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RESEARCH ARTICLE

ANTIBACTERIAL ACTIVITY AND PHYTOCHEMICAL SCREENING OF WILD EDIBLE MUSHROOM *PLEUROTUS OSTREATUS*COLLECTED FROM HIMACHAL PRADESH.

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Abstract

Objective:-The objective of present study was to identify the unknown wild edible mushroom based on morphological characteristics and DNA based sequencing by using universal primers ITS 1and ITS 4, antibacterial activity, phytochemical screening and FTIR analysis of mushroom extract spectra.

Methods:- The wild edible mushroom specimen was tentatively identified morphologically and was cultured on 2% MEA. The isolate thus obtained was screened for its antimicrobial activity. Methanol extract was prepared in order to carry out antibacterial activity. The extract was subjected to qualitative phytochemicaland FTIR analysis.

Results:-The isolates on the basis of morphological and molecular characterization was identified as *P. ostreatus*. Agar-well diffusion method was used to carry out antimicrobial assay. The methanolextract of*P. ostreatus* showed antimicrobial activity against *E.coli* having zone inhibition diameter 24.3±0.5, against *S. aureus* having zone inhibition diameter 26.6±0.5,against*P. aeruginosa* having zone inhibition diameter20.3±0.5 and against *K.pneumoniae* having zone inhibition diameter 28.6±0.5. Fourier transform infrared (FT-IR) analysis indicated that all mushroom's extract spectra showed typical carbohydrate pattern with strong and broad absorption near 3300 cm^{-1}which indicates the presence of OH group.

Conclusion:-The isolate analysis have been showed to be good source of antimicrobial agent and phytoconstituents, therefore could be potential source of bioactive compounds with beneficial biological activities.
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Introduction:-

The growing emergence of drug resistance microorganism now a days has become a serious threat to effective treatment of infections (Klein *et al.*, 2007). Resistance to drug happens usually due to long term indiscriminate use of antimicrobial agentswhich results in acquisition of mutation in the genome and genes of disease causing microorganisms that assist their survival.(Mazodier and Davies, 1991, Gao*et al.*, 2003).Whilst it is not possible to prevent the bacterial evolution, but it is important to choose the most appropriate and reliable antibiotics and use them properly to minimize the development of drug resistant strains.(Alves*et al.*, 2012).

Mushrooms are source of nutritionally functional food as well as physiologically beneficial medicines. (Sagakami*etal.*, 1991, Wasser and Weis, 1999). The fruiting body and mycelium constitutes the compounds with a

wide range of antimicrobial activity. In mushrooms the cell wall glucans are well known for their immunomodulatory properties along with many of the externalized secondary metabolites that combat bacteria and viruses, hence are known to be rich source of antibiotics (Benedict and Brady, 1972,Kupra*et al.*, 1979,Suzuki*et al.*, 1990, Collins and Ng, 1997, Eo*et al.*, 1999, Brandt and Piraino , 2000. It is also believed that many mushrooms species consist of antimicrobial compounds that help them to protect themselves and survive in their natural environment and some of them proved to be beneficial for humans (Lindequist, 2005).The effect of mushroom's extract against pathogenic bacteria are studied in different parts of the world by a number of researchers (Gbolagade*et al.*, 2007,Turkoglu *et al.*, 2007).The active domain of present study was to investigate the antimicrobial properties of wild edible mushroom having variety of beneficial biological properties which may play important role in the human health maintenance and to search for new products having these properties.

Material and methods:-

Collection and isolation of sample:-

Mushroom fruiting body was collected from the forest of Badu Sahib,Distt, Sirmour,Himachal Pradesh and was labelled as sample no. 105. The fruiting body was brought to laboratory in a sterile plastic bag washed thoroughly with sterile distilled water and 0.001% mercuric chloride and with the aid of sterile blade it was aseptically break lengthwise exposing the inner tissue. A small piece of tissue was aseptically transferred on to the plates of Malt extract agar.

Identification of sample:-

Morphological identification

The identification of fruiting body of wild mushroom was based upon sporocarp morphology and macroscopic characteristics.

Molecular identification:-

Genomic DNA isolation and PCR amplification:-

Genomic DNA isolation was done by CTAB(Cetyltrimethyl ammonium lysis buffer) method(Zolan and Pukkila 1986). The entire region of isolate 105 was amplified by using universal primers ITS 1 and ITS 4. PCR reaction were performed in 50 µl reaction mixture containing 1µl of DNA, 1 X PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each dNTPs , 2.5 units of Taq DNA polymerase, 0.5 µM of each primers i.e., ITS1(5'TCCGGTAGGTGAACCTGCGG 3') ITS 4 (5'TCCTCCGCTTATTGATATGC 3'). The tubes were placed in a thermal cycler (Agilent sure cycler 8800) for amplification under following conditions: One cycle Initial denaturation at 94 ºC for five minutes, 35 cycles of denaturation at 94 ºC for 30 seconds , annealing at 62 ºC for 30 seconds, elongation at 72 ºC for one minute and final extension at 72 ºC for five minutes. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with ITS1 and ITS4 primers using BDT v3.1 Cycle sequencing kit on ABI 3730xl Genetic Analyser at Eurofins genomics, Bangalore.The 18S rDNA gene sequence was used to carry out BLAST with the database of NCBI gene bank database. Based on maximum identity score first ten sequences were selected and aligned using multiple alignment software program Clustal W. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4.

Antimicrobial activity:-

Source of test microorganism used:-

Four bacterial strains*Klebsiella pneumoniae* MTCC109,*Pseudomonasaeruginosa*MTCC741, *Staphylococcusaureus*MTCC 737and*E. coli*MTCC739were obtained from Microbial Type Culture Collection (MTCC) IMTECH, Chandigarh (INDIA) and stored at 4^{o}C in refrigerator and sub cultured at regular intervals of 48 h until use.

Extract Preparation:-

The mycelia grown on MEA plates were inoculated in a conical flasks having liquid medium (pH 6.6) culture containing glucose 2%, peptone 1%, and yeast extract 2%. The flasks were shaked at 150 rpm at 25°C. The culture fluid was separated from pellets by filtration after 7 days of fermentation and then extract three times with methanol solution. Themethanol fractions was evaporated and the extracted material was dissolved in DMSO (Merck). All extracts and fractions from mycelial cultures were stored at 4°C until use for assaying their activities (Rojas *et al.*, 2006).

Antibacterial activity:-

Muller Hinton agar plates with 4% NaCl supplementation were prepared. Sterilized swabs were dipped in standardized bacterial suspension with inoculums size of 1.5 X 10^8cfu/ml prepared above and excess culture was removed by turning the swab against the side of the tube. Inoculum was spread evenly over the entire surface of Muller Hinton agar plates. These plates were allowed to dry for at least 15 min and thenwell (7mm diameter) were made on petri dish using sterile cork borer. About 25µl extract was introduced into bore agar wells 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 for antibacterial activities of extracts after 24 hours of incubation at 37°C.Antimicrobial activity was determined by measuring the diameter zone of inhibition in mm(Moshi *et al.,* 2006).

Preliminary phytochemical screening:-
The freshly prepared extract was subjected to phytochemical analysis in order to ensure the presence of phytochemical constituents.

Test for alkaloids:-
Mayer's test:-5ml of extract was taken and few drops of Mayer's reagent was added by the side of test tube. White creamy precipitate were observed indicating positive results.

Wagner's test:-5ml of extract was taken and few drops of Wagner's reagent was added by the side of test tube. Reddish brown precipitate appeared confirming the test as positive.

Test for Carbohydrates:-
Molisch's test:-5ml of extract was mixed with two drop of alcoholic solution of α napthol and shaked well. 1 ml of concentrated H_2SO_4 was added along the side of the test tube and allowed to stand. The violet ring appeared indicating the presence of carbohydrates.

Benedict's test:-To the 3ml of extract 1 ml of Benedict's reagent was added. The mixture was heated on boiling water bath for two minutes. The characteristics colour precipitate was observed confirming the presence of sugar.

Test for detection of phenolic compounds and tannin:-
Ferric chloride test:-Few drops of neutral ferric chloride was added to 5ml of extract. The dark green colour was appeared.

Lead acetate test:-To the 5ml of extract 4 ml of 10 % lead acetate was added. Bulky white precipitate was appeared confirming the presence of phenolic compounds.

Test for flavonoids:-
Alkaline reagent test:-Few drops of NaOH was added to test solution intense colour was formed which turn colourless on the addition of dilute acid confirming the presence of flavonoids.

Fourier transform infrared spectroscopy (FTIR):-
FTIR spectroscopy of the samples was tested by using FTIR spectrometer (Aligent Cary- 630) (Grosve*et al.,* 2001). 10 mg of sample was put on mirror stage of spectrometer and spectrum was obtained by tightening the lens over sample. After analysis the lens was cleaned to remove the sample completely (Jouraiphy*et al.,* 2008).

Results:-

Identification of sample:-
Morphological characterization:-
Cap: 4-10 cm; convex, becoming flat or somewhat depressed; kidney-shaped.,pale brown to dark brown in colourhaving in-rolled margins.
Gills: Running down the stem; close; with a gray tinge.
Stem: Usually rudimentary and lateral.
Flesh: Thick; white.
Spores 6-9 x 3.5-5 µ; smooth; cylindrical. Mycelia are septate and consist of clamp connections as shown in Fig 1.
Molecular identification:-

In the present study genomic DNA was isolated from mycelial culture of isolate and was amplified with ITS 1 and ITS 4 primers. PCR products obtained from couple of primers ITS1 and ITS 4 provided a single band at 500 bp. The sequences for sample was recorded and shown in table 1. (http//blast.ncbi/nlm/.nih.gov/Blast.cgi), sequence of sample was homologous with partial sequence of 5.8 S ribosomal RNA gene, internal transcribed spacer 2 complete sequence and 28S ribosomal gene partial sequence of species.*Pleurotus ostreatus* accession number HM 770898.1, GU722280.1, KR908742.1, KF724525.1, KJ010873.1. The aligned nucleodite sequence of sample was submitted to NCBI and nucleotide sequence is provided with gene bank accession no. KU 892064.The phylogenetic tree constructed (Fig 2) also revealed that the sample 105 was classified in Kingdom Fungi, division Basidiomycota, Class Agaricomycetes, Order Agaricales, Family Pleurotaceae, Genus *Pleurotus* and Species *Pleurotus ostreatus*.

Antibacterial activity:-

Screening for antibacterial activity was done by agar well diffusion method. Results of antibacterial activity was shown in Table 2. Methanol was used as solvent for the preparation of extract and finally dissolved in DMSO. The extract showed varying degree of inhibition on test microorganisms taken for screening. It was observed from the results that extract was highly effective against *K. pneumoniae*having zone inhibition diameter 28.6±0.5 followed by *S. aureus*26.6±0.5,*E. coli*24.3±0.5 and *P. aeruginosa* 20.3±0.5 respectively.

Preliminary phytoconstituents analysis:-

The preliminary phytoconstituents analysis of *P. ostreatus* revealed that the extract contain alkaloid, carbohydrates, flavonoids, Phenolic compounds and Tannins as shown in Table3.

Fourier transform infrared spectroscopy (FTIR):-

An infrared spectrum of samples was recorded with an FT-IR spectrometer (Agilent technologies) in the range of 4000-800 cm^{-1}. The infrared spectrum helps to evaluate the vibrations of molecule and polar bonds present between the atoms of sample molecules. As shown in Figure 3 and table 4.The mushroom extract spectra showed typical carbohydrate pattern with strong and broad absorption near 3264 cm^{-1}, which indicates the presence of OH groups. Other regular features was also identified in FT-IR spectra of extract. The peaks at 2921 cm^{-1} in sample 105, are interpreted due to CH_2 asymmetric stretch/ symmetric stretch. The signal at 1547cm^{-1} is indicative of Secondary amine N-H bending. The region around 1208 and 1156 cm^{-1} in sample is dominated by sugar ring vibrations overlapping with stretching vibration of (C-OH) side group and (C-O-C) glycosidic bond vibration. The peak near 1374 cm^{-1} corresponds to C-H in plane binding vibrations.

Discussion:-

Resistance to antibiotic among bacteria has been increasing drastically, therefore nutraceuticals and drugs having antibacterial activities are needed. Microorganisms such as *S. aureus*, *K. pneumoniae*, *E. coli*, *P. aeruginosa* are examples of resistance to antibiotics now a days. (Alves*et al.,* 2005) 14 mushroom isolates with higher antimicrobial activity against target microorganism was detected by Rosa *et al.,* in2003.In another study by Zjawiony, 2004 it was observed that about 75% of polypore fungi showed strong antimicrobial activity.In the present study the extract showed good antimicrobial activity against all the pathogenic bacteria taken which are similar to the results reported from extracts prepared from *P. ervngii*, *P. ostreatus*, *P. sajor-caju*, *L.squarrosulus* and *Agaricusbisporus*against *K. pneumoniae* and *S. aureus*. (Alves*et al.,* 2012). Yamac and Bilgili (2006) in his study found that mycelial extract of many fungi possess high inhibitory activity against gram negative bacteriaour results are in collaboration with this finding. The mycelial extract was found to have good inhibitory effect against *E. coli*, *P. aeruginosa* in the present study.

Phytochemical analysis of *P. ostreatus* has revealed that it consist of alkaloids, carbohydrates, flavonoids, phenolic compounds and tannins. The results are in accordance to the previous literature reported(Okwulehie and Ogoke, 2013). Inorder to investigate the molecular properties of extract of mushroom and to evaluate the co- relation between their structural characteristics and bioactivity in the present study FTIR spectra technique was used. The peak at 2925 is due to H-C-H stretching of alkane (Kozarskiet al., 2012). The 1200-1000 cm^{-1}region is dominated by sugar ring vibration overlapping with stretching vibration of (C-OH) side groups. The peaks near 1374cm^{-1}corresponds to C-H in plane bending vibration and at 1080$cm^{-1}$the stretching band is curved by the signal of pyranose form of the O substitute residue (C-O)(Mathlouthi and Koenig, 1986,He*et al.,* 2012). The band near 1030 cm^{-1} are indicative of α –linkage which correlates the study ofKozarski*et al.,* 2012. The extract therefore considering the criteria that it consist of both α and βglycosidic conformations.

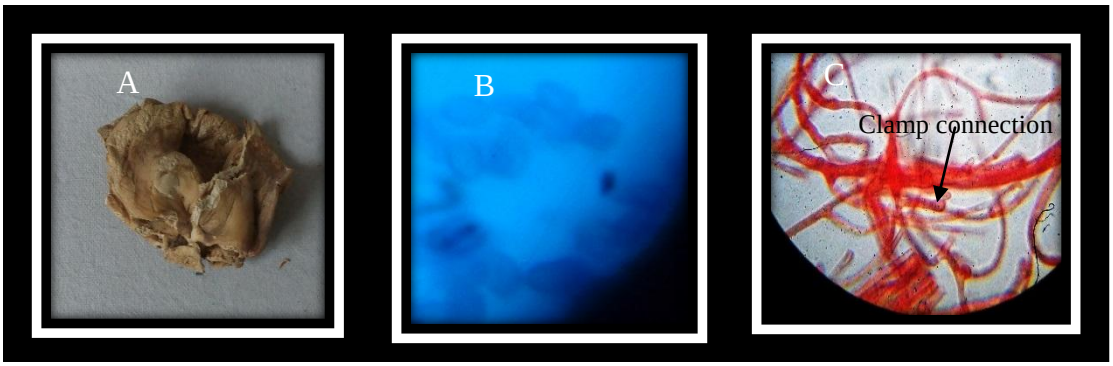

Fig 1: A) Fruit body of sample B) Endospore structure C) Mycelial strand.

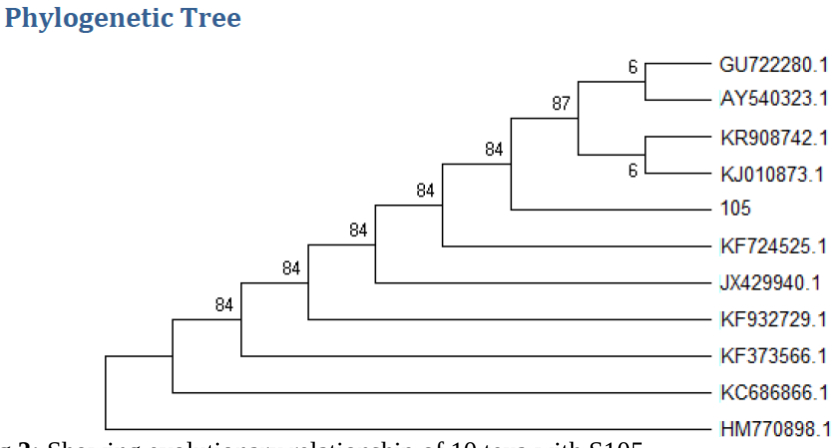

Fig 2: Showing evolutionary relationship of 10 texa with S105.

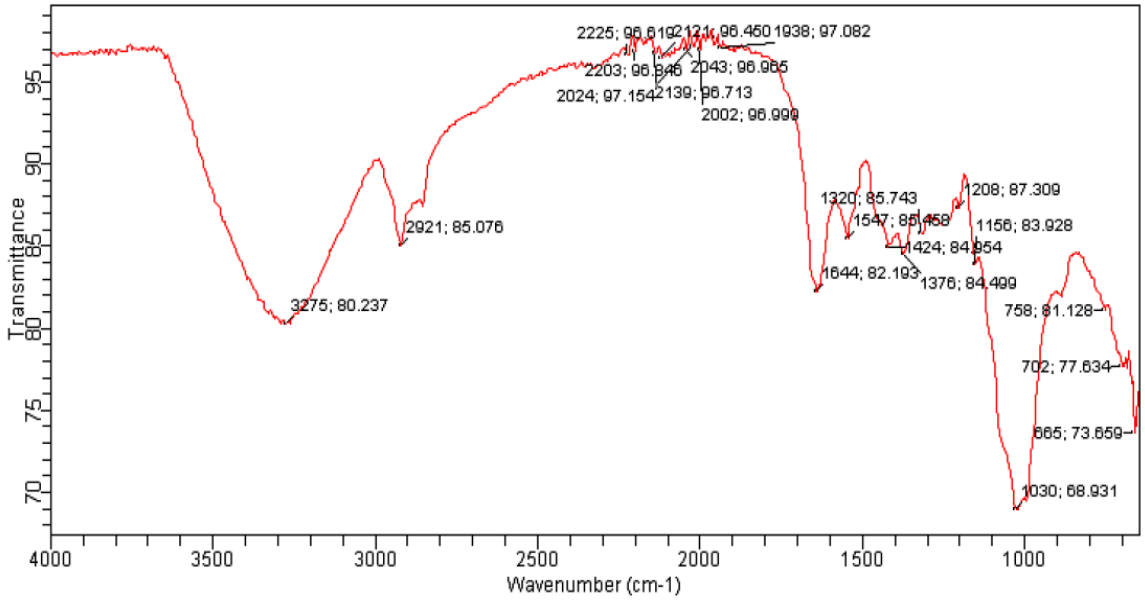

Fig 3: FT-IR spectrum of crude extract of *P. ostreatus*.

Table 1: Nucleotide sequence of wild edible mushroom.

P. ostreatus	GCTTTATCGTTCGTAGTGACCTGCGGAGGATCATTAATGAATTCACTATGGAGTT GTTGCTGGCCTCTAGGGGCATGTGCACGCTTCACTAGTCTTTCAACCACCTGTGA ACTTTTGATAGATCTGTGAAGTCGTCTCTCAAGTCGTCAGACTTGGTTGCTGGGA TTTAAACGTCTCGGTGTGACTACGCAGTCTATTTACTTACACACCCCAAATGTAT GTCTACGAATGTCATTTAATGGGCCTTGTGCCTATAAACCATAATACAACTTTCA ACAACGGATCTCTTGGCTCTCGCATCGATGAAGAACGCAGCGAAATGCGATAAG TAATGTGAATTGCAGAATTCAGTGAATCATCCGAATCTTTGAACGCACCTTGCG CCCCTTGGTATTCCGAGGGGCATGCCTGTTTGAGTGTCATTAAATTCTCAAACTC ACTTTGGTTTCTTTCCAATTGTGATGTTTGGATTGTTGGGGGCTGCTGGCCTTGA CAGGTCGGCTCCTCTTAAATGCATTAGCAGGACTTCTCATTGCCTCTGCGCATGA TGTGATAATTATCACTCATCAATAGCACGCATGAATAGAGTCCAGCTCTCTAAT CGTCCGCAAGGACAATTTGACAATTGACCTCAA

Table 2: Zone inhibition of methanolic extract of P.ostreatus against pathogenic bacteria.

Antibacterial activity of Methanol extract Zone inhibition diameter in mm			
Microorganisms	Extract	DMSO (-ve control)	Ciprofloxacin (+ve control)
E. coli	24.3±0.5	-	21.5±0.5
S.aureus	26.6±0.5	-	28.6±0.5
P. aeruginosa	20.3±0.5	-	22.3±0.5
K. pneumoniae	28.6±0.5	-	30.6±0.5

Each value is expressed as mean±SD of three replicates, - indicates no activity

Table 3. Phytochemical analysis of edible mushroom.

Phytochemicals	Test	Observation
Alkaloids	Mayer's test	+
	Wagner's test	+
Carbohydrates	Molisch's test	+
	Benedict's	+
Phenolic compounds and Tannin	Ferric chloride test	+
	Lead acetate test	+
Flavonoids	Alkaline reagent test	+

Table 4: Infrared spectroscopy correlation table.

Pleurotus ostreatus		
1.	3275	Hydroxyl group (O-H stretching)
2	2921	(H-C-H asymmetric stretch/ symmetric stretch) Alkane group
3	1547	Secondary amines (N-H bending)
4	1030	α –linkage
5	1156	(C-O-C) glycosidic bond vibration
6	1208	sugar ring vibrations overlapping with stretching vibration of (C-OH) side group
7.	1374	C-H in plane binding vibrations.
8.	1320	O-H group of phenolic compound
9.	1644	Amide bandindicating presence of some residual protein

Conclusion:-

The methanol extract of *P. ostreatus*found to have good antimicrobial activity against gram positive and gram negative bacteria and being a polar solvent it was found to extract maximum phytoconstituents. The molecular properties and compounds detected by FTIR analysis warrant investigation for the compounds having potential to improve human health, and application as dietary supplements.

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Reference:-

1. Alves, M.J.,Ferreira,I.C.F.R., Martins, A., Pintado, M. (2012): Antimicrobial activity of wild mushroom extracts against clinical isolatesresistanttodifferentantibiotics. Journal of Applied Microbiology., 113: 466–476.
2. Benedict, R. G. and Brady, L. R. (1972):Antimicrobial activity of mushroom metabolites. J Pharmacol Sci., 61:1820–1822.
3. Brandt, C. R. and Piraino, F. (2000): Recent Res DevAntimicrob AgentsChemother., 4:11–26.
4. Collins, R. A. and Ng, T. B. (1997): Polysaccharopeptide from *Coriolus versicolor*has potential for use against Human immunodeficiency virus type I infection.Life Sci., 60:383–387.
5. Eo, S. K., Kim, Y. S., Lee, C. K., Han, S. S. (1999): Antiviral activities of various water and methanol soluble substances isolated from *Ganodermalucidum*. J. Ethnopharmacol., 68:129–136.
6. Gao, Y., H.,Zhou,S.,Huang,M.,Xu,A. (2003): Antibacterialand antiviralvalueofthegenus *Ganoderma*P.Karst.Species:Areview. International Journalof Medicinal Mushrooms., 5: 235–246.
7. Gbolagade, J., Kigigha, L.,Ohimain, E.(2007): Antagonistic effects of extracts of some Nigerian higher fungi against selected pathogenic microorganisms. Am-Eurasian J Agric Environ Sci., 2: 364–368
8. He, J. Z.,Ru,Q. M.,Dong, D. D., Sun, P. L.(2012): Chemical characteristicsandantioxidantpropertiesofcrudewater soluble polysaccharidesfromfourcommonedible mushrooms. Molecules, 17: 4373–4387.
9. Jouraiphy,A., Amir,S.,Winterton,P.,ElGharous,M.,Revel, J. C., Hafidi, M. (2008): Structural study of the fulvic fraction during composting of activated sludge–plant matter: Elemental analysis,FTIRand13CNMR. Bio-resource Technology, 99: 1066–1072.
10. Klein,E.,Smith,D. L., Laxminarayan, R. (2007): Hospitalizations and deathscausedbymethicillin-resistant *Staphylococcus aureus*, UnitedStates,1999–2005. Emerging InfectiousDiseases., 13: 1840–1846.
11. Kozarski,M.,Klaus,A.,Niksic, M., Vrvic, M.M.,Todorovic, N., Jakovljevic, D.(2012): Antioxidativeactivitiesand chemical characterizationofpolysaccharideextractsfromthe widely usedmushrooms *Ganodermaapplanatum*, *Ganodermalucidum*, *Lentinusedodes* and *Trametes versicolor*. Journal ofFood Composition andAnalysis., 26: 144–153.
12. Kupra, J., Anke, T., Oberwinkler, G., Schramn, G., Steglich, W. (1979): J Antibiotics 32:130–135
13. Lindequist, U. (2005): The pharmacological potential of mushrooms. Evidence-Based Complementary and Alternative Medicine., 2: 285-299.
14. Mathlouthi, M. and Koenig,J.L. (1986): Vibrational spectra of carbohydrates. AdvancesinCarbohydrateChemistryand Biochemistry., 44: 7–89.
15. Mazodier, P. and Davies, J.(1991): Genetransferbetweendistantly related bacteria. Annual Reviewof Genetics., 25: 147–171.
16. Moshi, M.J., Mbwambo, Z.H., Kapingu, M.C., Mhozya, V. H. and Marwa, C. (2006): Antimicrobial and Brine Shrimp Lethality of extracts of *Terminaliamollis*Laws. Afr. J. Trad. CAM., 3: 1-10.
17. Okwulehie, I. C. and Ogoke, J. A. (2013). Bioactive , nutritional and heavy metal constituents some edible mushrooms found in Abia State of Nigeria. International Journal of Applied Microbiology and Biotechnology research., 1:7-15.
18. Rojas, J.J., Ochoa, V.J., Ocampo, S.A., Munoz, J. F. (2006): Screening for antimicrobial activity of ten medicinal plants used in Colombian folkloric medicine: A possible alternative in the treatment of non-nosocomial infections. BMC Complement. Altern. Med., 6: 2.
19. Rosa, L. E., Machado, K. M. G., Jacob, C. C., Capelari, M. Rosa, C,.A., Zani, C. L. (2003): Screening of Brazilian Basidiomycetes for antimicrobial activity. Memórias do Instituto Oswald Cruz., 98: 967-974.
20. Sagakami, H., Aohi, T., Simpson, A., Tanuma, S. (1991): AnticancerRes 11:993–1000some mushrooms isolates. Pharmaceutical biology. 44, 2006, 660-667.

21. Suzuki, H., Iiyama, K., Yoshida, O., Yamazaki, S., Yamamoto, N., (1990): Structural characterization of the immune active and antiviral water solubilized lignin in an extract of the culture medium of *Lentinusedodes*, mycelia., AgricBiolChem 54:479–487
22. Turkoglu, A., Duru, M.E., Mercan, N., Kivrak, I., Gezer, K. (2007). Antioxidant and antimicrobial activities of *Laetiporussulphureus* (Bull.) Murill. Food Chem., 101: 267-273.
23. Wasser, S. P., Weis, A. L. (1999):Medicinal properties of substances occurring in higher Basidiomycetes mushrooms., Int J Med Mushrooms 1:31–62.
24. Yamac, M. and Bilgili, F.(2006): Antimicrobial activity of fruit bodies of some mushrooms isolates.Pharmaceutical biology., 44: 660-667.
25. Zjawiony, J. K. (2004). Biologically active compounds from Aphyllophorales (polypore) fungi. Journal of Natural Products., 67: 300-310.
26. Zolan, M. E. and Pukkila, P.J. (1986): Inheritance of DNA methylation in *Coprinuscinereus*. Mol Cell Biol.,6: 195-200.