised the pain threshold) in four methods acetic acid induced writhing, hot-plate tail flick and tail clip method (Suseem *et al.*, 2011) authors suggested *P. eous* potent analgesic property. Methanol extracts showed better inhibition than aqueous and ethyl acetate extracts. Sniderle, 2008 *Pleurotes pulmonarius* showed upto 85% of inhibition in acetic acid induce writing methods in test animals. Ethanolic extracts

showed considerable analgesic activity in hot plate method (Ede *et al.*, 2012). Kim *et al*. (2004) worked on analgesic effect of *Phellinus linteus* which showed inhibition upto 68 per cent and also the inhibition was dose dependent.

Least number of writhings was recorded by *P. tremulae* 21 and *E. speculum* 22. With inhibition 71 and 70 per cent respectively at 500 mg/kg concentration but *P. florida* at 500 mg/kg reported 40 writhings (Ganesh Purkar and Rai, 2013). Vijaymitra Raj *et al*. (2011) worked on methanol extract of *Clerodendrum phlomidis* was examined for analgesic activity by acetic acid induced writhing method. Methanol extract show 10 writhings at 100 mg/kg dose in 30 min and 5.4 writhings at 200 mg/kg dose with 63 and 80 per cent protection by 100 mg/kg and 200 mg/kg respectively. *Termitomyces albuminosus* significantly reduced the number of writhings of rats in acetic acid induced writhing (Lu *et al*., 2008).

Samuel *et al*. (2012) reported methanol extract of *Ganoderma applenatum* significantly prolonged reaction time in hot plate test of analgesic activity. At 150 and 180 min reaction time of rat was 80 and 85 seconds respectively at the dose of 600 mg/kg which is greater than the standard Pentazocine reaction time which is 64 and 69 seconds at 150 and 180 min respectively. Methanol extract of *Pleurotus ecus* inhibited pain by 53 per cent at 1000 mg/kg dose in the present study at 500 mg/kg dose of *P. linteus, P. tremulae* and *E. speculum* inhibited 63, 71 and 70 per cent respectively (Table 16 and Fig. 17). This proves *P. eous* is weaker than selected mushrooms.

Some medicines and compounds with antitumour and immunomodulatory activities have many side effects in which the inhibition of ovulation and increase in the

oocyte degeneration which leads to embryotoxic activity and result in abortion, but the polysaccharopeptide of *Coriolus versicolor* alleviated pain in rats without affecting the embryo and also female reproductive system (Ng and Chan, 1997). This is the evidence for mushroom has less or no side effects on patients. The test mushroom also may have no side effect on patient, so there is a need for detail evaluation with histological studies.

Amylase inhibition

Diabetes mellitus is of two types, type-1 and type-2 (UK Hypoglycaemia Study Group, 2007) and α-amylase deals with the type-2 diabetes where α-amylase increase blood sugar level by breaking down the carbohydrates into simple sugars. If α-amylase is de-activated or inhibited, the raise in blood sugar level may be reducing till the normal metabolisms takesplace. As the use of α-amylase inhibitor is increasing instead of using other drugs in recent year (Jumpaeng *et al*., 2013). Therefore, to study the anti-diabetic activity the of test mushrooms extracts *in vitro* α-amylase method is choosen.

In the present study, three different mushroom studies for anti-diabetic activity *P. linteus, P. tremulae* and *E. speculum*. The extracts were tested in three different concentrations 100 and 500 μg/ml. All the extracts showed significant inhibition *P. linteus* being heighst followed by *P. tremulae* and *E. speculum*. *P. linteus* methanol extracts showed upto 90.69 per cent inhibition at 500 μg/ml concentration and 65.68 per cent at 250 μg/ml and 49.43 per cent at 100 μg/ml concentration. *P. tremulae* showed 43.8 per cent inhibition at 100 μg/ml concentration at 81.93 per cent inhibition at 250 μg/ml and 89.44 per cent at 500 μg/ml concentration (Table 17 and Fig. 18). *E. speculum* showed weaker inhibition compared to other two *E. speculum* at 100 μg/ml concentration

12.55 per cent inhibition was observed and in 250 μg/ml, concentration 45.68 per cent and at 500 μg/ml 80.06 per cent inhibition was observed.

In vitro alpha-amylase activity of *Psidium guajava* inhibited diabetic upto 96.3 per cent in present study *P. lintus* inhibited upto 90.69 per cent. In *in vitro* α-amylase inhibition activity of several plants extracts *Nigella sativa* showed 84 per cent of inhibition at 100 mg/ml conc. in present study (Sathivelu *et al.*, 2013). Ethyl acetate and ethanol extract of leaf and callus of *Costus pictus* showed α-amyalse inhibitory effect upto 77.53 per cent (Sindhu *et al.*, 2012), in the present study 80.06 per cent inhibition was seen by *E. speculum* at 500 μg concentration. The hexane extract of stem of *Bouhinia purpurea* inhibited *in vitro* diabetes by 93.5 per cent at 100 μg/ml concentration (Choudhari *et al.*, 2013). In the present study, at the same concentration the extract of *P. lintus, P. tremulae* and *E. speculum* at 49, 43 and 12 per cent respectively. Abdullah *et al*. (2012) water extract of *Termitomyces heimii* showed only 16.04 per cent inhibition of α-amylase, in present study the least inhibition was recorded is 12.55 per cent by *E. speculum* extract in 100 μg concentration. Prabhu *et al*. (2014) worked on *in vitro* diabetic activity of the methanolic extract of *Calocybe indica* by α-amylase inhibition method. *C. indica* inhibited 89.48±3.54% at 1000 μg concentration, whereas in the present study 500 μg was the maximum dose of all extracts and showed 90.69, 89.44 and 80.06% by *P. lintus, P. tremulae* and *E. speculum* respectively. By the result obtained, we can say *C. indica* is weaker anti-diabetic in nature than the test mushrooms.

Kim *et al*. (2001) investigated the hypoglycemic effect of the exo-polymer of *Phellinus linteus* in rats with other four mushrooms *Lentinus edodes, Auricularia*

polytricha, *Cordeyceps militaris* and *Agrocybe cylindracea*. *P. linteus* significantly reduced plasma glucose level upto 18 per cent, but in present study *P. linteus* inhibited α-amylase upto 90 per cent the result vary a *in vivo* and *in vitro* due to many reasons like enzymes inside and external factors activity on them etc. Like-wise *P. tremulae* not behind in inhibiting α-amylase, 89 per cent inhibition was shows by *P. tremulae* at 500 μg/ml concentration. Imam *et al*. (2013) reported methanol extract of *Lawsonia inermis* inhibited α-amylase upto 60.97 per cent at low concentration 10 μg/ml (dose), but in the present investigation methanol extract *P. linteus* in 250 μg/ml dose showed 65.68 per cent inhibition dose of *L. innermis* is lower than the *P. linteus* still it is so effective compared to test organisms (Table 17 and Fig. 18).

Nair *et al.* (2013) reported that *Artocarpus altilis, Cinnamum zeylanicum, Piper betel* and *Artocarpus heterophyllus*, inhibited α-amylase dose dependent manner. 60% inhibition was highest 100 μg/ml concentration among the experiment by methanol extract of *A. heterophyllus*. In the present study, 100 μg/ml dose of test mushroom extract showed 12-49 per cent inhibition of α-amylase. Kazeem *et al*. (2013) reported that aqueous extract of *Morinda lucida* inhibited α-amylase 80 per cent at 500 μg/ml dose. In the present study, 500 μg/ml of *P. linteus* inhibited 90% of α-amylase, *E. speculum* 500 μg/ml inhibited 80% being stronger than *M. lucida* as reported by Kazeem *et al*. in 2013. The present studied mushroom may prove potent candidates to develop the anti-diabetic drug in future with less or no side effects.

Cytotoxic activity

Present research has demonstrated that the tested mushrooms may be useful in protecting against virally induced cancers through enhancement of natural killer cells, and may also play a role in the prevention of cancers induced by diet and poor lifestyle choices. Thus mushrooms may have a significant role in cancer treatment (Monro, 2003).

Nitha *et al*. (2007) worked on anti-tumour activity of mycelia of *Morchella esculenta* against DLA and EAC cell line in mice and inhibition was upto 54.90 per cent at 1000 μg/kg concentration. In the present study, 200 μg concentration 58, 100 and 70 per cent of inhibition was seen by *P. tremulae, P. linteus* and *E. speculum* respectively against DLA cells (Fig. 21a, 21b, 21c) and against EAC cells 68, 100 and 62 per cent by *P. tremulae, P. linteus* and *E. speculum* respectively inhibition was observed (Fig. 20). Sun *et al*. (2011) *Inonotus obliquus* inhibited the tumour cell growth by 74.6% on lung cancer cell line *in vitro* by MTCC assay, in the present study, *P. linteus* at 100 μg concentration inhibited the 78±1.5 DLA cells by tryphan blue exclusion method. Froufe *et al*. (2010) proved the low molecular weight compounds from wild mushroom by docking three compounds Aromatase, Estrone sulfatase and 17β-HSD1 which proved to inhibit the breast cancer.

Toa *et al*. (2006) reported antitumour activity of polysaccharides of *Pleurotus tuberregium*, polysaccharides named as TM36 and sulphate polysaccharides named as S-TM3b, S-TM3b was stronger than TM3b in inhibiting liver cancer cells upto 70 per cent whereas TM3b of *Pleurotus tuberregium* inhibited only 35 per cent of the liver cancer cells *in vitro* compared to *Pleurotus tuberregium* both *Phellinus* species in the

present study inhibited tumour cells significantly. *Phellinus* inhibited both DLA and EAC cells 100% this result is in agreement with the Song *et al*. (2011), who reported the antitumour cells activity of *P. linteus* against human colon cancer cells. *Entoloma speculum* exhibited significant cytotoxic activity/antitumour activity, this agrees with the result of Chung *et al*. reported *Inonotus obliquus* inhibited four types of human cancer cells lung, breast, stomach and cervical cancer cells. *P. linteus* mushroom and mycelia both inhibited cancer by inducing the apoptosis neuroblastoma cells. Combination of drugs with low dose of *P. linteus* extract may produce apoptosis in cancer cells (Daniel Sliva, 2010).

Ajith and Janardhan (2003) reported ethyl acetate extract of *Phellinus ramous* significantly inhibited DLA and EAC cell line while methanol was weaker in inhibition but in present investigation methanol extract of *P. linteus* and *P. tremulae* showed excellent inhibition against DLA and EAC both cell line. Grube (2001) reported *Agaricus bisporus* inhibited breast cancer by suppressing aromatase, whereas mycelia of *Morechella esculenta* significantly exhibited antitumour activity against DLA and EAC solid tumour *in vivo* (Nita *et al*., 2007). Lee *et al*. (2009) reported antitumour activity of water and ethanol extract of *Inonotus obliquus*, *Iobliquus* inhibited HT-29 human colon cancer. Water extract was stronger than ethanol extract in inhibiting colon cancer cells. Ethanol extract inhibited 25-30 per cent whereas water extract 60 per cent of cancer cells *in vivo*. In the present study, methanol extract of *E. speculum* inhibited DLA and EAC cells by 70 and 62 per cent respectively (Fig. 19, 20, 21 and Table 18, 19).

We observed that cytotoxic activity was significant in all the three test mushrooms. The present result is differing from the findings of Rajesh Dhanasekaran,

2014. Methanol extract of *P. linteus* inhibited EAC and DLA tumour cells 100 per cent. This is in agreement with the results of Rajeshwari and Krishnapriya (2011). The present study of cytotoxic activity is tested against DLA and EAC cell lines, but there is need to test the cytotoxic activity against wide range of cancer cells *in vitro*, that may prove test mushrooms are potential to inhibit many types of cancer cells.

Anti-inflammatory

Carragenan induce acute inflammation in animals, which is short-term reaction, resulting in pain, redness, swelling and generation of heat. As carragenan induced model is very sensitive to non-steroidal anti-inflammatory drugs, it is very important tool for evaluating new drugs from natural source (Wen *et al*., 2011).

In the control group the paw volume range from 0.5-1.15 ml from 0-6 hr time. the standard drug reduced the paw volume range from 0.45-0.51 ml from 0-6 hr. Methanol extracts of *Phellinus linteus* showed significant reduction of paw volume to 0.86-0.75 ml in 4^{th} and 6^{th} hrs respectively, in 250 mg/kg dose same extract in 500 mg/kg. Significantly inhibited inflammation upto 0.73 ml at 6 hrs. However the maximum effect was observed in methanol extract of *Entoloma speculum* at 500 mg/kg dose at 6 hr time. the P value is <0.0001. *P. linteus* and *E. speculum* showed significant activity compared to that of *Phellinus tremulae* (Fig. 22a & b and Plate-9).

Literature review stated that carrageenan induced acute inflammation model is most suitable test the screen anti-inflammatory agents (Joseph *et al*., 2009).We observed that in 4^{th} and 6^{th} hour after carr injection the inflammation was reduced, this is with the agreement of Huang *et al.* in 2012 (Table 20, 21).

Following results suggest that the methanol extracts of test mushroom represent a potential anti-inflammatory agents and this beneficial effects may give way for future researches to purify bioactive compound from mushroom and work on anti-inflammatory properties. Lee *et al.* (2012) worked on the anti-inflammatory of cold water extract of *Lignosus rhinocerus* by carrageenan induced paw edema test. *L. rhinocerus* extract showed significant inhibition in 200 mg/kg dose. Also the results were dose dependent manner.

Anti-inflammatory activity of *Phellinus linteus* by Croton oil induced ear edema in mice was examined by Kim *et al*. (2004). Authors conforms *P. linteus* contain active anti-inflammatory components and exhibit strong activity. Moro *et al*. (2012), the phenol content of mushroom is responsible for the anti-inflammatory activity as these inhibit lipopolysaccharides (LPS), induced nitric oxide (NO) production which is the main reason for inflammation in organisms (Lin *et al*., 2014; Kim, 2006; Ma *et al*., 2013). Activity may be due to the presence of erogsterol compound which showed considerable anti-inflammatory activity. Flavonoids which target prostaglandins involved in inhibiting acute inflammation (Suseem *et al.*, 2011).

Taofiq *et al*. (2016), reported that mushrooms inhibit inflammation *in vivo* and *in vitro*. Phenolic compounds of mushroom inhibit NO (Nitric Oxide) which are responsible for inflammation *Phellinus linteus* inhibit inflammation in dose dependent manner. In tilone, hydroxyl benzaldehyde, hispolon, hispidin and caffeic acid are phenolic compounds that are isolated from *P. linteus*. Linoleic acid a active compound of *Agaricus brasiliensis* inhibited no production and suppressed the inflammation (Saiki *et al*., 2017), in the present study GCMS analysis of test mushrooms reported to have many

compounds which are responsible for anti-inflammatory action (Table 3, 4 and 5). Protein (PEP) isolated from *Pleurotus cryngii* inhibited inflammatory mediators including NO resulting in inhibition of inflammation (Yuan *et al*., 2017). *Phellinus pini* significantly inhibited inflammation in carragenan induced hind paw edema in rats). *P. linteus* and *E. speculum* significantly inhibited inflammation compared to *P. tremulae* this is in agreement with the Im *et al*. (2016).

The high dose inhibited stronger than low dose this is in agreement with Ganesh Purkar and Rai (2013). *P. florida* also inhibited inflammation dose dependently. 500 μg/ml at fourth hour the volume of paw was 0.15 ml within the present work the volume was 0.71, 0.91 and 0.61 ml at fourth hour of *P. linteus, P. tremulae* and *E. speculum* respectively. Ganesh Parkar and Rai (2013) reported anti-inflammatory activity of *Pleurotus florida* by carragenan induced paw edema methanol. At third and fourth hour of experiment the inhibition was more than initial hours. In the present study, also the inhibition was more in fourth and sixth hour as the extract inhibit prostaglandin which are responsible for second phase of oedema. For the first phase of oedema histamine and serotonin are responsible, so the mechanism of action may be by inhibiting histamine, serotonin or by inhibiting the synthesis of prostaglandis. This is in agreement with Huang *et al*. (2012).

Huang *et al*. (2012) isolated Inotilone from *P. linteus* and examined the *in vivo* and *in vitro* anti-inflammatory activity. Inotilone decreased the production of nitric oxide production and inhibited the oedema in rats. In the present work, the methanol extract of *P. linteus* and *P. tremulae* both inhibited inflammation but GCMs analysis did not show

the presence of inotilona. The other compound or combination of compounds may be acted on inflammation mechanisms.

Some mushroom may induce inhibition of production of nitric oxide (NO) and inducible nitric oxide synthase (iNOS), but some may only inhibit production of NO (Moro *et al*., 2012). Moro studied the six edible mushrooms where only three induced inhibition of NO and iNOS both (*A. bisporus, Cantherellus cibarius* and *Lactarius deliciosus*) and only one (*C. cornucopioides*) inhibited only NO production, the other two *P. ostreatus* and *Boletus edulis* did not show any anti-inflammatory effect. *Termitomyces albuminosus* polysaccharides extracts at 200 μg/ml dose inhibited 81.6 and 40.5 per cent of inflammation in xylene induced ear edema and carrageenan induced paw edema respectively. Crude saponin extract inhibited inflammation by 79 and 55.6 per cent in same method (Lu *et al*., 2008). Nguyen *et al*. (2013) studied on methanol and hot water extract of *Dictyophora indusiata* suppressed inflammation in carragenan induced rat paw edema after 6 hrs of inducing in rats. Test mushrooms are potent to inhibit inflammation *in vitro*. These may lend a way to develop new anti-inflammatory medicine which may not show any side effects or show lesser side effects in future.

Chapter - 6

Summary

Phellinus linteus was collected from Devara Kaadu (sacred groove) near Jambekoppa village of Sagar taluk. *Entoloma speculum* and *Phellinus linteus* were identified by morphological characters. *Entoloma speculum* was collected from medicinal plants garden of Kuvempu University campus between July and August of 2014 in Bhadravathi taluk.

They were characterized based on morphology. Three sporocarps were shade dried and known quantity of powder was subjected to extraction using petroleum ether, chloroform and methanol by Soxhlet method. Methanol yield was more followed by chloroform and pet ether. Moisture content was more in *Phellinus tremulae* 16.6% followed by *P. linteus* 7.3% and *E. speculum* 5.3%, water soluble extraction was 38.16% in *P. tremulae* and 8.88% in *P. linteus* and only 6.64% in *E. speculum*. Alcohol soluble extraction was almost equal in both *E. speculum* and *P. tremulae* 12.8 and 12.5% respectively. pH was neutral in *E. speculum* and *P. tremulae* is acidic (5.9%) and pH of *P. linteus* is slightly acidic by 6.64% (Table 1).

Pet ether extract showed positive result for steroids. Alkaloids, tannins and flavonoids were present in all the extract of *P. tremulae*. Steroids, glycosides and phenols were present only in chloroform extract of *P. tremulae*. Methanol extracts showed positive result for glycosides, triterpenoids and phenols. Pet ether extracts showed positive to only alkaloids and tannins but chloroform and methanol extracts of *P. linteus* showed positive to all test except saponins (Table 2).

The methanol extract showed the presence of more mycochemicals, so it was subjected to GCMS analysic. Ergosterol was present in all the three selected mushrooms

other than ergosterol hydroxyproponic acid and palmitic acid were major compounds found in *E. speculum*. Ergosta-5, 22-trien-3-01 was major compound in GCMS analysis of *P. tremulae*. 3-(p-hydroxy-m-methoxyphenyl)-2-propenol 2-methoxy-5-formyl-1, 3(2H)-benzoxodiole were major compounds in *P. linteus*. Mycelia of *P. linteus* and *E. speculum* were cultured with the help of spore print in potato dextrose broth. Mycelia was subjected for extraction with methanol and subjected to anti-microbial activity. Mycelia proved potent antimicrobial agent.

The extracts of all test mushrooms are subjected to antimicrobial activity. Antimicrobial test was against 8 pathogenic fungi and 8 pathogenic bacteria. In all the mushroom pet ether extract showed weaker and methanol proved stronger in inhibition of fungi and bacteria. Pet ether extract of *E. speculum* showed maximum inhibition against *S. aureus* and minimum against *C. merdarium*, while chloroform extract of *E. speculum* showed minimum zone of inhibition against *T. rubrum* and *X. citric*. Methanol extract of *E. speculum* inhibited *F. solani* and *P. aeruginosa* maximum and minimum *T. rubrum* and *S. typhi* (Table 12, 13 and Fig. 13, 14).

Mycelia of *Phellinus linteus* inhibited *K. pneumonia, X. campestris* and *A. tumefaciens* maximum almost equal to standard with 27 mm and *E. coli* was least inhibited with 18 mm. Sporocarp inhibited *K. pneumonia* maximum with 29mm greater standard and *E. coli* was inhibited with 20mm zone as least (Table 7). In antifungal activity mycelia suppressed the growth of *C. merdarium* 28 mm to its maximum and *Fusarium solani* was inhibited with only 20 mm zone reporting least zone of inhibition, but mushroom inhibited *Fusarium solani* with maximum zone 27 mm and least reported was 12mm against *T. rubrum* (Fig. 7, 8 and Table 8).

Mycelial extract of *Entoloma speculum* inhibited *T. rubrum* maximum. *C. keratinophilum* and *P. crysogenum* were least inhibited. *A. tumefaciens* showed maximum zone of inhibition and *S. aureus* were resistant to mycelial extract and showed least inhibition zone (19 mm) (Table 14, 15 and Fig. 15, 16).

Mycelial extract of *P. linteus* inhibited *K. pneumonia* and *X. campestris* maximum and *E. coli* was least inhibited. *C. meradarium* was maximum inhibited with 28 mm and minimum inhibition was observed against *F. solani* and *A. flavus* (Table 8, 9 and Fig. 9, 10).

Petroleum ether extract of *P. linteus* inhibited maximum *C. kerotinophyllum* and *K. pneumonia*. Chloroform extract inhibit *F. solani* and *Xanthomonas crysosporium*. Methanol extract inhibited all the test organisms. *Candida albicans* and *A. tumefaciens* were strongly inhibited while *T. rubrum* and *Xanthomonas crysosporium* showed resistance (Table 6, 7 and Fig. 7, 8).

Extracts of *P. tremulae* was weaker compared to *P. linteus* and *E. speculum* in antimicrobial activity. Pet ether extracts of *P. tremulae* inhibited only *C. albicans* and *X. campestris* other all test organisms showed resistance chloroform inhibited. *A. flavus*, *P. syringe* maximum while *T. rubrum* and *S. typhi* were inhibited minimum. Chloroform extract of inhibited *K. pneumonia* and *P. syringae* maximum by exhibiting 15 mm and least inhibition was observed against *P. aeruginosa* 10 mm (Table 10). *Fusarium solani*, *A. flavus* and *A. niger* were inhibited equally by 19 mm. *T. rubrum* and *P. chrysogenum* were least inhibited with 10 mm (Table 10). Methanol extract inhibited *K. pneumonia* maximum 20 mm and *S. typhi* is least inhibited by 11 mm zone but against *Fusarium solani* methanol showed 27 mm considerable zone, but *T. rubrum* were was least

inhibited 12 mm by methanol extract (Table 11 and Fig. 10). To evaluate the analgesic activity acetic acid induced writhing method was adopted. Ibruprofen was used as standard drug and the methanol extract of the three test mushrooms were administered in two dosage 250 mg/kg and 500 mg/kg to *Wistar* mice. *P. tremulae* (71%) and *E. speculum* (70%) were stronger in pain inhibition compared to *P. linteus* 63% results were significant when $P<0.0001$ (Table 16 and Fig. 17).

Amylase inhibition activity was performed *in vitro*. Methanol extract of test mushrooms were tested in three dosage 100, 250 and 500 μg/ml. *Entoloma speculum* was weaker when consider to *P. tremulae* and *P. linteus*, the percentage inhibition was 90, 89 and 80% by *P. linteus, P. tremulae* and *E. speculum* respectively (Table 17 and Fig. 18).

Cytotoxic test was evaluated *in vitro* against Ehrlich Ascites Carcinoma (EAC) and Dalton's lymphoma ascites (DLA) cell line from infected mice by Tryphon blue exclusion method. The methanol extracts were tested for cytotoxic activity in five different dose 10, 20, 50, 100 and 200 μg/ml. DLA cells were significantly inhibited by *P. linteus* (100%) followed by *E. speculum* (70%) and *P. tremulae* (58%) followed by *E. speculum* and *P. tremulae*. EAC cells were inhibited by *P. linteus* (100%) followed by *P. tremulae* (68%) and then by *E. speculum* (62%) (Table 18, 19 and Fig. 19, 20).

Anti-inflammatory activity was test *in vivo* in rats by carrageenan induced paw edema methods. Methanol extracts were administered to rats in two dose 250 and 500 mg/kg. Paw edema was measured at 0, 1, 2, 4 and 6 hour after inducing edema. *E. speculum* and *P. linteus* inhibited inflammation significantly compared to the *P. tremulae* (Table 20 and Fig. 22a, 22b).

Conclusion

Research studies available in literature, on both edible and non-edible wild mushroom species, highlighting their pharmacological activities of extracts and only few on the bioactive compounds, mushroom nutrients and their secondary metabolites exhibit various favourable effects on the functioning of the immune system because of valuable therapeutic compounds whose mechanism of action need to be fully understood by conducting clinical trials.. Even when the same mushroom species used for the same experiment, result might be different as the release of bioactive compounds are depending upon the cultivation method and environment, extraction procedure, time, solvent used and mushroom maturation. There are different methodologies to evaluate pharmacological activities of mushroom extracts and isolated compounds, and there is little information available about its action there is necessary to study the mode of action of compounds.

Phellinus linteus, P. tremulae and *Entoloma speculum* can serve as potential candidates for essential new antibiotics. Further work is needed toward the evaluation of their antimicrobial potential against a wider range of microorganisms. The identification and isolation of the active compounds responsible for this activity could provide new starting material for the development of novel antibiotics, which may provide a better source for developing new therapeutic agents.

Phellinus linteus, P. tremulae and *Entoloma speculum* possesses antimicrobial, analgesic, anti- inflammatory in vivo. As the in vitro activity are necessary to confirm the basic activity of compounds which are needed for the clear application of compounds

in vivo therefore cytotoxic, and alpha amylase inhibition potential was tested *in vitro* Analgesic, anti- inflammatory cytotoxic, and alpha amylase inhibition potential of test mushroom which might be due to presence of myochemicals like flavonoids, phenolics and polysaccharides. GCMS analysis of extracts showed the presence of several compounds, in them only few compounds are known for their action but biological activity of many compounds are yet to be explored, to know their biological activity and mechanism of action. Further, characterization of secondary metabolites is required for their scale-up of their production.

Increased application of mushrooms in medical field is the purpose of continuous production of mushrooms, as the mushroom are short lived but mycelia may be cultured in large amounts by standard procedures. Mycelia also harbour equal secondary metabolites that of fruiting body. Mycelia may be cultured whole year irrespective of season and temperature variation and extracted for bioactive compounds. In conclusion, the analysed species (*Phellinus linteus, P. tremulae* and *Entoloma speculum)* represent an important contribution to the knowledge of wild mushroom species and their possible beneficial effect for human health.

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Annexure

Chromous Biotech

Spectrum of Innovation

PROJECT COMPLETION CERTIFICATE

PROJECT TITLE	Cloning based Identification
SCIENTIST NAME	Jayashree K Kodiyalmath
INSTITUTE	Kuvempu University
PROJECT CODE	PCS – 93
DATE	22.11.16

Chromous Biotech certifies that the above service has been carried out and completed in accordance to the project requirement, in line with scientists information.

All information generated and input provided will be kept confidential.

All relevant information, instruction and deliverables has been provided to the scientist

Authorized Signatory

Asma Nazreen. k.A.

[ASMA NAZREEN. k.A]

Company Seal

CHROMOUS BIOTECH PVT. LTD. * Estd. 2006. * BANGALORE-92

Annexure-2

Estd. 1979

T.M.A.E. Society's

S.C.S. COLLEGE OF PHARMACY, HARAPANAHALLI.

Dist : Davanagere. State : Karnataka.

(Affiliated to Rajiv Gandhi University of Health Sciences, Bengaluru.
Approved by AICTE and Recognised by Pharmacy Council of India, New-Delhi)

Ref. No. SCSCP /IAEC /11/ 2016-17 Date 15-04-2017

Certificate of Ethical Clearance

This is to certify that **Mrs. Jayashree K Kodiyalmath** has submitted the synopsis entitled **"Bioprospecting of Selected Macrofungi in Taluks of Shimoga District"** under the guidance of **Prof. M Krishnappa** is scrutinized and approved by the institutional animal ethics committee of in the meeting held on **15.04.2017.**

On behalf of the institutional animal ethics committee, we the undersigned hereby certify and issue the ethical clearance of above said research proposal.

Mice: 24 (Two four)

Rats: 24 (Two four)

Member Secretary,
Institutional animal ethics committee,
S.C.S. College of Pharmacy,
Harapanahalli-583131.

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Annexure-3

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To,

Ms. Jayashree K. Kodiyalmath
Research Scholar
Dept. of Botany
Kuvempu University
Shankarghatta

REPORT OF ANALYSIS

Physico-Chemical Analysis

Report Code: CR781A	**Date:**26.08.2016
No. of samples: 5	**Sample Code:** JKK1, JKK2, JKK3

Amylase Inhibition Assay

Report Code: CR781B	**Date:**26.08.2016
No. of samples: 3	**Sample Code:** JKK4, JKK5, JKK6,

Annexure-4

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Amala Cancer Research Centre

(A Society Registered T. C Act, XII of 1955 sl. No. 56 of 1984)

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RESEARCH DIRECTOR : DR. RAMADASAN KUTTAN, Ph.D

AMALANAGAR - 680 555, THRISSUR
KERALA, INDIA

Ref :

Date: 27.3.17

***IN VITRO* CYTOTOXICITY STUDY**

The test compounds were studied for short term *in vitro* cytotoxicity using Dalton's lymphoma ascites cells (DLA) or ~~Ehrlich Ascites Carcinoma (EAC)~~ cells.

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