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Antimicrobial Evaluation of Different Wild and Commercial Mushroom Species

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Antimicrobial potential of different mushrooms extracts sequentially isolated by *n*- hexane, dichloromethane, ethyl acetate and water from four cultivated, *Pleurotus ostreatus*, *Lentinus edodes*, *Volvoriella volvacea*, *Hericeium erinaceus* and one wild *Ganoderma lucidum* were evaluated for their ability to inhibit the growth of different microbial species. Antimicrobial activity was assayed using disc diffusion and micro dilution methods. Antimicrobial potential was screened against two Gram negative bacterial species (*Escherichia coli, Pasturelaa multocida*) two Gram positive (*Bacillus subtilis, Staphylococcus aureus*) and four fungal species (*Aspergillus niger, Aspergillus flavus, Fusarium solani, Helmenthosporium mydis*), all the fractions possessed considerable antimicrobial activity, the fractions of *V. volvacea* and *H. erinaceus* were more effective against tested microbial species and can be compared favorably with positive controls (Rifampicin and fluconazole). Ethyl acetate and water fractions of *V. volvacea* and *H. erinaceus* showed zone of inhibition 15 ± 0.4 mm and 16 ± 0.6 mm against *P. Multocida* and *B. subtilis* respectively with minimum inhibitory concentration values of 294 and 255 ug/mL respectively. The maximum antifungal was again observed in *H. erinaceus* and *V. volvacea.* The fractions were more effective against *A. flavus* as compare to other fungal species with maximum zone of inhibition ranging from 9 ± 0.5-14 ± 0.9 mm and MIC values 302 ± 0.53-660 ± 0.73 ug/mL. In conclusion, the analyzed mushrooms extract are promising source of antimicrobial agents.

Key words: Mushrooms, Antimicrobial activity. *Pleurotus ostreatus, Volvoriella volvacea Lentinus edodes.*

Edible mushrooms are considered to be beneficial in improving the nutritive condition of diet whereas medicinal use of mushrooms goes back to very old times having long standing past of use in traditional ancient therapies (K1vrak *et al*., 2014). In developing countries infectious diseases are major threat for health problems. Several antimicrobial agents have been synthesized but random use of these antimicrobial drugs lead to the development of antibiotic resistance (Modi *et al*., 2014). Long term misuse of antibiotics causes drug resistance and as a consequence mutation in bacterial genome and genes helps in bacterial survival, drug resistant bacteria are serious threat to the effective treatment of infections (Klein *et al*., 2007). The application of antibiotics in animal feed has been reduced to avoid new antibiotic resistant strains. So, there is a need for noval antimicrobial compounds to beat bacteria and other pathogens (Ren *et al*., 2014). Numerous antibiotics

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such as Penicillin and griseofulvin have been isolated from microfungi. The diversity in fungal species offers a potential source of new antibiotics (Yamac and Bilgili, 2006). Such approach may be used to examine the antimicrobial properties of some exotic mushrooms as novel source of antimicrobial agents as well as the employment of these novel agents, and thus limit the use of conventional antibiotics to cases of life threatening infections, thus minimizing the development of resistant to such agents (Hearst *et al*., 2009). Mushrooms also possess phytochemicals having antimicrobial activity and can also be applied for food and medicinal purposes (Smolskait *et al*., 2014).

Natural products with biological potential are usually present in plants, mushrooms and some other sources, therefore the extraction techniques are important for the selection of substances or group of compounds of interest. Therefore the aim of this study was to investigate the antimicrobial activity of different fractions obtained from ethanolic extracts of selected mushrooms.

MATERIAL AND METHODS

Sample Collection and Preparation

The raw material used in this work consisted of mushrooms collected from different habitats and taxonomic identification was made from Mushroom Lab, Institute of Horticultural Sciences, University of Agriculture Faisalabad, Pakistan. The collected mushrooms were shade dried and cut in to small pieces. The specimen of each species was grounded in a domestic blender immediately, reduced to fine dried powder and stored at 4 ^{o}C before the extractions.

Classical organic solvent extraction (COSE)

Different solvents, *n*-hexane (Hx), dichloromethane (DCM) and ethyl acetate (EtAc), in ascending polarity of 0, 3.1 and 4.4, were used to fractionate the soluble compounds from selected mushrooms (Mahjoor, 2005). The COSE method used to obtain the extracts consist in a cold maceration of the mushrooms to avoid thermal degradation. The extraction was performed with dried mushrooms powder (100 g) placed in ethanol for six days. The resulting extract was evaporated at reduced pressure up to 10% of the initial volume to obtain the crude extract (CE), the ethanolic fraction. Then, the CE was partitioned with *n*-hexane, dichloromethane, ethyl acetate and water using 60 mL each. The organic solvents used were 99% pure (Sigma-Aldrich, USA) (Kitzberger *et al*., 2007).

Antimicrobial activity

The mushrooms fractions obtained with COSE were submitted to evaluation of antimicrobial activity with the bacteria strains: *Escherichia coli*, *Staphylococcus aureus*, *Bacillus subtilis* and *Pasteurella multocida*. The cultures were incubated at 37 ^{o}C for 24 h and then diluted in culture broth to contain 10^6 CFU/mL. Agar Mueller–Hinton and culture broth were used for the bacterial growing. All bacterial cultures were incubated in aerobic conditions.

The fungal strains: *Aspergillus niger*, *Aspergillus flavus*, *Fusarium solani* and *helmmenthosporium myedis* were also challenged in this study to ascertain the antimicrobial activity of mushrooms fractions. The cultures were incubated at 28 ^{o}C for 48 h and then diluted in culture broth to contain 10^6 CFU/mL. Agar potato–dextrose and culture broth were used for the fungal growing. All fungal cultures were incubated in aerobic conditions (Sharif *et al*., 2013).

Disk diffusion method

The agar diffusion method was performed using filter paper disks of size 10 mm for each bacterial suspension (10^6 CFU/mL) and inoculated in plates, where the bacteria' were spread uniformly on the agar surface. The disks were aseptically filled with the various mushrooms fractions. The fractions were used in the concentration of 10 mg extract/mL of DMSO (Dimethylsulphoxide) because DMSO does not offer inhibition to the microorganism growth. The plates were incubated at 37 ^{o}C for 24 h and next, examined to verify the inhibition. A positive result was defined as an inhibition zone around the disks, therefore indicating the presence of antibacterial substance in the extracts tested (Zubair *et al*., 2013).

Minimum inhibition concentration (MIC)

The antimicrobial activity of the extracts was evaluated through the determination of the minimum inhibition concentration (MIC) by the microdilution method in culture broth. The mushrooms fractions that present inhibition zone in the disk diffusion method were dissolved in 200 uL of DMSO and the solution added to 1800 uL of Muller–Hinton broth for the bacteria growth and

nutritive broth for fungi. Plates were prepared under aseptic condition. A sterile 96 well plate was labeled. A volume of 100 uL of test material was pipetted into the first row of the plate. To all other wells 50 uL of nutrient broth was added. Serial dilutions were performed. Tips were discarded after use such that each well had 50 uL of the test material in serially descending concentration. To each well 10 uL of resazurin indicator solution was added. Finally, 10 uL of bacterial suspension ($5x10^6$ CFU/mL) to achieve a concentration of $5x10^5$ CFU/mL. Each plate had a set of control: A column with a broad spectrum antibiotic as positive control. A column with all solution except test compound and a column with all solutions except of bacterial solution adding 10 uL of broth instead. Each plate was wrapped to ensure that bacteria did not become dehydrated. The plates were then incubated at 28 ^oC for fungus for 48 hours and 37 ^oC for bacteria for 24 hours. The absorbance was measured at 620 nm by micro quant for fungus and at 500 nm for bacteria. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at witch color changes occur was taken as MIC value. The results were expressed in ug/mL (Tanveer *et al*., 2013).

RESULTS AND DISCUSSION

Antibiotic resistance among bacteria increasing day by day, nutraceuticals with antibacterial activities is needed. Mushrooms could be an alternative source of antibacterial drugs. Keeping this in mind this study was conducted to examine the antimicrobial potential of some selected wild and commercial mushrooms. Preliminary screening of antimicrobial properties of mushrooms fractions was tested against four bacterial species *E. cloi, P. moltucida, B. subtilis and S. aureus* and four fungal species *A. niger, A. flavus, F. solani* and *H. mydis*. Our data quantitatively showed that the mushrooms had moderate antimicrobial activity against these selected microorganisms tested on. The mushrooms fractions gave zone of inhibition ranging from 9-16 mm against selected bacteria.

Table 1. Antibacterial activity of the mushrooms extracts against the selected bacterial strains by disc diffusion method

Mushrooms	Solvents	*E. coli*	*P. multocida*	*B. subtilis*	*S. aureus*
L. edodes	*n*-hexane	0	11 ± 0.5	12 ± 0.5	0
	Dichloromethane	10 ± 1.3	10 ± 0.3	0	12 ± 0.6
	Ethyl acetate	11 ± 1.0	13 ± 0.2	10 ± 0.9	10 ± 0.5
	Water	11± 0.3	12 ± 0.3	9 ± 0.4	0
P. ostreatus	*n*-hexane	14 ± 1.2	0	0	12 ± 0.5
	Dichloromethane	0	10 ± 0.7	14 ± 0.5	13 ± 0.7
	Ethyl acetate	14 ± 0.6	15 ± 1.0	14 ± 1.0	11 ± 0.3
	Water	14 ± 1.0	11 ± 0.9	0	12 ± 0.5
H. erinaceus	*n*-hexane	12 ± 0.7	14 ± 0.7	0	0
	Dichloromethane	10 ± 0.5	13 ± 0.8	12 ± 0.3	10 ± 0.5
	Ethyl acetate	14 ± 0.3	16 ± 1.0	15 ± 0.3	11 ± 1.0
	Water	10 ± 0.4	11 ± 0.5	16 ± 0.9	10 ± 0.5
V. volvacea	*n*-hexane	11 ± 0.6	0	12 ± 1.0	10 ± 0.4
	Dichloromethane	13 ± 0.4	14 ± 1.3	0	12 ± 1.04
	Ethyl acetate	14 ± 0.8	16 ± 0.5	12 ± 0.4	14 ± 0.7
	Water	12± 0.7	10 ± 0.4	12 ± 0.6	14 ± 0.9
G. lucidum	*n*-hexane	11 ± 0.3	0	12 ±0.5	0
	Dichloromethane	0	13 ± 0.6	14 ± 1.0	10 ± 1.1
	Ethyl acetate	14 ± 0.8	12 ± 0.5	11 ±0.3	12 ± 0.4
	Water	12 ± 0.2	0	11± 1.0	0
	Rifampicin	24 ± 0.6	22 ± 0.5	26 ± 1.0	20 ± 0.5

Results are expressed as a means ± standard deviation (n=3). The values are diameter (mm) of inhibition zone. 0 mm = reflecting no inhibition of growth. The maximum zone of inhibition was observed for ethyl acetate fractions against *p. multocida* and *B. subtilis*.

Table 2. Antifungal activity of the mushrooms extracts against panel of fungal species using disc diffusion method

Mushrooms	Fractions	*A. niger*	*A. flavus*	*F. solani*	*H. mydis*
L. edodes	*n*-hexane	10 ± 0.8	10 ± 0.5	0	10 ± 0.7
	Dichloromethane	12 ± 0.9	12 ± 0.6	12 ± 1.2	0
	Ethyl acetate	0	14 ± 0.6	11 ± 0.9	12 ± 0.9
	Water	9 ± 0.6	9 ± 0.5	10 ± 0.8	0
P. ostreatus	*n*-hexane	12 ± 0.8	0	14 ± 0.5	13 ± 0.3
	Dichloromethane	10 ± 0.4	12 ± 1.0	0	0
	Ethyl acetate	9 ± 0.3	11 ± 0.2	12 ± 0.7	8 ± 0.6
	Water	0	12 ± 0.3	9 ±0.2	10 ± 0.5
H. erinaceus	*n*-hexane	13 ± 1.0	0	12 ± 0.3	10 ± 0.1
	Dichloromethane	14 ± 0.9	13 ± 0.9	10 ± 1.0	11 ± 0.4
	Ethyl acetate	12 ± 0.6	12 ± 0.8	13 ± 1.2	16 ± 0.5
	Water	11 ± 0.1	0	14 ± 0.8	12 ± 0.2
V. volvacea	*n*-hexane	13 ± 0.4	0	12 ±0.4	0
	Dichloromethane	10 ± 1.0	14 ± 1.0	12 ± 0.2	11 ± 0.7
	Ethyl acetate	14±0.63	12 ± 0.3	9 ± 0.4	14 ± 0.5
	Water	0	0	14 ± 1.3	12 ± 0.5
G. lucidum	*n*-hexane	11 ±0.4	9 ±0.5	12 ±0.32	0
	Dichloromethane	0	9 ±0.26	0	0
	Ethyl acetate	8 ±0.4	11 ±1.1	0	10 ±0.42
	Water	0	11 ±0.6	12 ±0.7	0
	Fluconazole	22±0.26	24±0.34	24±1.0	26±0.61

Results are expressed as a means ± standard deviation (n=3). The values are diameter (mm) of inhibition zone. 0 mm = reflecting no inhibition of growth. Maximum zone of inhibition was observed for water and ethyl acetate fractions of *H. erinaceus* and *V. volvacea* against *H. mydis* and *F. solani*

Table 3. Minimum inhibitory concentration of mushrooms fractions against bacterial species (ug/mL)

Mushrooms	Solvents	*E. coli*	*P. multocida*	*B. subtilis*	*S. aureus*
L. edodes	*n*-hexane	0	348±0.74	330±0.44	0
	Dichloromethane	526±0.26	527±0.36	0	330±0.46
	Ethyl acetate	348±0.71	317±1.1	526±0.86	526±0.33
	Water	0	330±0.46	580±0.48	
P. ostreatus	*n*-hexane	307±0.46	0	0	332±1.2
	Dichloromethane	0	526±0.47	305±0.47	317±0.56
	Ethyl acetate	295±0.86	302±0.53	306±0.71	348±0.43
	Water	346±0.46	349±0.26	0	330±0.92
H. erinaceus	*n*-hexane	330±1.2	306±0.71	0	0
	Dichloromethane	525±0.39	318±0.9	332±0.58	550±0.48
	Ethyl acetate	306±0.9	296±1.6	301±1.4	348±0.68
	Water	546±0.83	524±0.70	294±0.27	546±0.83
V. volvacea	*n*-hexane	544±0.67	0	348±1.05	526±0.34
	Dichloromethane	342±0.48	306±0.41	0	525±0.40
	Ethyl acetate	306±0.72	296±0.8	526±0.43	306±0.78
	Water	524± 0.3	548±0.56	520±0.90	308±0.93
G. lucidum	*n*-hexane	348±1.03	0	526±0.56	0
	Dichloromethane	0	352±0.67	306±0.43	548±0.51
	Ethyl acetate	306±0.64	526±0.49	548±0.90	526±0.42
	Water	526±0.80	0	548±0.41	0
	Rifampicin	112±0.47	196±0.27	84±0.54	201±0.46

Results are expressed as a means ± standard deviation: least values of the MIC's were observed for ethyl acetate and water fractions of *H. erinaceus, V. volvacea* against *P. mulocida* and *B. subtilis*.

The maximum activity was observed in ethyl acetate and water fractions 16 mm against *P. multocida* and *S. aureus* as compared to dichloromethane and *n*-hexane fractions. The order of antibacterial activity of different extracts was that ethyl acetate> water> dichloromethane> *n*. hexane. The significant activity was shown by the fractions against *P. multocida* followed by *S. aureus, E. coli* and *B. subtilis*. The *n*-hexane fractions showed the weak inhibitory activity for all selected microbial species. When these fractions were tested against fungal species also showed moderate activity, the zone of inhibition was in the range 9-14 mm. The maximum antifungal activity was observed by ethylacetate fractions of *H. erinaceus* and *P. ostreatus* against *F. solani,* poor activity was observed against *A. flavus* and *A. niger.* Overall, the least antimicrobial activity was shown by dichloromethane and n- hexane fractions against all selected microbes. Refampicin and Fluconazol were used as positive control for bacterial and fungal species respectively. Comparing mushrooms species. Wild *G. lucidum* showed very low activity as compared to the commercial mushrooms *P. ostreatus, L. edodes, H. erinaceus, V. volvacea*. There was considerable difference between them. The order of antimicrobial activity of selected musrooms was that *H. ernaceus> V. volvacea > P. ostreatus > G. lucidum*. Our results can be compared with the Hearst *et al.,* (2009), they tested aqueous extracts qualitatively against 29 bacterial and 10 fungal species, and it showed *L.edodes* extracts had extensive antimicrobial activity against 85% of the organisms in the trial. Smolskait *et al*. (2014) screened antimicrobial properties of mushrooms extracts sequentially isolated by cyclohexane dichloromethane, methanol and water from eight mushrooms species. It was observed that the fractions isolated from *I. hispidus* possessed antimicrobial activity against two bacterial and one yeast species, the fractions of *P. schweinitzii* inhibited tested microorganism, methanol fractions showed the largest zone of inhibition ranging from 15-17 mm against *P. aeruginosa and B. cereus.* Nowacka *et al.* (2014) investigated the antimicrobial potential of 19 polished wild edible mushrooms; the results showed the mushrooms extracts

Table 4. Minimum inhibitory concentration of mushrooms fractions against fungal species (ug/mL)

Mushrooms	Fractions	*A. niger*	*A. flavus*	*F. solani*	*H. mydis*
L. edodes	*n*-hexane	526±0.71	526±0.56	0	548±0.67
	Dichloromethane	548±0.37	526±0.44	486±0.73	0
	Ethyl acetate	0	308±0.58	302±0.6	526±0.53
	Water	580±1.4	546±0.74	525±0.74	0
P. ostreatus	*n*-hexane	524±1.1	0	346±0.38	317±0.45
	Dichloromethane	548±0.49	524±1.2	0	0
	Ethyl acetate	580±0.81	480±0.49	520±0.52	660±0.73
	Water	0	330±0.84	580±0.36	548±0.48
H. erinaceus	*n*-hexane	306±0.36	0	526±0.9	306±0.58
	Dichloromethane	306±0.48	302±1.0	548±1.2	482±0.83
	Ethyl acetate	306±0.63	306±0.45	480±0.41	295±0.41
	Water	480±0.46	0	306±0.53	520±0.92
V. volvacea	*n*-hexane	308±0.82	0	520±0.81	0
	Dichloromethane	542±0.76	306±0.9	330±0.38	347±0.74
	Ethyl acetate	348±0.63	330±0.84	580±0.61	307±0.43
	Water	0	0	307±0.35	327±0.46
G. lucidum	*n*-hexane	348±0.91	580±0.6	329±0.54	0
	Dichloromethane	0	578±0.94	0	0
	Ethyl acetate	626±0.87	346±0.76	0	520±0.63
	Water	0	416±0.83	346±0.46	0
	Fluconazole	190±0.34	174±0.45	112±0.34	96±0.47

Results are expressed as a means ± standard deviation: least values of the MIC's were observed for ethyl acetate and water fractions of *H. erinaceus, V. volvacea* and *L. edodes* against *A. flavus* and *F. solani*

showed the moderate antimicrobial activity, *B. subtilis* and *P. aeruginosa* were quite sensitive to mushrooms extracts, no significant difference was observes against gram positive and gram negative bacteria.

Microdilution assay was performed to measure the MIC values of the fractions that demonstrated antimicrobial activity. Minimum inhibitory concentration (MIC) was evaluated by diluting the fractions, measuring the MIC are the important indicator of antimicrobial activity; the lowest MIC's values were observed for ethyl acetate and water fractions. Most MIC's values were ranging from 294-580 µg/mL. The minimum inhibitory concentration was also observed in ethyl acetate and water against *P. multocida* and*B. subtilis*. In a previous study the MIC values for *P. ostreatus* ethanolic extracts were 1.25 mg/mL against *C. albicans*, 2.5-20 mg/mL against *P. aeruginosa*, 2.5-12.5 mg/mL against *B. cereus* (Vamanu, 2012). MIC's values for *C. sinensis* against *B. subtilis* were determined as 938 ¼g/ mL and for both *C. sinensis* and *P. australis* against *S. epidermidis* was 469 ¼g/ mL. The aquoues extracts of *G. lucidum* showed MIC 750 ¼g/ mL against *Micrococcus luteus* (Ren *et al*., 2014).

CONCLUSION

The fractions isolated with ethyl acetate, water and dichloromethane having antimicrobial potential against all the selected microbes whereas n- hexane fractions showed weak activity. Antimicrobial activity was expressed in minimum inhibitory concentration was also found to be in wide range. Our study suggested that antimicrobial constituents may be present in studied mushrooms and the fractions isolated with different polarity solvents also indicate the presence of mycochemicals. Further studies of mushroom species aimed at searching of new bioactive compound such as ingredients of functional food, pharmaceutical and cosmetics.

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