CHAPTER-3

Study of antibacterial potential, characterisation and efficacy enhancement of mushroom extract

3.1. Introduction

Infectious diseases (EIDs) are one of the major burdens on public health. However, most of the infectious diseases are of bacterial and rickettsial origin. The occurrence of antimicrobial resistance shown by the bacterial pathogen is the prime cause of these incidences (Jones et al. 2008). Although there are many commercial drugs in market to fight against microbes and free radical induced diseases but they are proved to be ineffective gradually (Nwachukwu and Uzoeto 2011). WHO had warned all the countries to control the use of antimicrobial compounds to avoid the cases of development of pathogenic MDR strains due to the lack of alternative therapies (WHO2012). To avoid the resistance development of synthetic chemotherapeutics attention has been given on natural products from alternative sources in search of novel bioactive agents.

Natural bioactive products have been utilised in the last few years and mushrooms has played an important role in that respect of development of new antimicrobial compounds. Several mushrooms are reported to exhibit both antibacterial and antifungal activity effectively against multiple drug resistant pathogens (Sharma et al. 2014).

Nanotechnology based science have been an interesting field of research and gained much importance from last two decades. It is the application of engineering techniques of functional systems at the molecular level. The term "*nano*" is derived from the Greek word "*nanos*" that means dwarf and is denoted as the measurement on the scale of one-billionth (10^9) of a metre in size (Narayanan and Sakthivel 2010). In recent years, nanotechnology is enlisted as one of the most attractive subjects of substantial research activities in modern

therapeutic sciences and hence metal nanoparticles are of great scientific and industrial importancedue to their unique optoelectronic and physiochemical properties with applications in diverse areas such as electronics, catalysis and drug delivery.

Silver nanoparticles (AgNPs) have been shown in numerous studies to display antibacterial activity (Duran et al. 2007; Guzman et al. 2009; Krishnaraj et al. 2010). Nanoparticles have higher antibacterial activity as they can easily penetrate into the nuclear content of bacteria by activating the DNA and enzymes of host leading to cell death. They possess a larger surface area for stronger bactericidal or bacteriostatic interactions (Pal et al. 2007). Thus the uses and application of AgNPs are considered as one of the leading and promising approaches for overcoming AMR (antimicrobial resistance) conditions.

The major drawbacks of popular chemical and physical synthesis methods of metal nanoparticles lead to the accumulation of fatal and toxic chemicals adsorbed on the surface portions of nanoparticles that creates many unfavourable effects after applications. To overcome these drawbacks natural resources such as bacteria, fungi, plants, and algae are presently used for the biosynthesis of metal nanoparticles. Due to high content of reducing and stabilising agent for synthesis of nanoparticles, fungi specially mushrooms are now used for this purpose.

Besides antibacterial activity mushroom showed cytotoxicity and antitumor effects also. The mushroom's cell-wall is made up of carbohydrates, proteins and lipids (Kapteyn et al. 1995; Sanjuan et al. 1995). The glucans and heteroglycans types of polysaccharides isolated from *Termitomyces eurhizus* (Mondal et al. 2004), *T. striatus* (Mondal et al. 2006), *T. robustus* (Chandra et al. 2007), *T. microcarpus,* and *T. clypeatus* (Pattanayak et al. 2015) have been reported. Glucans are recognized as biological response modifier and effective therapeutic agents for the cure of cancer and other dreadful infectious bacterial diseases (Chan et al. 2009). These are known to be potent immunomodulator (Wasser and Weis 1999),

antioxidant compounds (Blokhina et al. 2003; Kozarskiet al. 2011; Patra et al. 2013) and anti-tumor (Wasser 2002) agents.

Mushrooms possess a diverse range of secondary metabolites including phenolic compounds. Mushroom phenolics are the prime bioactive compounds for health benefits (Mallavadhani et al. 2006). Atherosclerosis and cancer could be controlled by the mushroom derived dietary components (John 2005). The most common phenolic acids are the derivatives of benzoic acid that includes gallic acid, protocatechuic acid, syringic acid, vanillic acid. Other than these cinnamic acid derivatives including p-coumaric acid, caffeic acid, chlorogenic acid, ferulic acid, *o*-coumaric acid are also reported to be the most prevalent phenolic compounds found in nature (Khoddami et al. 2013).

Malignancy and finally leading to development of cancerous tumours is the prime cause of death of a huge percentage of people worldwide per year (Jemal et al. 2011). The cancer management technique includes the surgery of tumours, application of chemotherapeutic drugs either in the form of oral medicine or injection and exposure of malignant cell mass into radiation. The treatments are applied depending upon the type of cancer, location of tumour formation and grade or stage of the cancer as well as the person's health, wealth and wishes (Cassileth and Deng 2004). Treatment options often are expensive and have side effects. In most of the cases the mode of action of chemotherapeutic drugs includes the destruction of tumours leading to the blockage of malignant cells progression but in that process, they cause damage to healthy cells and tissues. The situation has triggered the search of new antitumor substances from diverse natural sources for the development of safer anticancer agents (Chung et al. 2010). Ethyl acetate fraction from shiitake mushroom effectively induced apoptosis in human cancer cell lines (Fang et al. 2006). The protein extracts from *Clavatia lilacina*, *Pleurotus ostreatus* and *Volvariella volvacea* have showed anticancer properties (Wu et al. 2011).

Colon cancer accounts for third most popular cancer in males and second most common one in females worldwide. In India, out of 64,000 diagnosed cases 49,000 patients died due to colon cancer (IARC 2013). Though there are effective treatments present like chemotherapy, radiotherapy and immunotherapy, the survival rate of patients with malignant colon carcinomas remains poor. This could be either due to the resistance developed by tumour cells or their associated adverse side-effects. Therefore, screening of new therapeutic strategies is immediately required for successful management of this disease.

In this scenario, the present study provided an overview about the antibacterial potentials of some selected mushrooms against some target bacterial pathogens causes some important diseases of human. Among all the selected mushroom species for the study, *Termitomyces heimii* (Durgachhatu) is the most popular one that abundantly occur and sold in the nearby rural and urban markets of Gurguripal ecoforest during the month September and October. So far, there are no remarkable reports regarding the antibacterial activity by *T. heimii* occurring in this region. Moreover, the green synthesis of silver nanoparticles using *T. heimii* and *V. volvacea* extract, HPLC analysis for bioactive molecules and cytotoxicity through MTT assay have also been done in this study.

3.2. Materials and Methods

3.2.1. Preparation of mushroom extracts

The fleshy fruit bodies of mushroom samples were properly cleaned and dried in a food dehydrator (Presto, USA) followed by grinding to obtain powdered form. Dried and powdered mushroom samples were mixed with extracting solvents like hot water (W), acetone (A), ethanol (E) and methanol (M) in a ratio 1:5 (w/v). Samples were then placed on a shaker for 24 hrs agitation at 130 rpm at 37 °C. Whatman no. 1 filter paper were used for filtration purposes and stored at 4 °C for future use.

3.2.2. Antibacterial activity of mushroom extracts

The antibacterial activity of hot water, acetone, ethanol and methanol extracts from 7 selected mushroom species (Section 1.3.3) were studied by agar well diffusion method (Jagadeesh et al. 2010) against five Gram positive (*Micrococcus luteus*ATCC9341, *Streptococcus faecalis*MTCC5383, *Staphylococcus aureus* MTCC96, *Bacillus subtilis* MTCC441, *Bacillus cereus* MTTC3610) and five Gram negative (*Escherichia coli MTCC118, Shigella flexneri*MTCC7061, *Salmonella typhi*MTCC734, *Klebsiella pneumoniae* MTCC109, *Enterobacter aeruginosa* MTCC111, *Vibrio cholerae*MTCC3906 pathogenic bacteria were considered for the study. Antibacterial activity of extracts was determined in terms of clear zone of inhibition (CZOI) (Jagadeesh et al. 2010).

3.2.3. Biosynthesis and characterisation of silver Nanoparticles

Biosynthesis of silver nanoparticles was done by the procedure of Sujata et al. (2015). About 10 gm dried powder of *Termitomyces heimii* was boiled with 100ml two times (double) distilled water for a time of 10 min and then filtered using Whatman No. 1 filter paper. For the biosynthesis of silver nanoparticles, the filtrate was mixed to 100 ml of 0.001M $AgNO_3$ (prepared in deionized water) and the pH of the reaction mixture was finally adjusted at pH 10. Then the mix solution was incubated at 40°C with 150 rpm for 24 hrs. The gradual change in colour after 12 hrs was observed and the formation of silver nanoparticles (AgNPs) were then characterized by UV-Visible spectroscopy under the range of 200-600 nm using a TECHCOMP (Japan) UV-Vis spectrophotometer. Furthermore, A portion of the residual solution after incubation was centrifuged (REMI, India) at 10,000 rpm for 15 min and the suspension that was obtained immediately washed with deionized water to get pure nanoparticles free of proteins and enzymes (Philip 2009). After that the nanoparticles were analyzed through FTIR (PerkinElmer, India) and the spectra were recorded from 400 to 4000 cm^{-1}, with a resolution of 4 cm^{-1}.

3.2.4. Antibacterial efficacy by silver nanoparticles

The antibacterial activity of the synthesised silver nanoparticles was evaluated by agar well diffusion methods (Jagadeesh et al. 2010). The determination of antibacterial activity of AgNPs was conducted against two bacterial pathogens *S. aureus* and *S. flexneri*.

3.2.5. Partial purification by column chromatography and fraction collection

The methanolic extract of *T. heimii* was purified partially by column chromatography [Silica Gel230-400 mesh (40-63µm), column diameter 1cm]. Elution gradient mixtures of methanol (polar) and chloroform (non-polar) are used for this purpose and the collected fractions were further tested for their antibacterial activity against Gram positive and Gram negative pathogenic bacteria.

3.2.6. HPLC analysis

20µl sample (highest active methanolic fraction after antibacterial activity study) was loaded on the HPLC system (Agilent, USA). Sample with a flow rate of 0.8 ml/min at 25 °C was run across Agilent C18 column (100mm×4.6mm, 3.5µm) for the separation (Mitra et al. 2015). The mobile phase consisted of eluent A and eluent B. Acetonitrile and aqueous phosphoric acid (0.1% v/v) were used as eluent A and eluent B. An inclined program was used for elution: 0-2 min, 5% A; 2-5 min, 15% A; 5-10 min, 40% A; 10-15 min, 60% A; 15-18 min, 90% A. The absorbance of sample solution was measured at 280 nm. Identification of sample compounds were done on the basis of the retention times and absorption spectra of the individual compounds in the reference to standard peaks (qualitative analysis).

3.2.7. Isolation and purification of polysaccharide from Termitomyces heimii

The isolation of crude polysaccharide was done using alkaline (4% NaOH) extract of powdered fruit bodies of *T. heimii* following standard protocol (Maity et al. 2014a). The bulk portion was freeze dried (Instrumentation India, Kolkata, India) and kept at - 20 °C for

further analysis. Purification of a portion (50 mg) of the crude polysaccharide was performed by gel permeation chromatography (GPC) on DEAE Sephadex-100 column by water elution with little modifications of the procedure as adopted by Maity et al. (2014b). Two homogeneous fractions *Termitomyces heimii* polysaccharide (THP-I and THP-II)] were isolated and purified by GPC and further tested for antibacterial potentials against *Staphylococcus aureus*.

3.2.8. Characterization of THP-I through LC-MS and NMR study

A portion of THP-I was further analyzed through liquid chromatography-mass spectrometry (SQD2, Waters corporation, USA) (Futatsuyama et al. 1999) and NMR study (BRUKER corporation, USA) for their chemical characterization (Mursito et al. 2010).

3.2.9. Determination of MIC and MBC

To determine the antibacterial potentials of the polysaccharide fraction THP-I, the MIC and MBC assays was performed against two pathogenic bacteria namely *Staphylococcus aureus* (Gram positive) and *Shigella flexneri* (Gram negative) (Tambeker et al. 2006).

3.2.10. In vitro cytotoxicity assay

MTT [(3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrasodium bromide)], is a pale yellow substrate, cleaved by live cells to yield a dark-blue formazan product. This process is controlled by the active mitochondria of living cells. Here the amount of MTT cleaved is directly proportional to the number of viable cells. This assay is performed using standard protocol (kit) (Jedinak and Silva 2008). Here the THP-I of *T. heimii* was dissolved in DMSO and serially diluted with complete medium to get a range of test concentrations. Vero (kidney cells of green monkey) and HCT (Human colon cancer cell) cell lines were used in this study. Cell lines maintained in minimum essential DMEM medium with 10 % foetal bovine serum (FBS) at appropriate conditions were seeded in 96-well plates and treated with control, 25

µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml, 800 µg/ml and kept at 37°C in a CO_2 incubator (ESCO, USA) for 48 hrs. Later 10 µl of MTT reagent was added to every well and incubated for 2 to 4 hrs at 37°C. The formazan formation was confirmed by observing the appearance of dark-blue colour through microscope. Then 100 µl of soluble solution (kit) was added to each well under a safety cabinet (ESCO, USA) and measured the OD values at 570 nm in ELISA reader (Robonik, India). Percentage of cell viability is measured by following rule [Cell viability = (treated cells/ control cells) ×100]. The values are plotted against the concentrations of the sample.

3.3. Results and Discussions

3.3.1. Antibacterial potential of mushroom

Antibacterial activity (in terms of zone of inhibition) of selected mushroom extracts in four different solvents (hot water, methanol, ethanol and acetone) were tested against 10 human pathogenic bacterial strains in comparison with the standard antibiotics (Table-3.1). Comparative statistical analysis resulted that the methanolic extract of *Termitomyces heimii* showed the highest antibacterial activity against *Staphylococcus aureus* (18 mm ZOI) (Fig-3.9 C) which is a Gram positive bacteria and *Shigella flexneri* (16 mm ZOI) (Fig- 3.9 A) which is a Gram negative bacteria. Methanol and acetone extracts of *Pleurotus ostreatus* showed a higher ZOI (13 mm) against two Gram negative bacteria *E. coli* (Fig- 3.9 F) and *K. pneumoniae* (Fig-3.7A) respectively, while hot water extract of the same showed comparatively lesser ZOI against 2 Gram positive bacteria *B. cereus* and *B. subtilis*. Previously, Hearst et al. (2009) reported antibacterial activity of aqueous extract of *Pleurotus ostreatus* against *B. subtilis* (7 mm ZOI) and *B. cereus* (5 mm ZOI). Kalyoncu et al. (2010) found that the ethanolic extract of *P. ostreatus* showed 12 mm and 16 mm ZOI against *B. subtilis* and *B. cereus* respectively.

In the present investigation methanol extract of *Volvariella volvacea* showed 14 mm

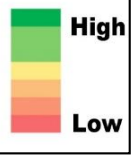
ZOI against *S. flexneri*, *S. typhi* and *S.aureus* (Fig- 3.9 A,B,C). Earlier, Jagadeesh et al. (2010) evaluated the antibacterial activity of *Volvariella bombycine* and reported that methanol extract showed highest ZOI against *Bacillus subtilis* (15 mm) followed by *Staphylococcus aureus*(14 mm),*Escherichia coli*(12 mm), *Klebsiella pneumoniae*(12 mm) and *Pseudomonas aeruginosa*(8 mm).Ethyl acetate extract of four different edible mushrooms (*P. sajor-caju, P. ostreatus, V. volvacea* and *A. bisporus*) were evaluated for their antibacterial activity against *B. subtilis, S. aureus, E. coli, Klebsiella pneumoniae* and *Proteus vulgaris* (Surekha et al. 2011) and it was found that only *A. bisporus* and *P. ostreatus* ,showed effective ZOI against all pathogenic strains.

Kamble et al. (2011) measured antibacterial activity of *Ganoderma lucidum* and reported that methanolic extract showed the highest in vitro antibacterial activity against *S. aureus* (11.5mm) followed by *B. subtilis* (10.5mm) and *E. coli* (10mm). Whereas, results of the present study exhibited higher antibacterial efficacy by methanol extract of *G. lucidum* against *S. aureus* (12 mm) followed by *B. subtilis* (13 mm) and *E. coli* (12 mm). These findings are concurring with the results of Sheena et al. (2003). As per the results of present study the antibacterial activity of *Schizophyllum commune* showed higher values (ZOI) than the results as reported by Deka et al. (2007). Variations of antibacterial activity by the same mushroom species were probably due to the effect of different solvents used for the preparation of extract. Earlier, Kumari et al. (2017) has measured the antibacterial activity of *T. heimii* using different concentrations of methanolic extract against 5 pathogenic bacteria (*S. aureus, E. coli, K. pneumoniae, S. pyogenes* and *Pseudomonas* sp.). In this scenario the present study is the first elaborative work on antibacterial activity of *T. heimii* using 4 different extracts (hot water, methanol, ethanol and acetone) against 10 pathogenic strains including both Gram positive and Gram negative bacteria. In this context, the studied mushrooms of Gurguripal ecoforest showed effective antibacterial efficacy and in particular

T. heimii had emerged as most potential species for future development of new antibacterial agents. The partially purified methanolic extracts of *T. heimii* were also tested for their antibacterial activity and the highest active methanolic fraction (F_{11}) (Fig 3.10 A, B)was then analysed through HPLC.

Table 3.1- Comparative analysis of antibacterial activity of different mushroom extracts against pathogenic bacteria

	V. volvacea	*P.ostreatus*	*A. auricula*	*A. hygrometricus*	*S. commune*	*T. heimii*	*G. lucidum*	
E. coli	7	8	8	7	7	8	5	Hot Water
S. flexneri	6	7	8	9	8	9	6	
S. typhi	7	7	8	9	8	9	7	
K. neumoniae	7	8	6	8	9	10	7	
V. cholerae	6	7	6	7	8	8	6	
M. luteus	7	8	9	8	7	10	8	
S. faecalis	7	8	9	8	9	10	8	
S. aureus	8	7	9	8	7	9	7	
B. subtilis	9	8	7	8	8	9	7	
B. cereus	9	8	8	7	7	9	7	
E. coli	12	13	11	9	8	14	12	Methanol Extracts
S. flexneri	14	11	9	10	11	16	13	
S. typhi	14	12	8	11	10	14	11	
K. neumoniae	13	10	10	11	12	13	11	
V. cholerae	11	12	8	9	10	12	12	
M. luteus	10	9	8	7	10	12	10	
S. faecalis	9	8	10	8	9	14	10	
S. aureus	14	10	11	11	10	18	12	
B. subtilis	12	12	13	11	14	15	13	
B. cereus	11	12	12	11	10	15	14	
E. coli	10	9	11	12	11	12	11	Ethanol Extracts
S. flexneri	9	9	10	11	11	11	9	
S. typhi	9	10	10	11	12	10	9	
K. neumoniae	10	9	11	12	12	9	10	
V. cholerae	9	10	10	11	12	11	10	
M. luteus	10	11	10	12	11	10	9	
S. faecalis	9	10	9	10	11	11	11	
S. aureus	10	11	11	10	9	11	10	
B. subtilis	10	10	11	10	11	12	11	
B. cereus	12	11	10	9	11	10	11	
E. coli	10	10	9	10	10	11	12	Acetone Extracts
S. flexneri	12	10	10	10	9	13	11	
S. typhi	12	11	11	11	11	12	13	
K. neumoniae	11	13	10	12	10	14	11	
V. cholerae	10	12	11	11	12	13	12	
M. luteus	11	12	12	12	13	14	9	
S. faecalis	13	11	10	10	9	14	13	
S. aureus	12	10	9	9	11	11	11	
B. subtilis	11	10	10	10	10	13	11	
B. cereus	10	11	10	9	9	12	10	



Maximum antibacterial activity (18 mm) was observed in Methanol extract (coloured in green) of wild mushroom *T. heimii* against *S. aureus*. Antibacterial activity of methanol extract of *T. heimii* was significantly higher than activity in hot water extract ($p < 0.001$), ethanol extract ($p < 0.001$), and acetone extract ($p < 0.05$) of *T. heimii*.

3.3.2. Antibacterial efficacy enhancement of mushroom extract throughAgNp$_s$

The mushroom extract was responsible for the reduction of silver ions and absorbed on the surface of silver nanoparticles accounting for their stabilization (Wang et al. 1998). In present experiment the reduction reaction was carried out at pH 10. It was found that, the reduction reaction occurs at very fast rate. The reduction of silver nitrate to silver nanoparticles was indicated by the colour change from pale or light yellow to reddish brown. The colour arised may be due to excitation of surface plasmon resonance (SPR) in the metal nanoparticles. The very fast rate of $AgNO_3$ reduction in presence of mushroom extract indicated with expectation of phenyl groups ionization.

The absorption spectrum of the silver nanoparticles is represented in Fig 3.1. The result of FTIR study was depicted in Fig 3.2 and revealed that the absorption bands were around at 3400 cm^{-1} and 690 cm^{-1}, which are characteristics of carbohydrate rings and halogen compounds respectively (Peng et al. 2003).

AgNPs synthesized using *T. heimii* extract exhibited increased antibacterial activity showing 18 mm and 19 mm clear ZOI against *S. flexneri* and *S. aureus* respectively (Table 3.2: Fig. 3.10 C and D). It was revealed that the antibacterial efficacy increased almost by 10% with silver nano-conjugates. Previously, Noginov et al.(2007) reported the formation of silver and gold based nanoparticles using 1mM solution of silver nitrate and auric acid, the results were finally confirmed by using UV-vis spectral analysis. AgNPs and AuNPs contain free electrons which give rise to a surface plasmon resonance (SPR) absorption band.

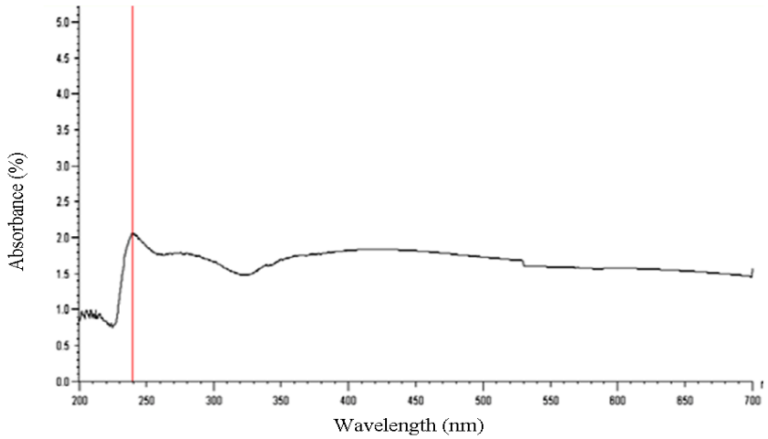


Fig 3.1- UV-Vis spectrum analysis of silver nanoparticles

FTIR measurement is one of the alternatives to identify the probable biomolecules of the nanoparticles synthesized using mushroom extract. Philip (2009) has investigated that the FTIR analysis were performed for the proper identification of the probable biomolecules responsible for capping and efficient stabilization of the metal nanoparticles synthesized using mushroom extract. The silver nanoparticles absorb strongly at 1679, 1539, 1452, 1430 and 1040 cm^{-1}. This type of obtained data with remarkable differences in their absorption value supports the scientific fact that the capping series could be different for gold and silver nanoparticles.

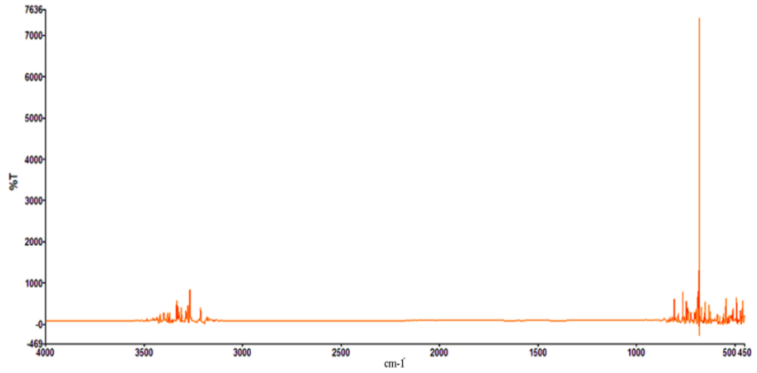


Fig 3.2- FT-IR study of silver nanoparticles

Table 3.2-Antibacterial activity of AgNPs synthesized by *T. heimii* extract (inhibition zone in mm)

Organism	Hot water	Acetone	Ethanol	Methanol	AgNPs	Antibiotic	Control
S. flexneri	9	13	9	16	18	22	-
S. aureus	9	11	10	18	19	22	-

3.3.3. Study of active constituents in T. heimii

HPLC analysis is useful to predict the constituents present in the methanolic extract of *T. heimii*. The result showed the presence of four carbohydrates like ribose, glucose, sucrose and xylose. Three major phenolic compounds namely gallic acid, p-coumaric acid and ferulic acid were also detected (Fig- 3.3).

The phenyl hydroxyl group in p-Coumaric acid, ferulic acid and gallic acid is the key components responsible for antioxidant activity (Mathew et al. 2015). Mitra et al. (2015) have reported two phenolic compounds (cinnamic acid and pyrogallol) from the ethanolic extract of *T. heimii*. Earlier Puttaraju et al. (2006) compared the antioxidative activities of water extract and methanolic extract from fruiting bodies of 23 species of edible mushrooms, among which *T. heimii, T. mammiformis* were superior in the expression of antioxidant activities and had a preponderant content of phenolic compounds. They also reported that *T. heimii* contains very high amount of *p*-coumaric acid (3700 mg Kg^{-1} DM) than cereals, pulses, fruits and vegetables. According to Lou et al. (2012), *p*-coumaric acid exhibits dual damage mechanisms to kill bacteria; cell membrane damage followed by DNA binding and inhibition. Therefore, *T. heimii* explored an opportunity for the discovery and utilization of natural bioactive compounds to improve our health status.

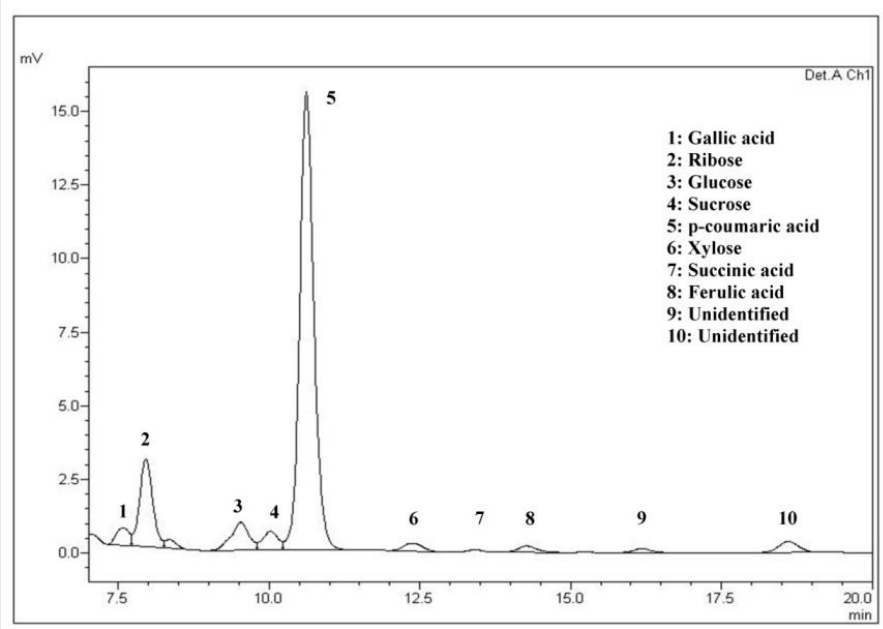


Fig 3.3- HPLC analysis of *Termitomycesheimii* extract

3.3.4. Isolation,purification and antibacterial activity of crude polysaccharide

Dried fruit bodies (100 gm) of *T. heimii* yields 380 mg of water soluble crude polysaccharide (pale white in colour). Two homogeneous fractions THP-I (20 mg) and THP-II (6 mg)) were purified and collected after gel permeation chromatography (GPC). THP-I showed higher antibacterial activity (16 mm ZOI) against the pathogenic bacteria *Staphylococcus aureus* (Fig-3.10 E).

Previously, immunomodulatory, anti-inflammatory, antioxidant and antitumor properties of mushroom derived polysaccharides has been reported by other workers. Mondal et al. (2006) has reported water-soluble heteropolysaccharide from *T. striatus* which enhance immune system through activating splenocytes. Lu et al. (2008) demonstrated that the dry matter of culture broth of *T. albuminosus* exhibited anti-inflammatory property. Both water soluble and insoluble β-glucan isolated from hot water extract of *T. robustus* exhibited immunomodulatory properties in significantly activating macrophages, splenocytes and thymocytes (Bhanja et al. 2012). Polysaccharides extracted from *T. albuminosus* have

reduced atrophy of both spleen and thymus of immunosuppressed mice (Wang 2013). Manna et al. (2015) reported about the antioxidant activity by the isolated water soluble β-glucan from *T. heimii.*

There are also some other reports regarding the bioactivity of polysaccharide extracts from *Termitomyces* mushroom (Hsieh and Ju 2018) but no particular study on antibacterial activity of the same has been done so far. In this regard this is the first report about the antibacterial activity of *T. heimii* derived polysaccharide against both Gram positive and Gram negativepathogenic bacteria.

3.3.5. Spectroscopical analysis

LC-MS analysis of THP-I fraction showed abundant presence of glucose molecule (180 g mol^{-1}) (Fig-3.4). Glucose as monomeric sugar unit forms β-glucans which is a polymer of carbohydrates (Futatsuyama et al. 1999).

The proton magnetic resonance spectrum (^{1}H NMR) of the THP-I fraction gave protons within chemical shifts (δH) between 3.40 to 4.0 indicating that the compound possesses CH and CH_2 functional groups that resonate with the oxygen atom of the -OH group. Five anomeric protons at δH 3.40, 3.42, 3.44, 3.45 and 3.94 ppm (Fig 3.5) revealed that the compound is a polysaccharide). Similar findings were reported earlier by Mursito et al. (2010)andManna et al. (2015).

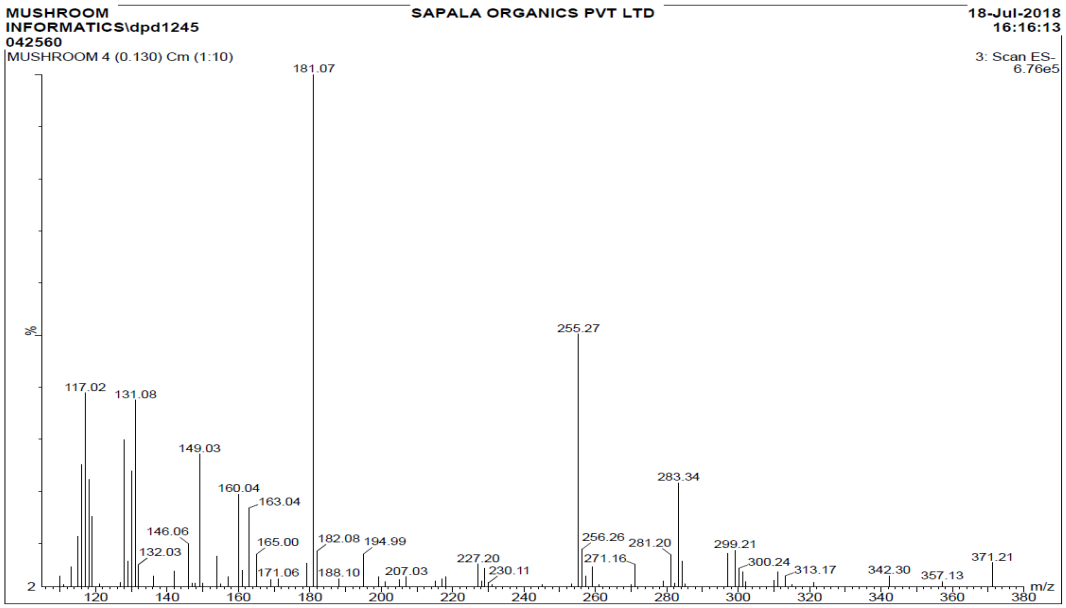
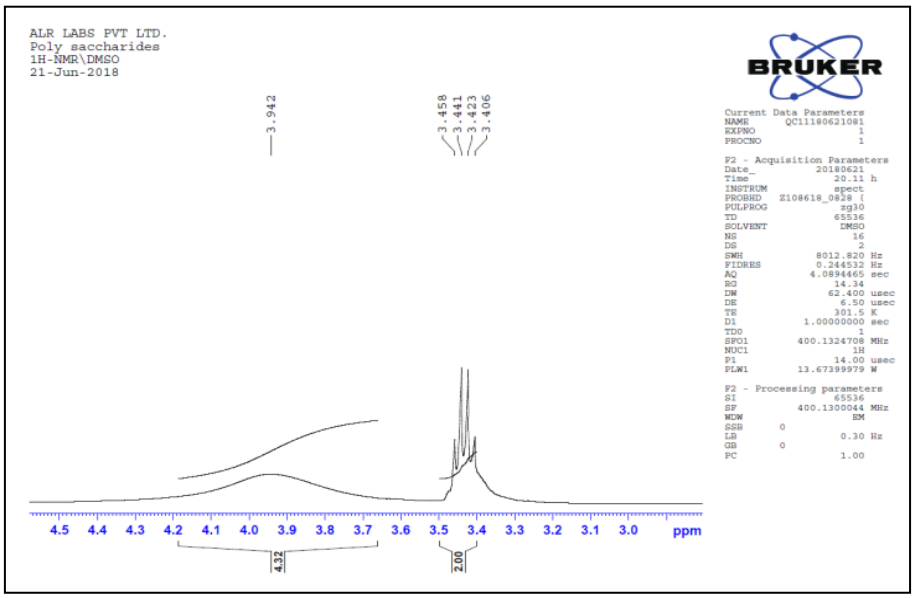


Fig 3.4- LC-MS analysis of THP-I from *T. heimii*



peak

ALR LABS PVT LTD.
Poly saccharides
1H-NMR\DMSO
21-Jun-2018

#	ADDRESS	FREQUENCY [Hz]	[PPM]	INTENSITY
1	40038.2	1577.473	3.9424	1.98
2	41623.5	1383.650	3.4580	4.34
3	41680.8	1376.647	3.4405	9.16
4	41738.3	1369.605	3.4229	9.02
5	41795.3	1362.643	3.4055	4.49
6	49489.5	421.897	1.0544	9.67
7	49546.8	414.895	1.0369	15.00
8	49604.4	407.853	1.0193	9.02

Fig 3.5- ^{1}H-NMR spectrum and chemical shifts of THP-I from *T. heimii*

3.3.6. MIC and MBC study of T. heimii extract

The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of the water soluble crude polysaccharide fraction from *T. heimii* (THP-1) were shown in Table 3.3. According to present study, THP-1 inhibited the growth of *Staphylococcus aureus* at a concentration of 62.5 µg/ml and exhibited bactericidal efficacy at 125 µg/ml. Whereas it showed 125 µg/ml and 250 µg/ml as MIC and MBC respectively against *Shigella flexneri*. Hence it was revealed that THP-1 exhibited higher antibacterial efficacy against Gram positive bacteria than Gram negative one. Earlier, Barros et al. (2008) has reported that the methanolic extract of *Cantharellus cibarius, Hypoloma fasciculare, Lepista nuda* showed the MIC value as 5 µg/ml against *Bacillus subtilis*, even lower than the standard ampicillin (MIC 12.5 µg/ml). Sharifi et al. (2012) has determined the MIC value of crude polysaccharide fractions (III A and III B) from *Ganoderma* sp. as 64 µg/ml against pathogenic *E. coli*. In their study, they have found the MBC value of same two fractions as 32 and 64 µg/ml against *Proteus mirabilis*. The present work has demonstrated a significant level of antibacterial activity of the studied extract against both Gram positive and Gram negative bacteria may be indicative of the presence of broad sprectum antibacterial compounds within *T. heimii*.

Table 3.3- MIC and MBC values of THP-I from *T. heimii*(µg/ml)

Pathogenic bacteria	MIC			MBC		
	mushroom	ciprofloxacin	water	mushroom	ciprofloxacin	water
S. aureus	62.5	15.625	_	125	31.25	_
S. flexneri	125	15.625	_	250	31.25	_

3.3.7.Cytotoxicity study of T. heimii extract

The cytotoxic effects of THP-I from *T. heimii* were studied on Vero and HCT cell lines at different concentrations ranging from 25 µg/ml to 800 µg/ml [Fig 3.6(A)]. The MTT assay revealed that the sample has no considerable cytotoxic effects on Vero cell line. But interestingly it showed significant cellular toxicity against Human Colorectal Carcinoma cell line (HCT) at a dose concentration 200 µg/ml and showed more significant effects at a dose of 600 µg/ml and 800 µg/ml gradually.

Earlier reports showed that the proliferation of colon cancer cells (HCT-116) markedly decreased with increasing concentration of methanolic extracts of *Pleurotus ostreatus* (Jedinak and Silva 2008). Manna et al. (2015) reported cytotoxic effect of polysaccharide fraction isolated from *T. heimii* against Human blood lymphocyte at the dose of 400 µg/ml. Zhang et al. (2007) opined that polysaccharides are the most potent mushroom derived substances having anti-tumor and immunomodulatary properties. The present study evidently showed that in vitro application of the polysaccharide fraction (THP-I) isolated from *T. heimii* has restricted the growth of colon cancer cells without any noticeable adverse effect on normal cells [Fig 3.6(B), (C)]. The results suggested that the polysaccharide fraction of *T. heimii* could be used as a natural antitumor agent.

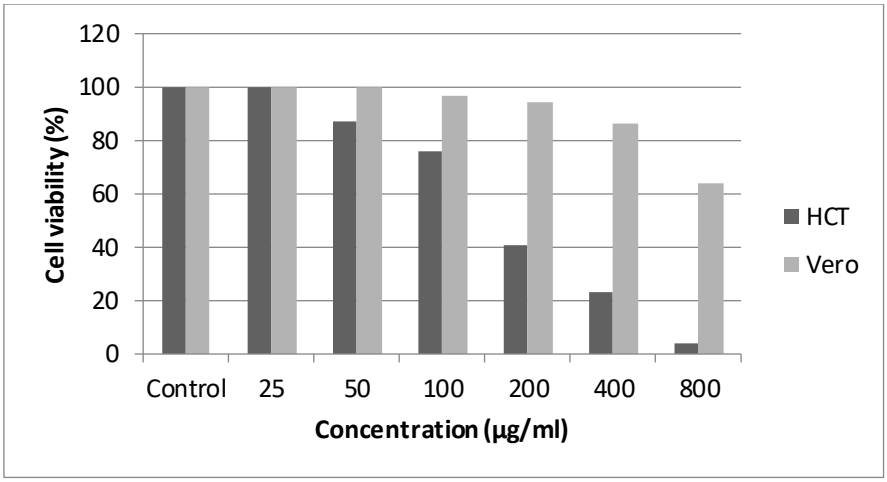


Fig3.6(A)- Cytotoxic effects of THP-Ifrom *T. heimii* on Vero and HCT cell lines

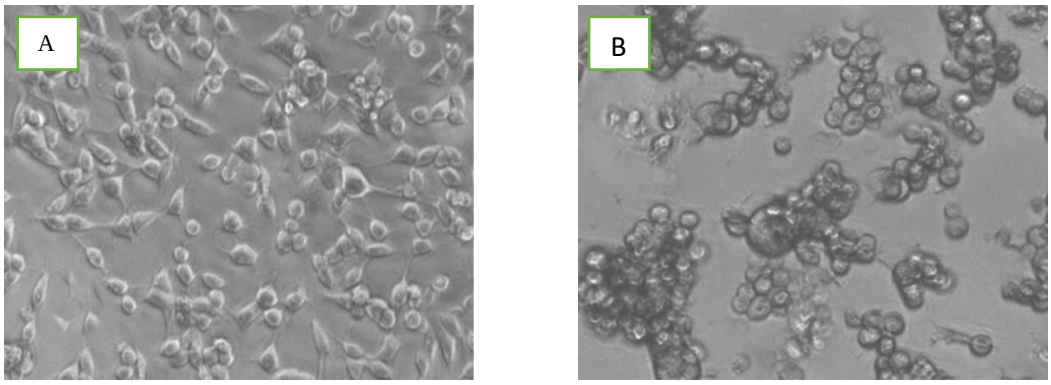


Fig 3.6(B)-HCT cells before treatment (A), cell death after treatment (B)

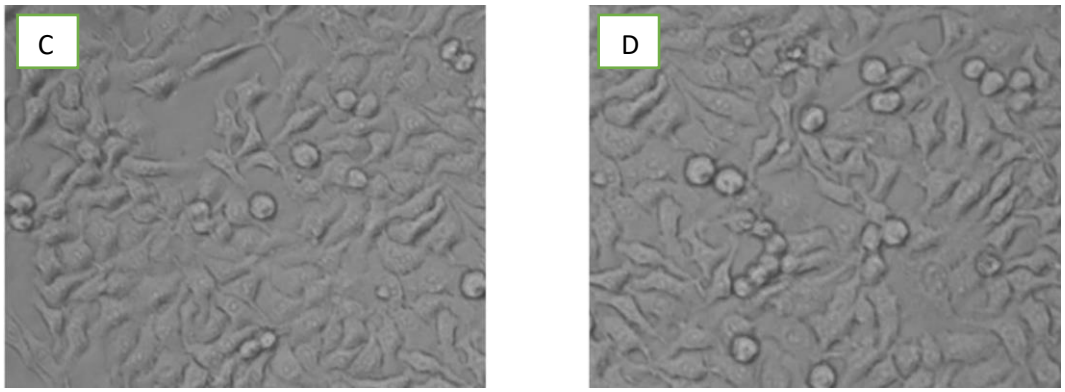
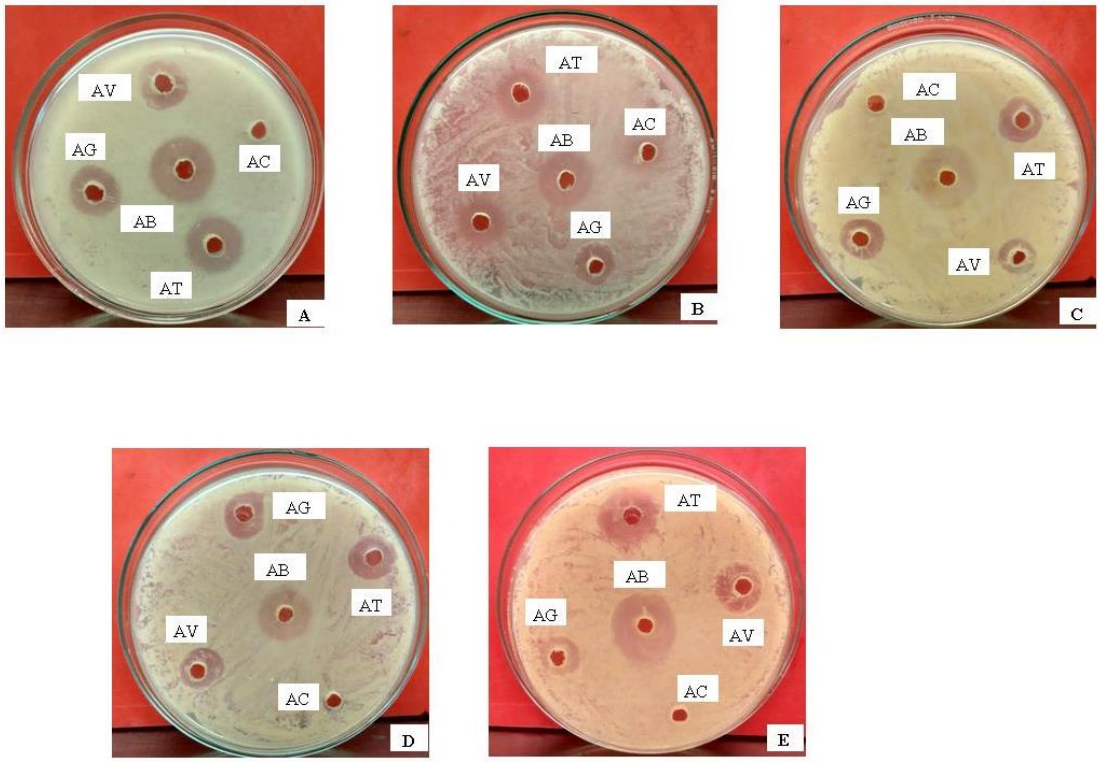


Fig 3.6(C)- Vero cells before treatment (C), after treatment (D)

3.4. Conclusion

The present study revealed that methanolic extract of the wild edible mushroom *Termitomyces heimii* occurring in Gurguripal ecoforest has shown broad spectrum antibacterial activity against both Gram positive and Gram negative bacterial pathogens and hence can be employed for the development of alternative antibacterial drug against human diseases. *T. heimii* could be a good agent for the biosynthesis of silver nanoparticles (AgNPs) exhibited enhanced antibacterial activity. HPLC analysis has confirmed the presence of major phenolic compounds like ferulic acid, gallic acid, *p*-coumaric acid have strong synergistic effects as antioxidant and thus can solve our health problems due to oxidative stress. Liquid chromatography and mass spectrometry analysis have showed the presence of polysaccharide compounds having effective antibacterial activity. This is the first report about cytotoxic effects of polysaccharide fraction from *T. heimii* against colon cancer cell lines (HCT). Presently several companies are developing medicines from different mushrooms, and these medicines although expensive, have promising health benefits including fighting against cancer. In this regard the present findings may boost the future prospects of *T. heimii* in solving our health problems including cancer therapy. Further it has been also concluded that the concentration of ***p*-CA** was highest in the methanolic extract of *T. heimii* in comparison to other active compounds. The potential of that extract is thus majorly based upon the activity of ***p*-CA**. Additional in-depth analysis of ***p*-CA** activity may explore inventory ideas for the treatment of bacterial diseases.



A— Antibacterial activity of acetone extracts of WEM against *Klebsiella pneumoniae*

B— Antibacterial activity of acetone extracts of WEM against *Streptococcus faecalis*

C— Antibacterial activity of acetone extracts of WEM against *Vibrio cholerae*

D— Antibacterial activity of acetone extracts of WEM against *Shigella flexneri*

E— Antibacterial activity of acetone extracts of WEM against *Micrococcus luteus*

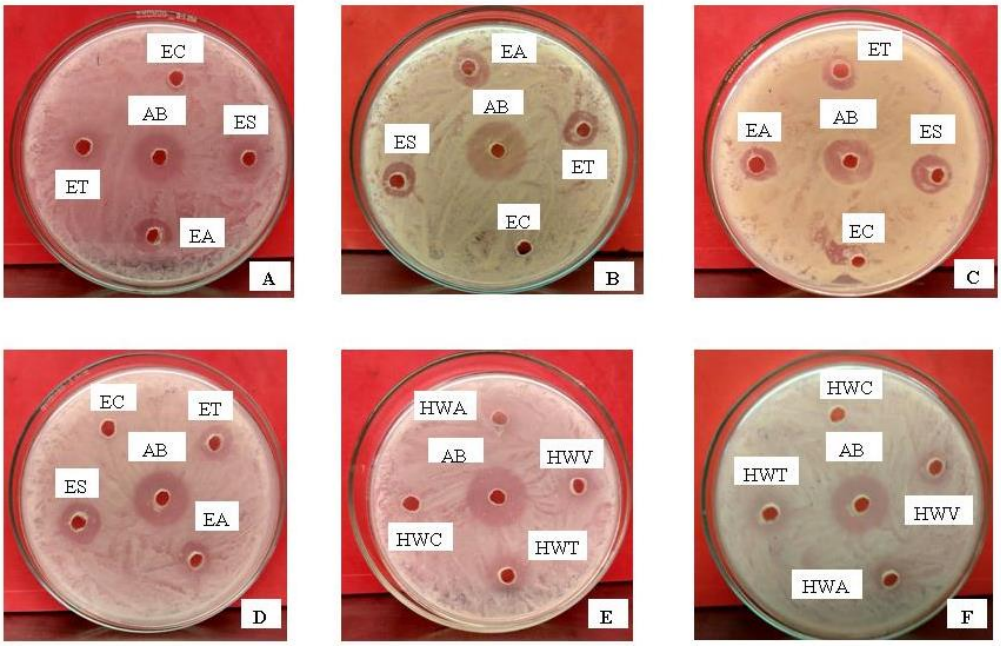
AB– Antibiotic

AG– Acetone extract of *Ganoderma lucidum*

AT- Acetone extract of *Termitomyces heimii*

AV– Acetone extract of *Volvariella volvacea*

Fig 3.7- Antibacterial activity of acetone extracts against the selected bacteria



A— Antibacterial activity of ethanolic extracts of WEM against *Klebsiella pneumoniae*

B— Antibacterial activity of ethanolic extracts of WEM against *Micrococcus luteus*

C— Antibacterial activity of ethanolic extracts of WEM against *Salmonella typhi*

D— Antibacterial activity of ethanolic extracts of WEM against *Vibrio cholerae*

E— Antibacterial activity of hot water extracts of WEM against *Klebsiella pneumoniae*

F— Antibacterial activity of hot water extracts of WEM against *Streptococcus faecalis*

AB– Antibiotic

EA– Ethanolic extract of *Astreaus hygrometricus*

ET- Ethanolic extract of *Termitomyces heimii*

ES- Ethanolic extract of *Schizophyllum commune*

EC– Ethanol control (only ethanol no mushroom extract)

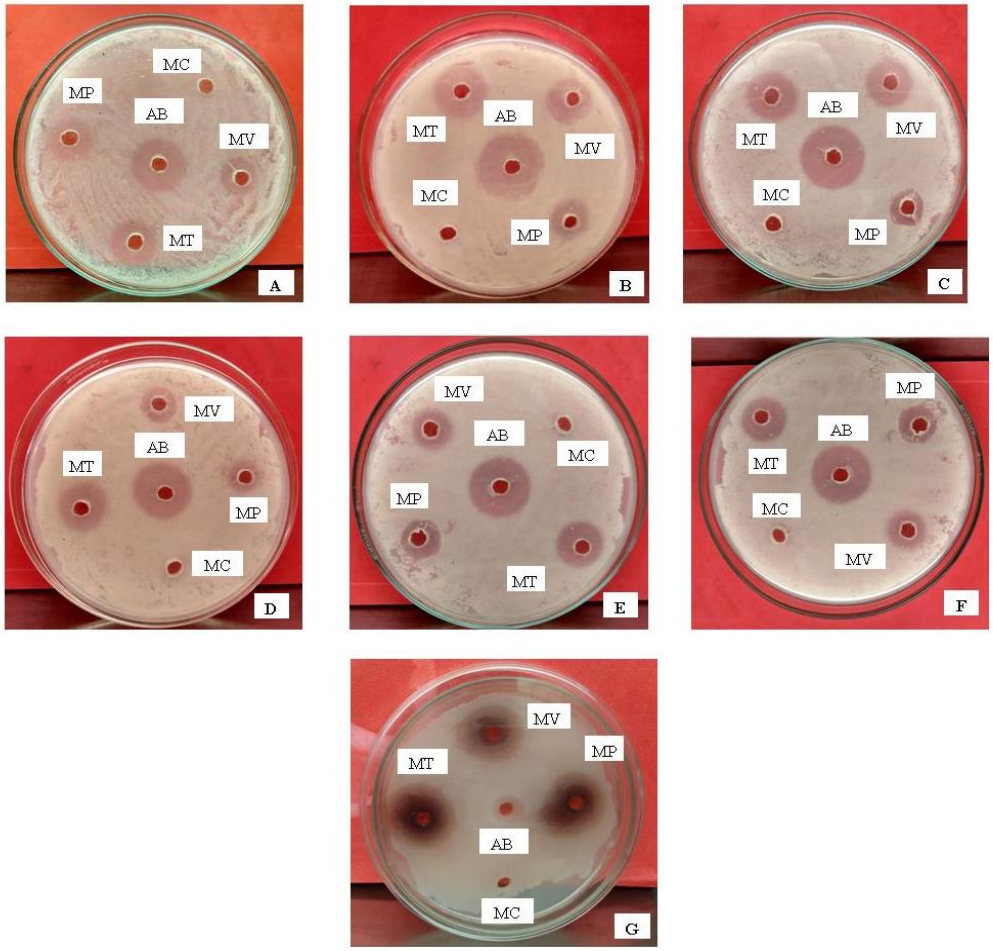
HWT– Hot water extract of *T.heimii*

HWV- Hot water extract of *Volvariella volvacea*

HWA– Hot water extract of *Auricularia auricula*

HWC– Hot water control

Fig 3.8- Antibacterial activity of ethanolic (A, B, C, D) and hot water (E, F) extracts against the selected bacteria



A— Antibacterial activity of methanolic extracts of WEM against *Shigella flexneri*

B— Antibacterial activity of methanolic extracts of WEM against *Salmonella typhi*

C— Antibacterial activity of methanolic extracts of WEM against *Staphylococcus aureus*

D— Antibacterial activity of methanolic extracts of WEM against *Bacillus cereus*

E— Antibacterial activity of methanolic extracts of WEM against *Bacillus subtilis*

F— Antibacterial activity of methanolic extracts of WEM against *Escherichia coli*

G— Antibacterial activity of methanolic extracts of WEM against *Klebsiella pneumoniae*

AB– Antibiotic

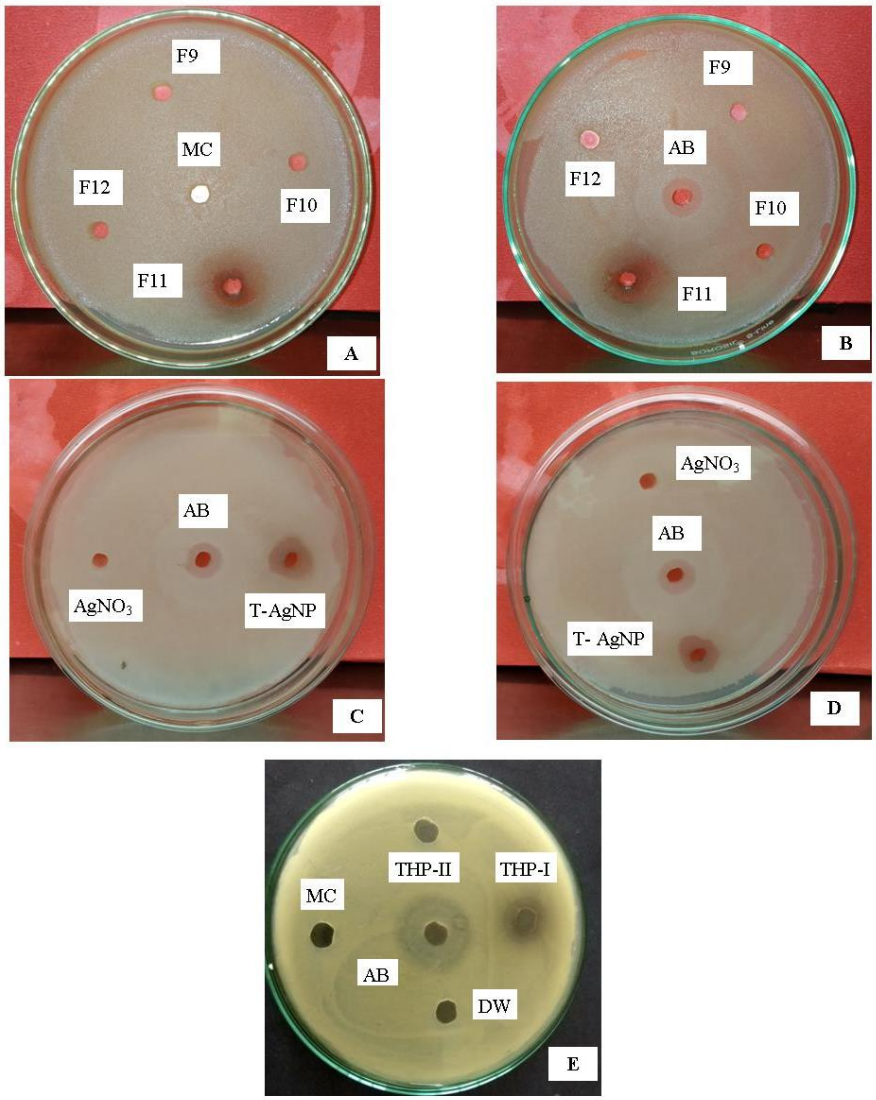
MP– Methanol extract of *Pleurotus ostreatus*

MT- Methanol extract of *Termitomyces heimii*

MV– Methanol extract of *Volvariella volvacea*

MC– Methanol control (only methanol, no mushroom extract)

Fig 3.9- Antibacterial activity of methanolic extracts against the selected bacteria



A– Antibacterial activity of fractions (F11) of *Termitomyces heimii* extracts against *Staphylococcus aureus*

B– Antibacterial activity of fractions (F11) of Termitomyces heimii extracts against *Shigella flexneri*

C– Antibacterial activity of AgNPs of *Termitomyces heimii* against *Staphylococcus aureus*

D- Antibacterial activity of AgNPs of *Termitomyces heimii* against *Shigella flexneri*

E-Antibacterial activity of *Termitomyces heimii* Polysaccharide against *Staphylococcus aureus*

MC-Methanol control, AB– Antibiotic, AgNO3– silver nitrate, AgNPs– Silver nano particles
F11– Fraction 11 THP-I and THP-II– *T. heimii* polysaccharide extract I,,II ; DW– Distilled water

Fig 3.10- Antibacterial activity of F_{11}, AgNPs and THP-I against the selected bacteria

Appendix II- Pathogenicity of thetarget pathogens for the study of antibacterial activity of selected mushroom extracts

Target pathogens	Pathogenicity
Micrococcus luteus	Colonizing in the surface of heart valves, causing endocarditis in humans.
Streptococcus faecalis	Urinary tract infections, septicaemia, endocarditis, meningitis
Staphylococcus aureus	Infective endocarditis, various skin infections such as pimple, impetigo, boils, cellulitis folliculitis, scalded skin.
Bacillus subtilis	Food poisoning.
Bacillus cereus	Food borne diseases, severe nausea, vomiting and diarrhoea, food poisoning.
Escherichia coli	Diarrhoea, UTIs (urinary tract infections), neonatal meningitis, gastroenteritis.
Shigella flexneri	Bacterial dysentery, shigellosis.
Salmonella typhi	Typhoid fever, abdominal pain, constipation, headache, nausea.
Klebsiella pneumoniae	Pneumonia, urinary tracts infection.
Vibrio cholerae	Cholera, a diarrhoeal disease.