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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF AQUEOUS EXTRACT OF SIX MUSHROOMS COLLECTED FROM HIMACHAL PRADESH

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ABSTRACT

Mushrooms have been used for prevention and treatment of multitude disorder. Macro fungi regarding to the development of novel safe antimicrobials and antioxidants has become attractive source for research from last decades. In the present study antimicrobial activity of aqueous extract of six mushrooms was carried out by agar well diffusion method while 1, 1 Diphenyl -2- picrylhydrazyl (DPPH) free radical scavenging assay was used to evaluate the antioxidant properties. The results thus obtained revealed that extracts from all six mushrooms showed good antimicrobial activity against *S. aureus, E. coli* and *K. pneumoniae* while *P. aeruginosa* is susceptible to extract of *P. floridanus*, *P. ostreatus* , *T. versicolor* and *Calocybe indica.* All extracts exhibited DPPH (1, 1 – diphenyl -2- picrylhydrazyl) free radical scavenging activities with *P. floridanus* having high antioxidant activity (246.71±1.88) and *Macrocybe* sp. having low antioxidant activity (1653.56±9.3). The mushroom aqueous extract investigated in the present study have antibacterial as well as antioxidant activities that warrant further studies as a potential dietary supplements in order to improve health and well being.

Keywords: Mushrooms, antimicrobial activity, antioxidant activity, DPPH

1. INTRODUCTION

The growing emergence of drug resistance towards microorganisms has become a serious threat to effective treatment of infections now days (Klein *et al.,* 2007). The drug resistance is mainly due to indiscriminate and in appropriate use of antibiotics (Gao *et al.,* 2003). The resistance to drugs has not only economic concern and drastic impact on patients only but also affects the pharmaceutical producers, physicians, health care administrators and

public (Mc Gowan, 2001). It has therefore become important to develop new effective therapeutic agents to counter resistant pathogens (Baratta *et al.,* 1998). Although there is a huge diversity of antibacterial compounds, bacterial resistance to first choice antibiotics has been significantly increasing. Microorganisms including *Klebsiella* spp., *E. coli* produce broad-spectrum beta-lactamase or present resistant to third generation cepahlosporins (Harbarth *et al.,* 2001). Another example are MRSA (Multidrug resistant *S. aureus*), resistant to vancomycin, *Acinetobacter* spp. having increasing resistance to carbapenems and colistin and *Pseudomonas* spp. resistance to carbapenemics, cephalosporins and amino-glycoside. (Kempf and Rolain, 2012). It was found in various studies by researchers that consumption of antibiotics in low dosage may not be effective against killing of microorganism causing disease but they stress them which results in the production of free radicals. These free radicals damage DNA and might cause mutation leading to the development of resistance (Kohanski *et al.,* 2010). The bacterial and fungal evolution is impossible to prevent, hence it is important to choose the most appropriate antibiotics and their appropriate use to minimize the development of bacterial strains (Alves *et al.,* 2012). Several plants and herbs are being used which are known to have antimicrobial properties. In addition to them mushrooms are also used as source of medicine against microorganisms which are resistant to antibiotics and against free radical damage. There are diverse range of Basidiomycetes possessing the macro-scopic reproductive structures and have been utilizing for medicinal as well as curative purpose not from the present but since from prehistoric times. Mushrooms are known to have a veritable treasure-house of several bio-active compounds possessing antimicrobial, anti-tumorgenic hypoglycaemic and hypolipidemic prop-erties (Venturini *et al.,* 2008). They have low fat contents and consist of vitamins, proteins, polysaccharides and many other nutritive compounds (Purkayastha *et al.,* 1985). It is believed that mushrooms need antimicrobial compounds in order to protect and survive in the environment. These compounds can be isolated from many mushroom species and some of them proved beneficial to human-being (Lindequist, 2005).

Mushrooms consist of bioactive compounds having wide range of activity against pathogenic microorganisms. They are known to be the rich sources of natural antibiotics, where the cell wall glucans are well known for their immune-modulatory properties, and for externalized secondary

metabolites combat fungi, bacteria and viruses (Collin and Ng, 1997; Bains and Tripathi, 2015). The active domain of present study was therefore to investigate the antimicrobial and antioxidant activity of aqueous extract of mushrooms collected from Himachal Pradesh.

2. MATERIAL AND METHOD

2.1 Source of Macro fungi

Three mushroom fruiting bodies namely *P. ostreatus*, *P. floridanus* and *T. versicolor* were collected from the forest of Badu Sahib, Distt, Sirmour, and Solan, Himachal Pradesh. Three cultivated mushrooms mycelial cultures *Mycrocybe* sp. *Agrocybe aegerita*, *Calocybe indica* were obtained from DMR (Directorate of Mushroom Research Centre), Solan.

2.2 Source of test organism and their maintenance:

Four bacterial strains *Klebsiella pneumonie* MTCC 109, *Pseudomonas aeruginosa* MTCC 741, *Staphylococcus aureus* MTCC 737 and *E. coli* MTCC 739 were obtained from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh (INDIA) and stored at 4ºC in refrigerator and sub cultured at regular intervals of 48 h until use.

2.3 Extract preparation:

Dry mycelial biomass was accurately weighed and 10 ml deionised water was added to it. The mixture was heated at 100°C for 20 minutes and allowed to cool for 20 minutes. After this, the filtrate was separated from the mycelial biomass by using No. 42 (x 2) Whatman filter paper. The process was repeated twice. The combined filtrate was then freeze-dried and the dry weight of sample was recorded.

2.4 Antibacterial activity:

Pure cultures of bacterial strains were seeded into nutrient agar plates. Wells (7mm diameter) were made on petri dishes using sterile cork borer and about 25µl extract were introduced into them using sterile dropping pipette. These plates were kept inside the refrigerator at 4°C for 6 hours to allow proper diffusion of extracts into medium. The plates were then examined after 24 hours of incubation at 37°C (Bains and Tripathi, 2015).

2.5 Minimal inhibitory concentration:

Each well of 96-well microtitre plate was aliquoted with 50 µl of Muller Hinton Broth (MHB);. 11th well (growth control) was added with MHB with 10% DMSO as negative control and 12th well (sterility control) was added with 100 µl of Ciprofloxacin antibiotic as positive control. 50 µl of mycelial extract initially dissolved in 10% DMSO to the concentration of 50 mg/ml was added into the first well and a serial 2-fold dilution was performed by transferring 50 µl of the suspension to the

subsequent wells up till the 10th well; the final 50 µl of the suspension was discarded. Then, 5 µl of bacterial suspension was added to each well and incubated at 37℃ for 24 hours. After 24 hours of incubation 5 µl of resazurin was added to each well. Plates were incubated at 37℃ for additional 1 hour. After one hour of incubation the plates were read for colour change from blue to purple/ pink. A blue coloured solution indicated the growth inhibition in the test wells, while pink to colourless solution indicated microbial growth or absence of inhibition.

2.6 Determination of Anti-oxidant activity:

2.6.1 DPPH free radical assay

Free radical scavenging activity of aqueous extract of all isolates of mushrooms was determined by DPPH method. 0.1 mM of DPPH solution was prepared in methanol and 0.5 ml was added to 0.5 ml of extract. The mixture was then vortexed thoroughly and left for 45 minutes in dark at room temperature. The absorbance was measured at 515 nm against blank. A lower absorbance represents higher DPPH scavenging activity. The capability of scavenging DPPH radicals was calculated by using following equation:

DPPH scavenging effect (%) = (1-As/Ac) X 100

Where, Ac is the absorbance of control containing DPPH solution and As is absorbance of extract solution containing DPPH (Hung and Morita, 2009).

3. RESULTS

3.1 Antibacterial activity:

Aqueous extract was used as solvent for the preparation of extract. Screening for antimicrobial activity against four pathogenic bacteria was done by agar well diffusion method (Table 1 and Fig 1- 4). Maximum inhibition of *E. coli* for aqueous extract was shown by *T. versicolor*, (28.6 ±0.6) followed by *Calocybe indica* (25.3 ±0.6), *P. ostreatus*, (25±1), *P. floridanus* (24.3±0.6), *Macrocybe* sp. (23.6±0.6) and *Agrocybe aegerita* (22.3±0.6), against *Pseudomonas aeruginosa* only four isolates *Pleurotus ostreatus* (24.6±0.6), *Pleurotus floridanus* (22.3±0.6), *Trametes versicolor* (21.6 ±1.5) and *Calocybe indica* (19±1) showed activity. Inhibition of growth of *K. pneumoniae* by hot water extract of *Pleurotus floridanus* (28±1) is maximum followed by *Trametes versicolor* (24±1), *Pleurotus ostreatus* (22.3±0.6), *Calocybe indica* (21.6±0.6), *Agrocybe aegerita* (18.6±0.6) and *Macrocybe* sp. (19.3±0.6) respectively. Against *S. aureus* hot water extract of *Pleurotus floridanus* showed maximum inhibition ranges (28.3±0.6), followed by isolate *Pleurotus ostreatus* (25.3±0.6), *Macrocybe* sp.

(25±1), *Trametes versicolor* (23±1), *Calocybe indica* (22.6±0.6) and *Agrocybe aegerita* (21±1).

3.2 Minimal inhibitory concentration of aqueous extract:

Resazurin is an oxidation-reduction indicator used for the evaluation of cell growth. It is a blue non-fluorescent and non-toxic dye that becomes pink and fluorescent when reduced to resorufin by the oxido-reductase enzymes within viable cells. Resorufin can be further reduced to hydro-resorufin which is colorless and non-fluorescent. The antibacterial and antifungal activity was assessed by MIC which was defined lowest concentration at which substance that prevents change in colour occurred. A microtiter plate based assay was carried out for each bacterial strain and results are shown in Table 2 and Fig 5-8. According to results all aqueous extract of isolates exhibit a broad antibacterial spectrum of activity and caused inhibition of growth of all bacterial strains. The results showed that the hot water extract (aqueous extract) of isolate *Pleurotus floridanus* also showed good antimicrobial activity against *P. aeruginosa* with MIC 1.56 mg/ml followed by isolate *Pleurotus ostreatus* with MIC 3.125, *T. versicolor* with MIC 6.25 mg/ml and *Calocybe indica* with MIC 12.5 mg/ml. Next in order of sensitivity is *K. pneumoniae*. Hot water extract of isolate *Pleurotus floridanus* also showed good potential for antimicrobial activity against *K. pneumoniae* with MIC 1.56 mg/ml, followed by *Pleurotus ostreatus*, *Trametes versicolor* and *Calocybe indica* with MIC 6.25 mg/ml each while least activity was shown by isolate *Agrocybe aegerita* and *Macrocybe* sp. with MIC 12.5 mg/ml each. Hot water extract of isolate *P. floridanus* showed good antimicrobial activity against *S aureus* with MIC 1.56 mg/ml each next to them is extract of *P. ostreatus* and *Macrocybe* sp. with MIC 3.125 followed by extract of isolate *T. versicolor*, *Calocybe indica* with MIC 6.25 and *Agrocybe aegerita* with MIC 12.5 each. Hot water (aqueous) extract of isolate *P. floridanus* and *T. versicolor* showed excellent potential of antimicrobial activity against *E. coli* with MIC 1.56 mg/ml followed *Calocybe indica* with MIC 3.125 mg/ml, *P. ostreatus* and *Macrocybe* sp. with MIC 6.25, extract prepared from *Agrocybe aegerita* showed least activity with MIC 12.5 mg/ml.

3.3 DPPH free radical scavenging assay:

The antioxidant activities of aqueous extracts were expressed as IC50 values of DPPH. Ascorbic acid was used as the reference compound (positive control) with concentration ranges from 100 to 1000 mg/ml for all the above spectroscopic

methods. In DPPH method IC50 for aqueous extract of *P. floridanus* was highest (246.71±1.88) followed by *P. ostreatus* (366.01±2.05), *Trametes versicolor* (479.94±1.94), *Calocybe indica* (1105.6±6.28), *Agrocybe aegerita* (1204.01±1.86) and *Macrocybe* sp. (1653.56±9.3) as shown in Table 3.

Table 1: Antimicrobial activities of aqueous extracts of isolates against various bacterial pathogens

S. No.	Isolates	Test microorganisms and concentration of extract used (100mg/ml)			
		P. aeruginosa	*S. aureus*	*K. pneumonia*	*E. coli*
1	104	24.6±0.6^{a}	28.3±0.6^{a}	28±1^{a}	25±1^{b}
2	105	22.3±0.6^{b}	25.3±0.6^{b}	22.3±0.6^{bc}	24.3±0.6^{b}
3	127	21.6±1.5^{b}	23±1^{cd}	24±1^{b}	28.6±0.6^{a}
4	*Calocybe indica*	19±1^{c}	22.6±0.6^{d}	21.6±0.6^{c}	25.3±1.5^{b}
5	*Agrocybe aegerita*	-	21±1^{d}	18.6±0.6^{d}	22.3±0.6^{c}
6	*Macrocybe* sp	-	25±1^{bc}	19.3±0.6^{d}	23.6±0.6^{bc}

(-) indicates no antimicrobial activity Values sharing a common letter within the row are not significant at P<0.05. Values are Mean± SEM (n=3)

Table 2: Minimal inhibitory concentrations of aqueous extracts of isolates against bacterial strains

S.no.	Isolates	Minimal inhibitory concentration (mg/ml)			
		P. aeruginosa	*S. aureus*	*E. coli*	*K. pneumoniae*
1.	104	1.56	1.56	1.56	1.56
2.	105	3.125	3.125	6.25	6.25
3.	127	6.25	6.25	1.56	6.25
4	*C. indica*	12.5	6.25	3.125	6.25
5.	*A. aegerita*	-	12.5	12.5	12.5
6.	*Macrocybe* sp.	-	3.125	6.25	12.5

Table 3: IC $_{50}$ value (mg/ml) calculated for the aqueous extract of samples

S. no.	Isolates	IC $_{50}$ value (mg/ml) for the aqueous extract of samples
		DPPH
1.	*P. ostreatus*	366.01±2.05
2	*P. floridanus*	246.71±1.88
3	*Trametes versicolor*	479.94±1.94
4	*Calocybe indica*	1105.6±6.28
5	*Agrocybe aegerita*	1204.01±1.86
6	*Macrocybe* sp.	1653.56±9.3

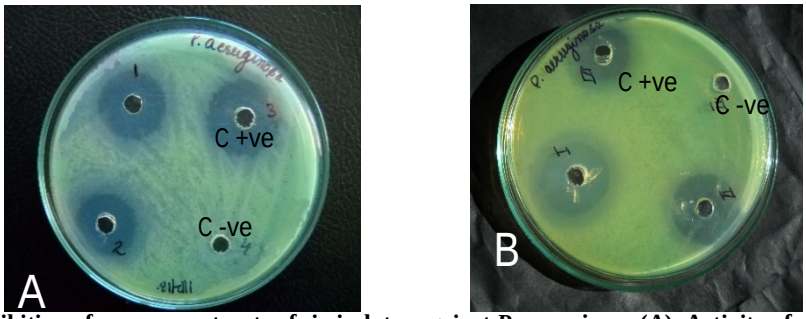

Fig 1: Zone of inhibition of aqueous extracts of six isolates against *P. aeruginosa* (A). Activity of extract from *P. floridanus, P. ostreatus* (B). Activity of extract from *T. versicolor* and *Calocybe indica*

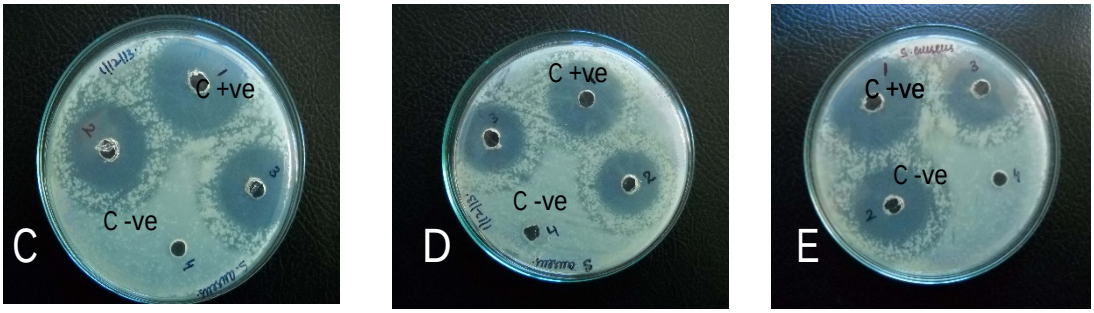

Fig 2: Zone of inhibition of aqueous extracts of six isolates against *S. aureus* (C). Activity of extract from *P. floridanus, P. ostreatus* (D). Activity of extract from *T. versicolor* and *Calocybe indica* (E). Activity of extract from isolate *Agrocybe aegerita* and *Macrocybe* sp.

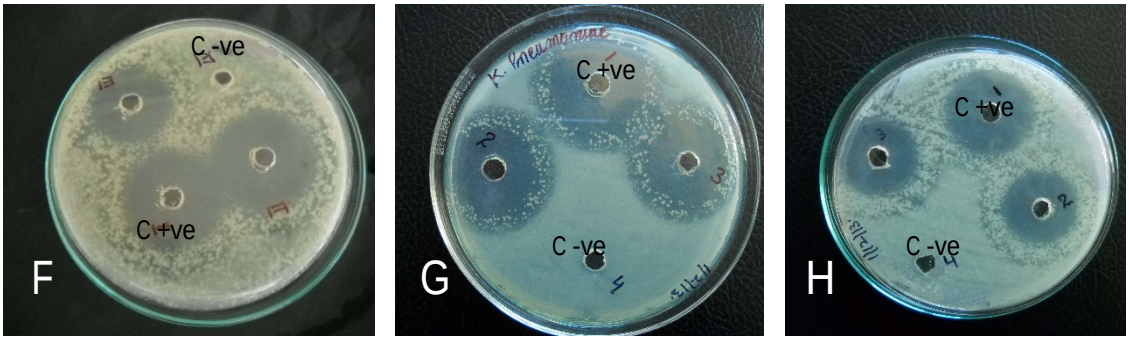

Fig 3: Zone of inhibition of aqueous extracts of six isolates against *K. pneumonia* (F). Activity of extract from *P. floridanus, P. ostreatus* (G). Activity of extract from *T. versicolor* and *Calocybe indica* (H). Activity of extract from isolate *Agrocybe aegerita* and *Macrocybe* sp.

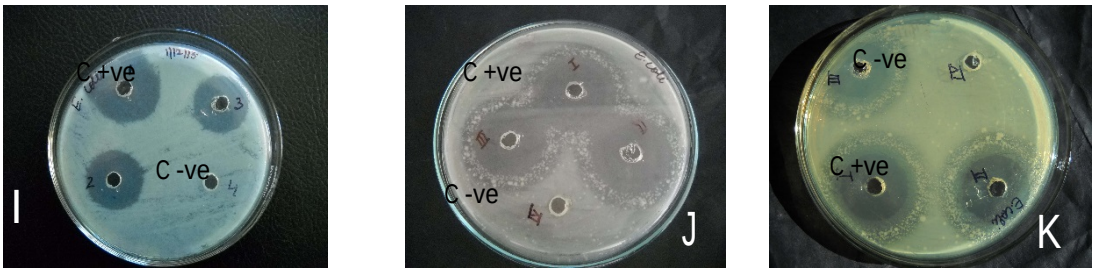

Fig 4: Zone of inhibition of methanol extracts of six isolates against *E. coli* (I). Activity of extract from isolates *P. floridanus, P. ostreatus* (J). Activity of extract from isolate *T. versicolor* and *Calocybe indica* (K).Activity of extract from isolate *Agrocybe aegerita* and *Macrocybe* sp

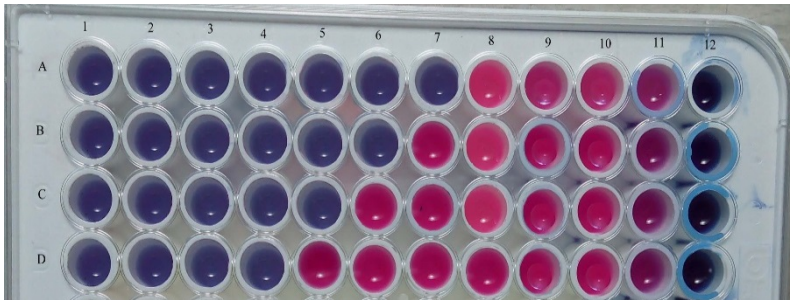

Fig 5: Minimal inhibitory concentration of aqueous extract of six isolates (Well 1-10 consist of extract and well 11 contains DMSO –ve control and 12 contains Ciprofloxacin +ve control) against *P. aeruginosa.* (A). MIC of extract from *P. floridanus* **(B).** MIC of extract from *P. ostreatus* **(C).** MIC of extract from *T. versicolor* **(D).** MIC of extract from *Calocybe indica.*

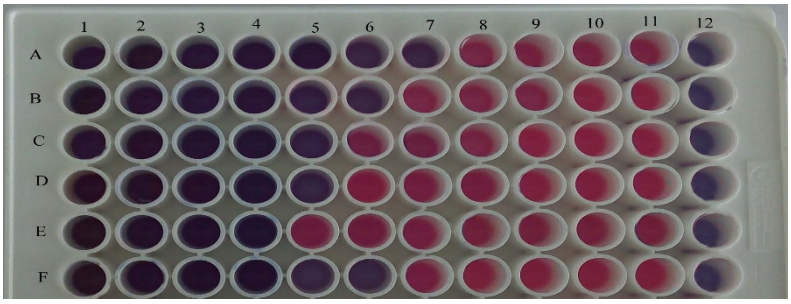

Fig 6. Minimal inhibitory concentration of aqueous extract of six isolates (Well 1-10 consist of extract and well 11 contains DMSO –ve control and 12 contains Ciprofloxacin +ve control) against *S. aureus*. (A). MIC of extract from *P. floridanus* (B). MIC of extract from isolate *P. ostreatus* (C). MIC of extract from isolate *T. Versicolor* (D). MIC of extract from *Calocybe indica* (E). MIC of extract from *Agrocybe aegerita* (F). MIC of extract from *Macrocybe* sp.

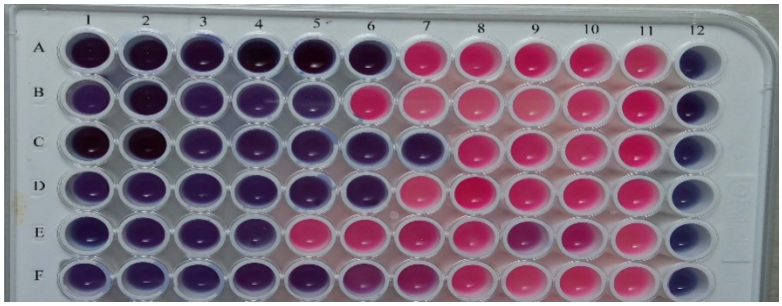

Fig 7. Minimal inhibitory concentration of aqueous extract of six isolates (Well 1-10 consist of extract and well 11 contains DMSO –ve control and 12 contains Ciprofloxacin +ve control) against *E. coli*. (A). MIC of extract from *P. floridanus* (B). MIC of extract from *P. ostreatus* (C). MIC of extract from *T. versicolor* (D). MIC of extract from *Calocybe indica* (E). MIC of extract from *Agrocybe aegerita* (F). MIC of extract from *Macrocybe* sp.

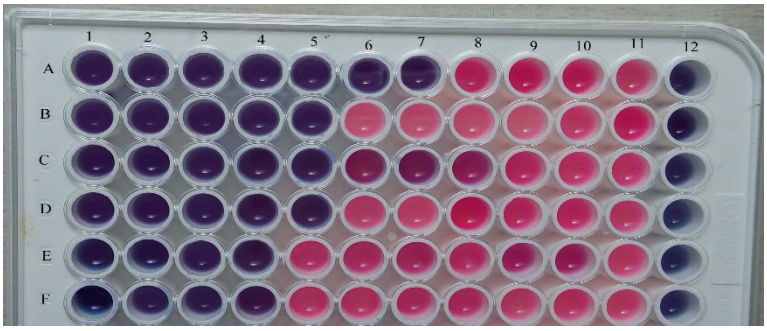

Fig 8. Minimal inhibitory concentration of aqueous extract of six isolates (Well 1-10 consist of extract and well 11contains DMSO –ve control and 12 contains Ciprofloxacin +ve control) against *K. pneumonia*. (A). MIC of extract from isolate *P. floridanus*.(B). MIC of extract from isolate *P. ostreatus* (C). MIC of extract from *T. versicolor* (D). MIC of extract from *Calocybe indica* (E). MIC of extract from *Agrocybe aegerita* (F). MIC of extract from *Macrocybe* sp.

4. DISCUSSION

Antimicrobial activity of aqueous extracts of mushrooms in the present study was carried out by agar well diffusion and it was revealed that extract of all mushrooms taken for study showed good antimicrobial activity against *S. aureus*, *E. coli*, *K. pneumoniae* while *P. aeruginosa* was susceptible to extracts of four isolates i.e. *P. floridanus*, *P. ostreatus* , *T. versicolor* and *Calocybe indica*. The sensitivity of *S. aureus*, *E. coli* and *K. pneumoniae* to mushroom extracts agrees with the previous studies of various researchers (Yamac and Bilgili, 2006; Bains and Tripathi, 2015, 2016). Unlike Gram negative bacteria, Gram positive bacteria have outer membrane and periplasmic space surrounding the cell wall, inner leaflet of this outer membrane consist of phospholipids whereas outer leaflet is mainly comprised of lip-polysaccharides (Holst *et al.*, 2007). Lip polysaccharides are mainly constructed from three parts namely, a proximal hydrophobic lipid A region, a core oligosaccharide region, connecting a distal O- antigen polysaccharide region to lipid A. The lipid polysaccharides molecules contains six or seven covalently linked

fatty acid chains which results in asymmetric bilayer of bacterial outer membrane from lipid –like cell walls and serve as efficient barrier against rapid penetration by chemotherapeutic agents and antibiotics and polysaccharides from various mushrooms extracts (Cohen, 2004). Periplasmic space majorly contained Murin a peptidoglycan. Peptidoglycan sacculus represents a rigid layer by which the form of bacterial cell wall was determined. Various molecules, such as mono and oligosaccharides and amino acids apart from peptidoglycan contained by the periplasmic space and their higher concentration results in making peri-plasmic space a gel like matrix with some holes (Holts *et al.,* 2009). The foreign molecules introduced from outside are break down by the periplasmic space therefore these act as protective barrier and are crucial for osmotic stability. In contrast the absence of outer membrane in Gram positive bacteria results in absence of membrane bound periplasm. They contain multi-layered peptidoglycan covalently substituted with anionic the anionic polymer teichoic acid or teichuronic acid. Gram positive walls possess the ability to retain large amount of proteins, lypo-glycans and cation, large water capacity (Hankok, 2002). Due to all these properties Gram positive bacteria are more permeable to antibiotics, chemotherapeutic agents as well as more vulnerable to attack of mushroom polysaccharides. The inability of the extracts to inhibit the growth of gram- negative bacteria like *P. aeruginosa* could be that the organisms possess a mechanism for detoxifying the active components (Chika *et al.,* 2007). To the best of knowledge, least studies were made to reveal that aqueous extracts prepared from mushrooms collected from Himachal Pradesh have antibiotic activity against bacteria. To assess free radical scavenging activity DPPH free radical scavenging assay is widely accepted model (Naik *et al.,* 2003). The assay avoid side reactions such as enzyme inhibition and metal ion chelation, which complicates the assay that are based on laboratory produced free radicals such as super oxide anion and hydroxyl radical (Amaroweiz *et al.,* 2004). The reaction of antioxidants to scavenge free radical of DPPH is caused by hydrogen donating ability of these compounds (Liu *et al.,* 2013). Polysaccharides isolated from mushrooms extracts have been proved for this ability, where the hydroxyl group of mono saccharaides unit can donate hydrogen to reduce DPPH radical. The patho physiology of wide range of diseases is related to reactive oxygen and nitrogen species. Oxidative damage to DNA results in triggering carcinogenesis (Ajith and

Janaradhanan, 2007). It has been suggested that the immunomodulation and antitumour activities of polysaccharides are largely related to their antioxidant properties (Russel and Patersom, 2006). The DPPH free radical scavenging assay performed in the present study is only a preliminary research tool to illustrate the anti-oxidant properties of aqueous extracts of mushrooms. Other methods are required to assess the additional type of antioxidant activity of these mushrooms extracts possess.

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