

Article

Validating Anti-Infective Activity of *Pleurotus Opuntiae* via Standardization of Its Bioactive Mycoconstituents through Multimodal Biochemical Approach

Aprajita Tiwari Pandey ¹, Ishan Pandey ², Anurag Kanase ³, Amita Verma ⁴, Beatriz Garcia-Canibano ⁵, Sarada Prasad Dakua ⁶, Shidin Balakrishnan ^{6,*} and Mohan Prasad Singh ^{1,*}

¹ Centre of Biotechnology, Nehru Science Complex, University of Allahabad, Prayagraj 211002, India; tiwari.aprajita2011@gmail.com

² Department of Pathology, Motilal Nehru Medical College, Prayagraj 211001, India; ishan.pandey411@gmail.com

³ Department of Bioengineering, Northeastern University, Boston, MA 02115, USA; kanase.a@northeastern.edu

⁴ Bioorganic and Medicinal Chemistry Research Laboratory, Department of Pharmaceutical Science, Sam Higginbottom University of Agriculture, Technology and Sciences, Prayagraj 211007, India; amitaverma.dr@gmail.com

⁵ Neurosciences Institute, Hamad Medical Corporation, Doha 3050, Qatar; BCanibano@hamad.qa

⁶ Department of Surgery, Hamad Medical Corporation, Doha 3050, Qatar; SDakua@hamad.qa

* Correspondence: sbalakrishnan1@hamad.qa (S.B.); mpsingh.16@gmail.com (M.P.S.)



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Abstract: Mushrooms produce a variety of bioactive compounds that are known to have anti-pathogenic properties with safer and effective therapeutic effects in human disease prognosis. The antibacterial activity of ethanol and methanol extracts of *Pleurotus opuntiae* were checked against pathogenic microorganisms viz. *Pseudomonas aeruginosa* ATCC 27853, *Proteus mirabilis* NCIM 2300, *Proteus vulgaris* NCIM 5266, *Serratia marcescens* NCIM 2078, *Shigella flexeneri* NCIM 5265, *Moraxella* sp. NCIM 2795, *Staphylococcus aureus* ATCC 25923 by agar well diffusion method at different concentrations of the extracts. Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of the extracts was determined by INT (Iodonitrotetrazolium chloride) colorimetric assay. Extracts were standardized by thin layer chromatography (TLC) in different solvent systems. The Retention factors (R_f) of different compounds were calculated by high performance TLC (HPTLC) fingerprinting at UV 254, 366, and 540 nm before and after derivatization. The ethanol and methanol extracts of *P. opuntiae* showed bactericidal activity against all the test pathogens at MIC values of 15.6 to 52.08 mg/mL and 20.81 to 52.08 mg/mL respectively. Whereas the MBC values for ethanol and methanol extract of *P. opuntiae* against all pathogens were recorded as 26.03 to 62.5 mg/mL and 125 mg/mL respectively. Preliminary mycochemical screening of both the extracts revealed high contents of bioactive compounds. Amongst all the solvent systems used in TLC, the best result was given by chloroform + hexane (8:2) which eluted out 5 different compounds (spots). HPTLC results revealed spots with different R_f values for all the 24 compounds present. Thus, it can be inferred from the present investigation that the mycoconstituents could be an alternative medication regimen and could play a role in new drug discoveries against different infections. Further, the antimicrobial components of these mushrooms can be transformed to bioengineered antimicrobial coatings for surfaces, drug and other hybrid systems for public health implications in combating persistent infections.

Keywords: antimicrobial activity; mushroom; *Pleurotus opuntiae*; mycoconstituents; microorganisms; compounds

1. Introduction

Antimicrobial resistance amongst infective bacterial communities poses a serious threat related to increased morbidity and mortality [1]. Resistance to popular antimicrobial

therapies and the emergence of multidrug-resistant bacteria is growing at an unprecedented pace. There are challenges in combating bacterial infections and associated diseases due to acute shortage of effective drugs, lack of successful preventive measures, and presence of few new antibiotics in the clinical pipeline, which has inspired the development of novel treatment options and alternative antimicrobial therapies [2]. According to WHO (World Health Organization), resistant microorganisms (such as bacteria, fungi, viruses, and parasites) are capable of resisting antimicrobial activity resulting in the existence and propagation of infections that cause numerous drug-resistant strains or organisms [3]. Centers for Disease Control and Prevention (CDC) report that nosocomial infections contribute to at least 5% of the hospital patients clinical setting is reported to have had more than 2 million illnesses and 99,000 deaths per year [4]. These morbid factors have cumulatively built the momentum towards the investigation of novel antimicrobial agents that are effective against pathogenic micro-organisms exhibiting resistant against conventional treatments [5]. Nowadays, novel antimicrobial agents from natural sources such as microbes, fungi, and plants are flourishing, and plant-based innovative therapeutics are steadily increasing [6].

Several natural resources have been explored for antimicrobial properties in the recent past, with mushrooms being one of the highly probed source [5]. The intrinsic properties of some mushrooms have been noted to exhibit healthy, safe and effective medicinal effects in the treatment of human disease. Moderate to good antimicrobial activity against bacterial and fungal infections has been demonstrated by some mushrooms, such as *P. ostreatus*, *P. sajor-caju*, *P. eryngii*, *P. florida* and *Agaricus bisporus* extract against several pathogenic bacteria [7]. A large number of bioactive compounds have been isolated and identified in mushrooms that include terpenoids, flavonoids, tannins, alkaloids, and polysaccharides, etc. [8]. To exploit the antimicrobial properties of mushroom, careful selection of solvents is critical, as they influence the type of bioactive components and the concentration that is extracted [9]. These bioactive compounds are generally produced as secondary metabolites for the growth and survival of the mushrooms in a specific environment [10]. Crude extracts usually contain more than one bioactive compound that tend to have synergistic effect thereby adding potential therapeutic value. Since mushrooms possess similar microbial antagonists to humans, bioprospecting mushrooms for antimicrobial agents could provide some novel therapeutics. The antibacterial activities detected here warrant investigation for their potential to improve human health and application as dietary supplements and also as nutraceuticals [11]. Different bioactives can be found during various stages of mushroom development. For instance, fruiting bodies and mycelium of mushrooms show distinct health-promoting values due to the nature and action of numerous bioactive compounds [6].

Pleurotus species belong to *Phylum Basidiomycota* that produce oyster shaped mushrooms (basidiocarps) and hence the name oyster mushrooms. They are edible and among the most common mushrooms worldwide [10]. The oyster mushroom, *Pleurotus* spp., is commonly grown on a broad variety of substrates consisting of lignin and cellulose. *Pleurotus* sp. is promising as a therapeutic mushroom with haematological, antiviral, anti-tumor, antifungal, antibacterial, hypocholesterolic, antioxidant and immunomodulation actions [12,13]. *Pleurotus opuntiae* is an essential mushroom of the xerophytic temperate regions of Mexico. Taxonomic relationships of *P. opuntiae* show that *P. agaves* is a synonym of *P. opuntiae* and *P. levis* are close species of *P. opuntiae*. Traditional uses of *P. opuntiae* as food and remedy for several health problems have been identified in literature [14,15]. The extensive cultivation of *Pleurotus* species is carried out for its consumption as food and for medicinal properties. Literature data are mostly available for the most common spp. such as *P. ostreatus* and *P. eryngii*, etc., while very little is known about the potential therapeutic properties of taxa, specially *P. opuntiae*. The medicinal value of *Pleurotus* taxa is still under-investigated in most of the Mediterranean countries [15]. In general, the bioprospecting studies concentrate almost exclusively on the screening of antibacterial properties of mushroom extracts without specifying the compounds responsible for their

action. However, some of the compounds previously reported for antimicrobial effect are sesquiterpenes and other terpenes, hormones, benzoic acid derivatives, anthraquinones and quinolines, and peptides and proteins [10]. The modes of action of organic compounds that may be associated with the antimicrobial function of mushroom extracts have been the subject of several studies [9].

Thus, determining the compounds or secondary metabolites of antimicrobial action of extracts obtained from medicinally important mushroom will help to design alternate or combinational treatment strategies to treat infections caused by antibiotic resistant bacteria. In line with this, the current study will focus on the lesser studied *Pleurotus opuntiae* for evaluating their medicinal value (antimicrobial). The antibacterial action of the mushroom extracts with two different solvent system will be determined and the extracts will further be characterized based on the types of mycoconstituents present. Thus, by understanding the type of bioactives present, the components responsible for antimicrobial properties can be identified. In future this could lead towards new drug discovery in the field of antimicrobial chemotherapy by isolation and identification of these antimicrobial compounds.

2. Materials and Methods

2.1. Collection of Mushroom

The culture of *Pleurotus opuntiae* (mushroom) was procured from Directorate of Mushroom Research (DMR), Solan (Himachal Pradesh, India), and was maintained on malt extract agar media at 25–28 °C and pH 6–6.5. The whole cultivation process was performed in-house at Centre of Biotechnology, University of Allahabad (Prayagraj, India). The fruiting bodies from the mushroom were washed thoroughly with distilled water. Following this, they were dried at room temperature, and ground to a fine powder and stored in airtight containers until further use.

2.2. Preparation of Mushroom Extracts

The extraction methodology of mushroom was similar to the one used by Oyetayo et al. [16], with minor modifications. A total of 10 g of powder was separately extracted with each 100 mL of ethanol and methanol by using a soxhlet extractor for 4 to 5 h at a temperature below the boiling point of the solvents. Using Whatman no. 1 filter paper, the extracts were filtered and the residual solvent was then removed by evaporation at 40 °C for 6–7 h using a rotatory evaporator. The residues obtained were stored in a sterile bottle under refrigeration (4 °C) prior to further analysis. The total percentage yield of extract was 40% w/w for methanol and 38.0% w/w for ethanol respectively [16]. The complete process for preparation of *P. opuntiae* extract is shown in Figure 1.

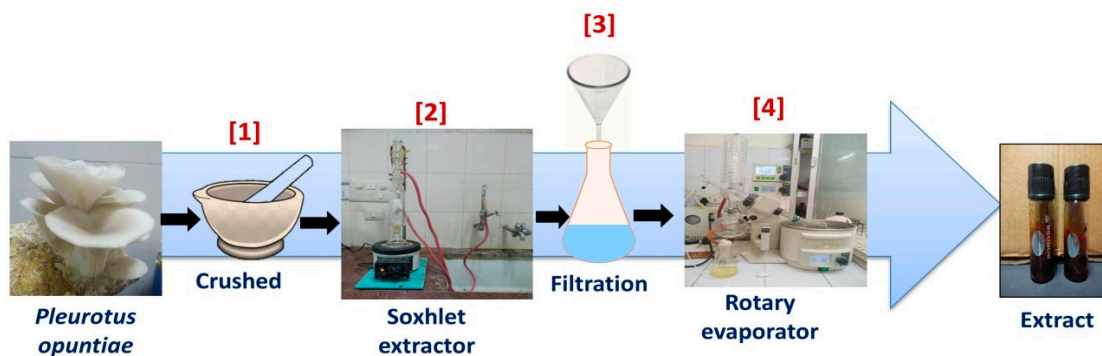


Figure 1. Schematic representation for the preparation of *P. opuntiae* extract.

2.3. Bacterial Tested Organisms

A total of one Gram-positive (*Staphylococcus aureus* ATCC-25923) and six Gram-negative (*Pseudomonas aeruginosa* ATCC-27853, *Proteus mirabilis* NCIM-2300, *Proteus vulgaris* NCIM-5266, *Serratia marcescens* NCIM-2078, *Shigella flexneri* NCIM-5265, *Moraxella* sp. NCIM 2795) pathogenic bacterial strains were used in this study for testing the antimicrobial activity of the crude mushroom extracts. The microorganisms were procured from the Department of Microbiology, IMS BHU (Varanasi), and NCIM (Pune), India respectively.

2.4. Determination of Antibacterial Screening Test

Antimicrobial activity of methanol and ethanol extract of *P. opuntiae* was determined by the agar well diffusion method [16] with slight modification. Briefly, the methanol and ethanol extracts were dissolved in 3% dimethylsulfoxide (DMSO) at different concentrations (i.e., 100, 125 and 150 mg/mL). An overnight culture of each microbial isolate was inoculated in nutrient broth to turbidity which was equivalent to 0.5 McFarland (10^5 CFU/mL). To determine the antimicrobial activity of the extracts, an aliquot of test culture was evenly swabbed over the surface of the solidified Muller Hinton agar with the help of a sterile swab. Next, small wells (5 mm in diameter) were made in the agar plates by sterile cork borer. Quantities of 100 μ L of both extracts were loaded into the different wells at different concentrations, respectively. Ciprofloxacin (5 μ g/mL) for Gram-negative and penicillin (10 μ g/mL) for Gram-positive dissolved in distilled water were used as the control for tested microorganisms. All the plates with respective extract and tested organisms were incubated at 37 °C, for 24 h. After 24 h, the zone of inhibition was measured in millimeters (mm) by the antibiotic zone scale. All the tests were carried out in triplicate and their means recorded.

2.5. Determination of Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentrations of the extracts were determined by using the INT microdilution colorimetric assay method described by [6,17] with slight modifications. The stock solution of each extract was prepared by dissolving 250 mg/mL of extract in dimethyl sulfoxide (DMSO). Briefly, 96-well microtiter plates were prepared by dispensing 100 μ L of nutrient broth into each well. A 100 μ L from the stock solution (250 mg/mL) of tested extract was added into the first row of the plates. Then, twofold serial dilutions were performed by transferring 100 μ L of solution from one row to another, using a pipette. The obtained concentration range was from 250 to 1.95 mg/mL for each extracts. Microbial inoculum size of 50 μ L (1.0×10^5 CFU/mL) was added to each well. Whereas in positive control 100 μ L of nutrient broth and 50 μ L of bacterial suspension were added and in the negative control, only the nutrient broth and extract was added. Then inoculated microtiter plates were incubated at 37 °C for 24 h. After 24 h the MIC was determined by adding 30 μ L (2 mg/mL) of 0.02% p-Iodonitrotetrazolium chloride (INT) and incubated at 37 °C for 30 min. INT was used as an indicator for bacterial growth as bacteria metabolizes INT and changes it to pink color. Lack of color change after the addition of INT indicated no growth of microorganisms and were chosen as MIC values.

2.6. Total Antibacterial Activity (TAA)

The total antibacterial activity (TAA), is a function of the extraction yield in milligram per 1 g of *P. opuntiae* and the minimal inhibitory concentration (MIC), expressed in milliliter per gram (mL/g) [18]. TAA indicates the volume of water or solvent that is added to 1 g of the extract that will still inhibit the growth of the pathogen [19].

2.7. Determination of Minimum Bactericidal Concentration (MBC)

The minimum bactericidal concentration of *Pleurotus opuntiae* extracts was determined by modifying microbroth dilution method described by Gebreyohannes and team [6]. Micro-titer plates (96-wells) were each filled with 100 μ L of sterile nutrient broth and serially diluted with extract concentrations within the range 250 to 1.95 mg/mL, respectively

following the same procedure described above for MIC determination. An inoculum size of 50 μL (1.0×10^5 CFU/mL) of test organisms were added to the appropriately labelled wells and incubated at 37 °C for 24 h. Post-incubation, a loopful of inoculum from each well of the microtiter plate with clear content (without any color change) was taken and streaked it on nutrient agar plates. Then plates were incubated for 24 h at 37 °C. Then, the MBCs were determined as the lowest concentration of the extract that permits no growth of bacteria. All tests were performed in triplicates to validate the results.

2.8. Mycochemical Screening

The methanol and ethanol extracts of *P. opuntiae* were screened to detect the presence of mycoconstituents such as alkaloids, carbohydrate, phenols, diterpenes, phytosterols (triterpenes), proteins, amino acids, tannins, anthraquinones, flavonoids, phlobatamins, terpenes, cardiac glycosides, saponins following standard protocols [20,21].

2.9. Thin-Layer Chromatography (TLC)

The methanol and ethanol extracts of *P. opuntiae* were investigated using TLC as described by [22]. Thin layer plates were prepared by spreading the mixture of silica gel (silica gel 60 GF254 (Merck) dissolved in distilled water (thick slurry) on clean glass slides. These plates were then dried and activated by heating in an oven for 30 min at 110 °C [23]. 100 mg of each extract was dissolved in 1 mL ethanol and 1 mL methanol, respectively, and applied as spots with the aid of capillary tubes on a silica gel coated plate about 1 cm from the base. The spotted plates were run in different solvent systems. Then developed plates were observed under the UV chamber and iodine chamber and were then sprayed with vanillin–sulfuric acid for the detection of the different spots after derivatization. Distances between the spots were measured and the Retention factor (Rf) values were calculated. The machine learning toolbox has recently helped decipher scientific data collected through a broad variety of medical information in the area of antimicrobial technology. In line with this, TLC chromatograms were analyzed using NIH ImageJ software [24].

Another method to develop prototype formulation was used with some modification known as bioautography method. In this method 100 mg of each extract was dissolved in 1 mL ethanol and applied as a coating layer (in the form of spot) on the silica gel coated plate at about 1 cm from the base. The spotted plates were run in developed solvent system (8 mL chloroform + 2 mL hexane). Then compounds in the extract got separated to form a thin coating layer (liquid coating) form in the TLC plate. The chromatogram (silica coated plate) was dried for up to 24 h at room temperature under a stream of air to remove the remaining solvent. The plates were then sprayed (spray coating) with bacterial suspension and incubated for 24 h. After 24 h it was further sprayed with a 2 mg/mL solution of p-Iodonitrotetrazolium violet (Sigma-Aldrich, St. Louis, MO, USA) (INT) and incubated for 4 to 6 h. Clear zone or white area of inhibition was observed against a purple background. White areas indicated reduction of INT to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of tested pathogen [25].

2.10. High-Performance Thin Layer Chromatography (HPTLC) Analysis

HPTLC analysis of ethanol extract of *P. opuntiae* was performed at CSIR-Central Institute of Medicinal and Aromatic Plants, CIMAP, Lucknow, India. The chromatographic analysis was performed using aluminum sheets precoated with silica gel 60 F₂₅₄ as a stationary phase. Aliquots of 2 μL of extract (100 mg of extract dissolved in 1 mL ethanol) were taken for sample loading. The mobile phase used was chloroform: hexane (8:2, v/v) and further derivatized in vanillin–sulfuric acid reagent for detection of different spots present in the extract. Densitometric scanning was performed at wavelengths 254, 366 and 540 nm.

2.11. Statistical Analysis

Data obtained from the study were analyzed with Graph Pad Prism Version 5.0 for Windows (Graph Pad Software, Inc., San Diego, CA, USA statistical package program. Values are expressed as mean \pm SEM (Standard Error Mean). Results obtained were statistically analyzed by using one-way ANOVA (Analysis of variance) followed by Bonferroni post hoc test, $p < 0.05$ was considered a significant value. The quantitative evaluation of different fractions of chromatograms (TLC) for both extracts was performed using the ImageJ software package.

3. Results

3.1. Antimicrobial Activity of *P. opuntiae*

In this work, antimicrobial activity of ethanol and methanol extract of *P. opuntiae* was tested against a prominent pathogenic Gram-positive bacteria (*S. aureus* ATCC 25923) and six Gram-negative bacteria (*P. aeruginosa* ATCC 27853, *P. mirabilis* NCIM 2300, *P. vulgaris* NCIM 5266, *Serratia marcescens* NCIM 2078, *Shigella flexneri* NCIM 5265, *Moraxella* sp. NCIM 2795).

Results of antimicrobial activity of the extracts suggest that both extracts show a broad range of antimicrobial activity against different pathogenic strains. Mushroom used in this analysis indicates varying degree of antimicrobial activity against the tested microorganisms. Antimicrobial activity of *P. opuntiae* extracts at varying concentrations is described in (Table 1 and Supplementary Material, Figure S1A,B. All the data obtained are statistically significant (p -value ≤ 0.05). It was observed that as the concentration of extract increases (100, 125, 150 mg/mL) the zone of inhibition also increases. Ethanol and methanol extracts of *P. opuntiae* were the effective in retarding microbial growth of all tested pathogenic bacteria by giving a clear zone of inhibition. The ethanol extract showed significantly higher antimicrobial activity against *P. vulgaris* (23.6 to 25.6 mm) and the least activity against *P. aeruginosa* and *Shigella* and *Serratia*. Methanol extract gave highest activity against *P. aeruginosa* (18.6 to 21.6 mm) and least activity against *Serratia* (10.0 to 15.3 mm) at all concentrations. The activity against *S. aureus* was negligible at concentration 100 mg/mL in methanol extract and gives the least activity at 150 mg/mL, whereas ethanol extract showed potent antimicrobial activity at all concentrations (100, 125, 150 mg/mL). *Moraxella* sp. shows no activity against both extracts of *P. opuntiae* at all concentrations. The standard antibiotic ciprofloxacin (5 μ g) for Gram-negative bacteria and penicillin (10 μ g) for Gram-positive bacteria was taken as control.

Table 1. Antimicrobial Activity of *P. opuntiae* at different concentrations against pathogenic strains.

Pathogens	Ethanol			Methanol			Antibiotics (Control)
	100 mg/mL	125 mg/mL	150 mg/mL	100 mg/mL	125 mg/mL	150 mg/mL	-
	Zone of Inhibition (mm)						
<i>S. aureus</i>	14.6 \pm 0.3	17.3 \pm 0.3	17.6 \pm 0.3	10.0 \pm 0.0	11.0 \pm 0.0	11.6 \pm 0.3	21.6 \pm 0.3
<i>P. aeruginosa</i>	11.3 \pm 0.3	12.6 \pm 0.3	14.3 \pm 0.3	18.6 \pm 0.3	19.6 \pm 0.3	21.6 \pm 0.3	20.6 \pm 0.3
<i>P. mirabilis</i>	14.6 \pm 0.3	16.3 \pm 0.3	19.3 \pm 0.3	15.6 \pm 0.3	17.6 \pm 0.3	19.0 \pm 0.0	24.3 \pm 0.3
<i>P. vulgaris</i>	23.6 \pm 0.3	24.6 \pm 0.3	25.6 \pm 0.3	16.3 \pm 0.3	18.6 \pm 0.3	19.6 \pm 0.3	33.6 \pm 0.3
<i>S. marcescens</i>	10.0 \pm 0.0	14.0 \pm 0.0	14.6 \pm 0.3	10.0 \pm 0.0	13.6 \pm 0.3	15.3 \pm 0.3	33.6 \pm 0.3
<i>S. flexneri</i>	11.6 \pm 0.3	13.6 \pm 0.3	16.3 \pm 0.3	17.6 \pm 0.3	19.6 \pm 0.3	19.6 \pm 0.3	25.6 \pm 0.3
<i>Moraxella</i> sp.	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	0.0 \pm 0.0	33.6 \pm 0.3

Results are shown as mean \pm SEM (n = 3); Values are significant at $p < 0.05$; followed by one way ANOVA then Bonferroni post hoc. Comparison; compared to control. Control: ciprofloxacin 5 μ g/mL (Gram-negative) and penicillin 10 μ g/mL (Gram-positive).

3.2. Minimum Inhibitory Concentration (MIC), Minimum Bactericidal Concentration (MBC), MBC/MIC Ratio and Total Antimicrobial Activity (TAA)

The MIC of methanol and ethanol extracts of *P. opuntiae* were employed by using INT colorimetric assay and MBC by microbroth dilution method to evaluate their bacteriostatic and bactericidal properties. The concentration effect of both the extracts were reported in Table 2 and illustrated in Figure 2. MIC values of ethanol extract were lower than those of methanol extract of *P. opuntiae*. Ethanol extract showed MIC between 15.6 and 52.08 mg/mL, whereas methanol extract show MIC between 20.81 and 52.08 mg/mL against different bacterial pathogens. Activity against *S. aureus* was noted at MIC 26.03 mg/mL for ethanol extract and 52.08 mg/mL for methanol respectively. *P. mirabilis* gave the lowest MIC value at 20.81 mg/mL for methanol and 15.6 mg/mL for ethanol extract. Similarly, ethanol extract showed the highest MIC value at 52.08 mg/mL against *Serratia* and *Shigella*.

Table 2. MIC, MBC and bactericidal potential (MBC/MIC) of methanol and ethanol extract of *P. opuntiae* against pathogens.

Pathogens	Ethanol			Methanol		
	MIC (mg/mL)	MBC (mg/mL)	* MBC/MIC	MIC (mg/mL)	MBC (mg/mL)	* MBC/MIC
<i>S. aureus</i>	26.03 ± 7.3	52.08 ± 14.7	2.00	52.08 ± 14.7	62.5 ± 0.0	1.20
<i>P. aeruginosa</i>	26.03 ± 7.3	26.03 ± 7.3	1.00	31.25 ± 0.0	52.08 ± 14.7	1.66
<i>P. mirabilis</i>	15.60 ± 0.0	26.03 ± 7.3	1.66	20.81 ± 7.3	52.08 ± 14.7	2.50
<i>P. vulgaris</i>	26.03 ± 7.3	62.5 ± 0.0	2.40	31.25 ± 0.0	52.08 ± 14.7	1.66
<i>S. marcescens</i>	52.08 ± 14.7	52.08 ± 14.7	1.00	52.08 ± 14.7	125.0 ± 0.0	2.40
<i>S. flexneri</i>	52.08 ± 14.7	62.5 ± 0.0	1.20	26.03 ± 7.3	52.08 ± 14.7	2.00
<i>Moraxella</i> sp.	NA	NA	–	NA	NA	–

MIC: minimum inhibitory concentrations; MBC: minimum bactericidal concentrations; NA: No Activity; Results are shown as mean ± SD ($n = 3$); Values are significant at $p < 0.05$; followed by one way ANOVA then Bonferroni post hoc. multiple Comparison; compared to control. Control: ciprofloxacin 5 µg/mL (Gram-negative) and penicillin 10 µg/mL (Gram-positive), * MBC/MIC ratio is ≤ 4 bactericidal and bacteriostatic if >4 .

MBC of the ethanol extract of *P. opuntiae* against all pathogens were found to fall between 26.03 and 62.5 mg/mL and for methanol from 52.08 to 125 mg/mL, respectively. The highest MBC value 125 mg/mL was by methanol extract against *Serratia*, whereas all other pathogens gave MBC value of 52.08 mg/mL. *S. aureus* showed 62.5 mg/mL MBC value for methanol and 52.08 mg/mL for ethanol extract, respectively. *P. aeruginosa* and *P. mirabilis* gave the lowest MBC value at 26.03 mg/mL for ethanol extract. Unlike MIC, ethanol extracts showed lower MBC values as compared to methanol extract. The obtained results are promising as both extracts were bactericidal as their MBC/MIC value of both extracts is ≤ 4 . MBC/MIC ratio is shown in Table 2. The value of total antibacterial activity (TAA) of both extracts of *P. opuntiae* was recorded against all the pathogens which show their broad antimicrobial potential. The higher the TAA value, the more efficacious the extract. For *S. aureus*, *Pseudomonas*, *P. mirabilis*, and *P. vulgaris* ethanol extract show high TAA as compared to methanol extract, whereas methanol extract showed high TAA against *Serratia* and *Shigella* (Figure 3).

3.3. Mycochemical Screening from *Pleurotus opuntiae*

In addition to the in vitro antimicrobial activity, mycochemical analysis was also carried for ethanol and methanol extracts of *P. opuntiae*. Tests for alkaloids, carbohydrate, phenols, diterpenes, phytosterols (triterpenes), proteins, amino acids, tannins, anthraquinones, flavonoids, phlobatamins, terpenes, cardiac glycosides, saponins were carried out and their results are shown in Table 3.

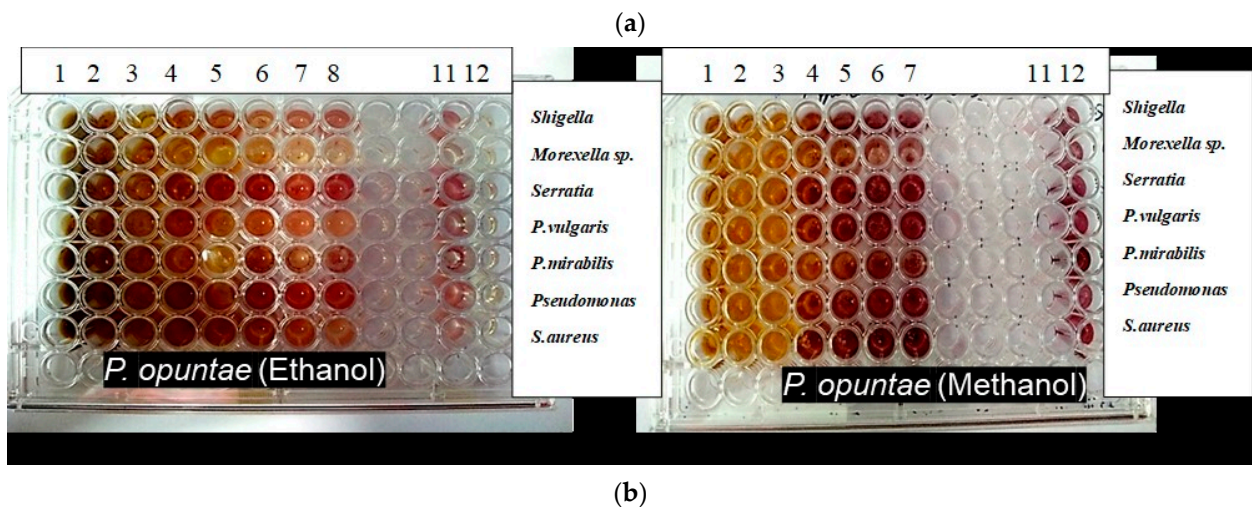
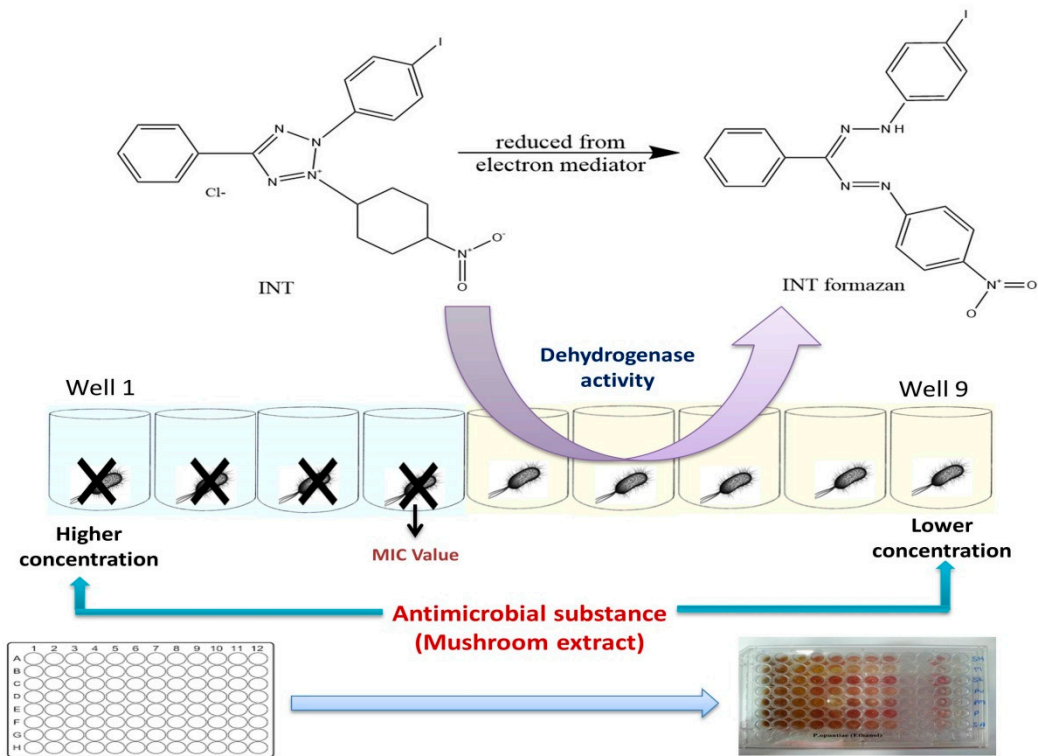


Figure 2. (a) Mechanism of formation of INT to INT formazan and determination of MIC value in INT colorimetric assay, (b) INT assay showing MIC values of methanol and ethanol extract of *P. opuntiae* against pathogens. 1—250 mg; 2—125 mg; 3—62.5 mg; 4—31.25 mg; 5—15.6 mg; 6—7.8 mg; 7—3.9 mg; 8—1.95 mg/mL; 11—positive (+ve) control and 12—negative (–ve) control for ethanol; 11—negative (–ve) control and 12—positive (+ve) control for methanol.

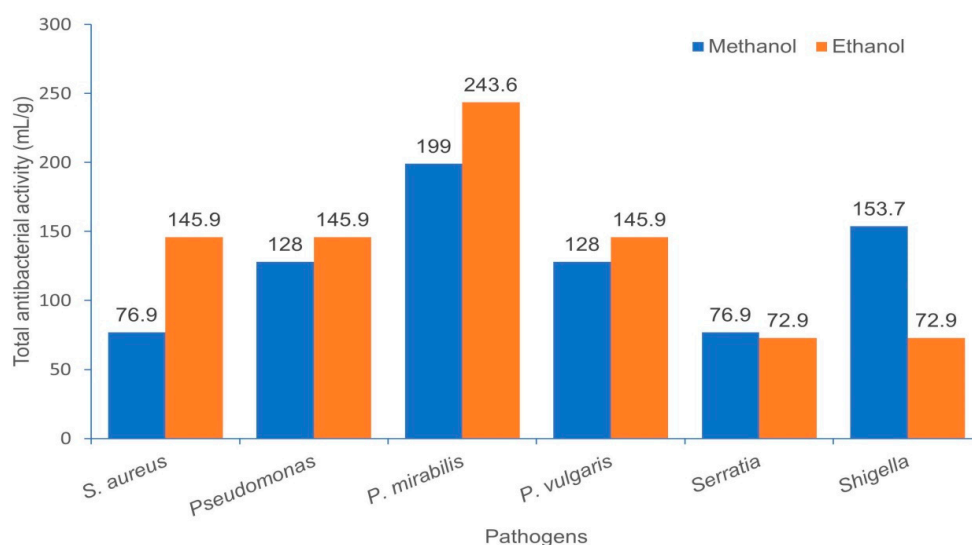


Figure 3. Total antibacterial activity (TAA) of ethanol and methanol extracts of *P. opuntiae* against the pathogens.

Table 3. Mycochemical screening of ethanol and methanol extract of *P. opuntiae*.

Mycoconstituents	Mycochemical Tests	<i>Pleurotus opuntiae</i> (Mushroom)	
		Ethanol	Methanol
Alkaloids	Mayer's test	++	+
	Wagner's test	++	+
	Dragendroff's test	++	+
Carbohydrate (Reducing sugar)	Benedict's test	+	+
	Barfold's test	+	+
Diterpenes	Copper acetate test	++	+
Phytosterol (triterpenes)	Salkowski's test	–	+
Phenols	Ferric chloride test	+	+
Flavonoids	Alkaline reagent test	–	–
Proteins	Xanthoproteic test	+	+
Amino acids	Ninhydrin test	+	+
Tannins	Gelatin test	–	–
	Braymer's test	–	–
Cardiac glycosides	Keller-Kilian's test	–	–
Anthraquinones	Borntrager's test	–	–
Phlobatamins	-	+	+
Terpens	Vanillin-sulfuric acid spray	+	+
Saponins	Froth test	+	+
	Foam test	+	+

++: Present in high concentration, +: Present in low concentration, –: Absent.

It was found that alkaloids and diterpenes are present in ethanol extract in a greater amount as compared to methanol extract. Triterpenes are only present in methanol extract. Tannins, cardiac glycosides, and anthraquinones are absent in both the extract. The antibacterial activity of the *P. opuntiae* extract in this study can be attributed to the presence of various bioactive components. Presence of different classes of secondary metabolites:

phenols, flavonoids, terpenoid, alkaloids, and saponins could be considered as the reason for the observed antimicrobial activities in this study.

3.4. Thin Layer Chromatography (TLC) Profile

Chemical constituents of both extracts were separated on TLC plates by standardizing in different solvent systems under saturated conditions, which are shown in Table 1, Supplementary Material. The R_f values of different eluted compounds were calculated. Amongst all the used solvent systems, the best result was given by Chloroform + Hexane (8:2) which eluted out 5 different compounds (spots) of *P. opuntiae* with different R_f values (Table 4). Apart from it, all the other solvent systems gave only 2 to 3 clear spots of separated compounds for both the extracts respectively. Although in 100% chloroform solvent system, 2 spots with the same R_f values (0.6, 0.7) in both extracts were observed which is also analyzed and confirmed by ImageJ software (Figures S2 and S3, Supplementary Material). In the solvent system, Chloroform + Hexane (8:2) ethanol extract gives 3 active bands (spots) with different R_f values 0.6, 0.3, 0.7, and methanol extract gives 2 active bands with R_f values 0.7 and 0.5 when visualized under UV and iodine chamber. In Vanillin-sulphuric acid, spray ethanol again gives 3 active bands with R_f values 0.7, 0.4, 0.2, and methanol gives 2 active bands with R_f values 0.7, 0.2. A total of five active bands were found in both the extract. Active bands of *P. opuntiae* in TLC result and their analysis and confirmation by ImageJ software is given in Table 4 and illustrated in Figure 4A–C). In the bioautography analysis of ethanol extract of *P. opuntiae*, only two pathogens *Shigella flexneri* and *Proteus vulgaris* gave positive antimicrobial activity results with R_f values 0.3 and 0.7, the supporting figure is given in Supplementary Material, Figure S4.

Table 4. TLC Results of Ethanol and Methanol Extract of *P. opuntiae*.

Solvent System	Extracts	R _f Values	Active Band	Total Band	
		Visualised in Iodine Chamber			
8 mL chloroform + 2 mL hexane	Ethanol	0.6, 0.3, 0.7	3	5	
	Methanol	0.7, 0.5	2		
	after Vanillin + Sulphuric Acid Spray				
	Ethanol	0.7, 0.4, 0.2	3	5	
Methanol	0.7, 0.2	2			

3.5. HPTLC Fingerprinting Analysis of Ethanol Extract of *P. opuntiae*

The HPTLC chromatogram showed that ethanol extract constituents were clearly separated without any tailing and or being diffuse. The chromatogram can be best observed under daylight, UV 254, 366 and 540 nm before and after derivatization (Figure 5). The HPTLC densitogram under UV–254, 366 and 540 nm of ethanol extract of *P. opuntiae* revealed several peaks which are presented in Figures 6 and 7. The densitometry results of extract for 10 µL sample size, under UV–254 nm (high energy zone) revealed 7 spots, for 25 µL sample size, under UV–366 nm revealed 3 spots and for 25 µL sample size, under 540 nm revealed 12 spots with R_f max values (Tables 5–7). For 254 nm major spot with the R_f max 0.33 along with the percentage area of 76.75%, for 366 nm major spot with the R_f max 0.34 along with the percentage area of 49% and for 540 nm major spot with the R_f max 0.2 along with the percentage area of 21.77% were recorded respectively. These are found to be principal components or marker compounds of mushroom extract. Thus, the developed chromatogram will be specific with selected solvent system chloroform:hexane (8:2) and serves as the better tool for standardization of the compound from mushroom (*P. opuntiae*).

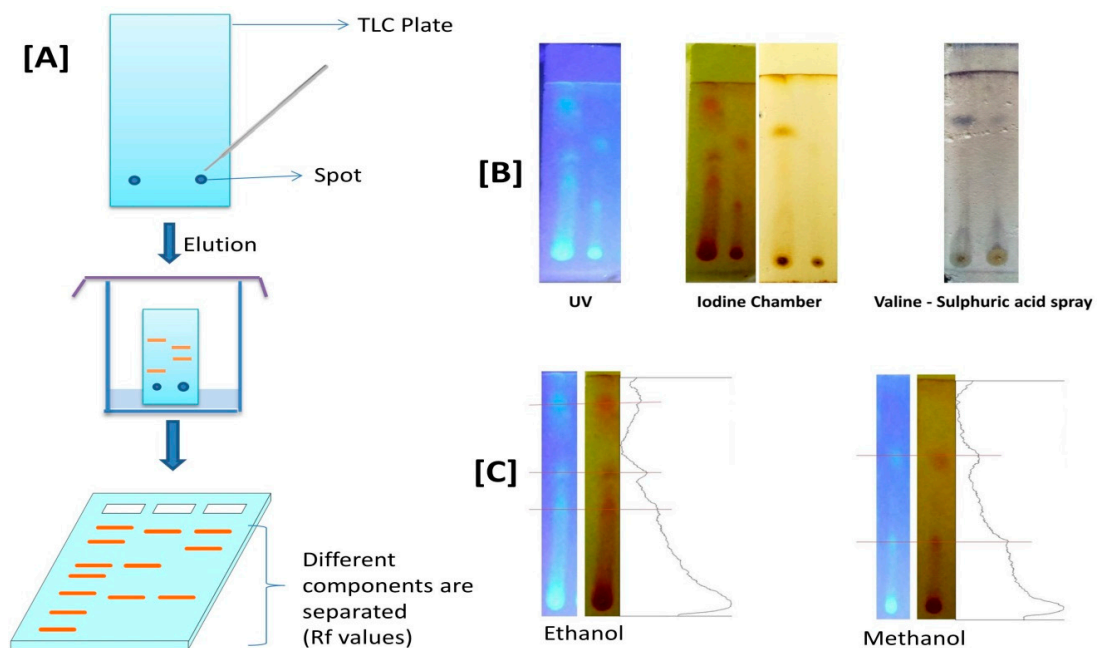


Figure 4. (A) Schematic representation of Thin Layer Chromatography (TLC), (B) TLC profile of ethanol and methanol extracts of *P. opuntiae* in Chloroform + Hexane (8:2) solvent system, (C) TLC and ImageJ-based chromatograms of ethanol extract and methanol extract fraction of *P. opuntiae* in Chloroform + Hexane (8:2) which shows 3 and 2 spots respectively.

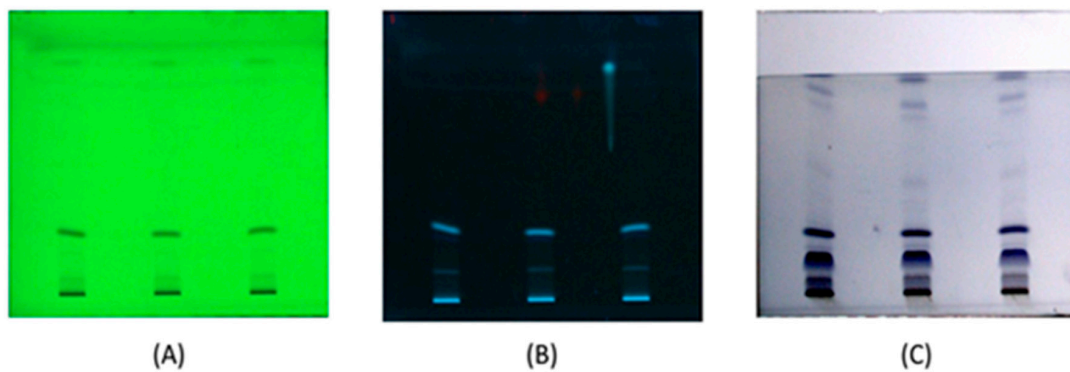


Figure 5. (A) HPTLC chromatogram image at 254 nm, (B) HPTLC chromatogram image at 366 nm and (C) HPTLC chromatogram image after derivatization.

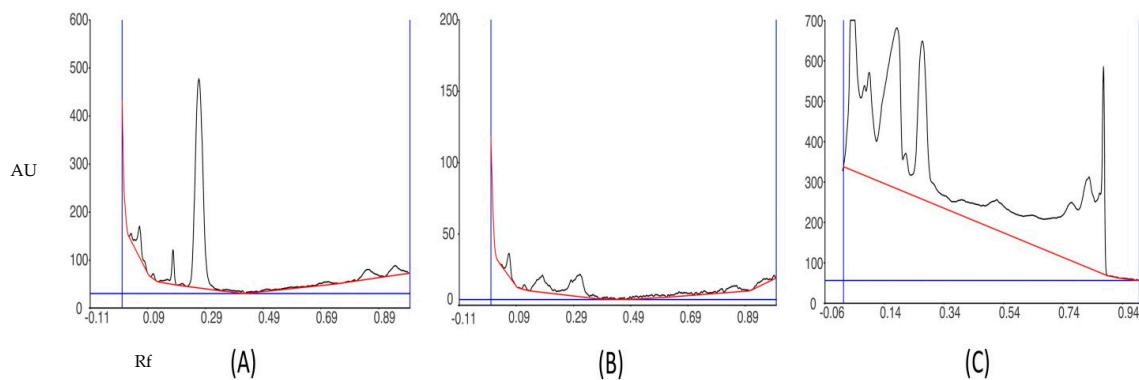


Figure 6. HPTLC densitogram of ethanol extracts of *P. opuntiae* at wavelength (A) 254 nm, (B) 366 nm and (C) 540 nm. *y*-axis shows AU- area under curve and *x*-axis shows Rf values of different compounds.

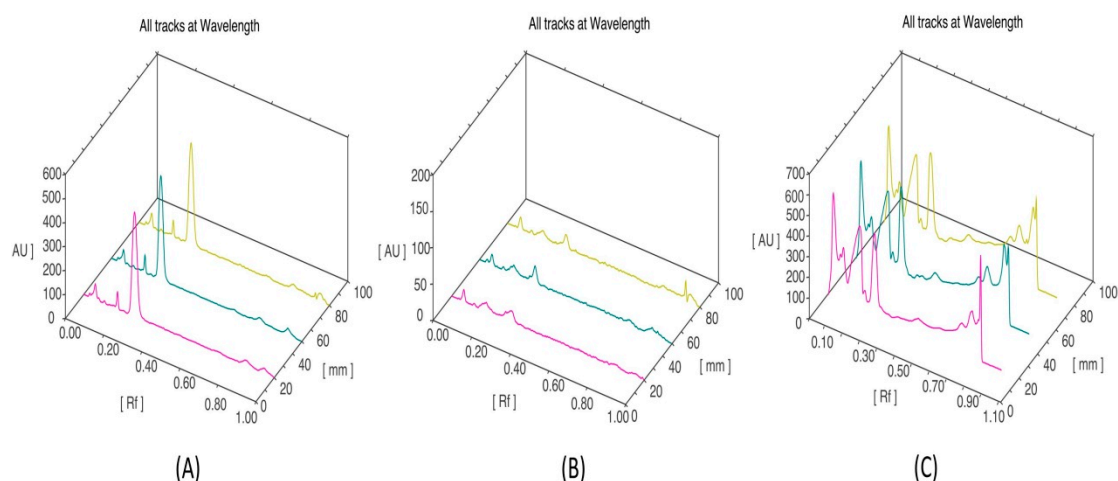


Figure 7. 3D HPTLC fingerprint of ethanol extracts of *P. opuntiae* at wavelength (A) 254 nm, (B) 366 nm and (C) 540 nm. AU—Area under curve (measured the uniform areas under the curves in between wavelengths 254, 366 and 540 nm, Rf—Retention factor (shows Rf values of different compounds), mm—shows length of chromatogram run.

Table 5. HPTLC profile of ethanol extract of *Pleurotus opuntiae* at 254 nm.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.02	0.5	0.03	15.7	2.46	0.04	9.6	105.1	0.83
2	0.04	10.2	0.06	65.0	10.18	0.09	0.2	867.6	6.87
3	0.10	0.2	0.11	10.5	1.64	0.12	0.1	77.3	0.61
4	0.14	3.6	0.18	71.9	11.24	0.19	0.2	628.2	4.98
5	0.23	0.3	0.27	435.2	68.09	0.33	2.4	9688.6	76.75
6	0.81	0.3	0.85	20.6	3.22	0.90	2.5	671.9	5.32
7	0.91	0.5	0.95	20.2	3.17	0.99	4.8	585.6	4.64

Table 6. HPTLC profile of ethanol extract of *Pleurotus opuntiae* at 366 nm.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.03	0.0	0.06	16.1	36.84	0.09	0.0	175.1	16.43
2	0.15	3.8	0.18	12.0	27.46	0.22	2.1	364.4	34.20
3	0.27	4.3	0.31	15.6	35.71	0.34	2.0	526.1	49.37

Table 7. HPTLC profile of ethanol extract of *Pleurotus opuntiae* at 540 nm.

Peak	Start Rf	Start Height	Max Rf	Max Height	Max %	End Rf	End Height	Area	Area %
1	0.00	5.2	0.03	501.3	17.05	0.06	187.9	12,608.3	13.74
2	0.06	188.5	0.07	221.7	7.54	0.08	210.3	3122.3	3.40
3	0.08	212.3	0.09	259.3	8.82	0.11	96.8	5401.7	5.89
4	0.12	96.8	0.18	398.0	13.54	0.20	78.3	19,978.2	21.77
5	0.20	80.3	0.21	96.3	3.28	0.23	47.9	1657.3	1.81
6	0.23	48.1	0.27	391.2	13.31	0.34	31.0	12,969.8	14.13
7	0.37	24.2	0.41	39.9	1.36	0.42	38.1	1402.5	1.53
8	0.48	47.3	0.52	75.0	2.55	0.55	61.9	4264.3	4.65
9	0.61	60.5	0.65	74.1	2.52	0.66	72.5	3313.7	3.61
10	0.67	73.6	0.77	144.6	4.92	0.79	132.9	10,724.0	11.68
11	0.79	133.1	0.83	225.1	7.66	0.85	170.0	9183.0	10.01
12	0.85	170.1	0.88	513.4	17.46	0.89	0.0	7153.8	7.79

4. Discussion

Mycoconstituents display a wide range of biological activities against pathogenic microorganisms [26]. The findings obtained from present study affirm this further with a focus on antibacterial activity. Similar antimicrobial potentials have been observed in *P. ostreatus* [27] and many other species [21] but have never been reported in *P. opuntiae* despite having medicinal properties. Bacterial strains included in this study were chosen based on their significance and etiology in clinical microbiology and in different infectious processes [28]. Eloff; 1998 [29] studied the antimicrobial activity of different plant extracts on different test organisms by using colorimetric assays of various tetrazolium salts as a reagent. The result of his experiment indicated that the formation of formazan product was most stable, particularly in the case of INT formazan tetrazolium salt assay. Hence, in our study, we rationally used INT tetrazolium salt assay for the MIC determination of *P. opuntiae*. Both extracts showed potential bactericidal activity against the tested pathogenic bacteria. Antimicrobial activity is usually regarded as bactericidal if the MBC/MIC ratio is ≤ 4 and bacteriostatic if $\text{MBC/MIC} > 4$ [30]. The obtained results from both the extracts were bactericidal as their MBC/MIC value were ≤ 4 .

Both extracts showed various degrees of antimicrobial activity against test pathogens. Variation in MIC of different extracts may arise from differences in their chemical constituents and volatile nature of their constituents [22]. Furthermore, the type of extraction solvent, the origin of the mushrooms, their age, species, as well as the type of bacterial strain, can be attributed to the differences in the antimicrobial activity [5]. The higher bioactive content in the mushroom extracts can also be one of the major factors correlating with different ranges of MIC values as some could exhibit synergistic effects as well. From the above result, it was concluded that ethanol extract gives the highest total antimicrobial activity with lower MIC and MBC values as compared to methanol extract of *P. opuntiae*. During mycochemical screening, the ethanol extract of *P. opuntiae* gave a large number of mycoconstituents compared to methanol extract. Such a difference between two extracts is generally correlated with the affinity of a particular bioactive compound towards a solvent which in turn depends on the polarity. By specific separation and purification strategies, the type of components specific to the solvents can be identified. The presence of a higher quantity of compounds in the ethanol extract of *P. opuntiae* is responsible for the antimicrobial activity at lower MIC values against test microorganisms [31,32]. The preliminary screening tests can be helpful in the identification of bioactive compounds in the mushroom and can help in the discovery and production of drugs [21]. Groups of mycochemical compounds are commonly associated with combating microbial resistance and having antimicrobial behavior in edible mushroom. Currently, many of the pathogenic bacteria exhibit multidrug-resistance to commonly used antibiotics causing different diseases [33]. Due to these contributory factors, the search for a new drug is important in current times, and *P. opuntiae* has been proven as a potential source of bioactive molecules. The isolation of these bioactive compounds needs different analytical methodologies which include thin layer chromatography as one of the procedures for preliminary screening [33].

Since silica gel retains more polar compounds, the nonpolar compounds are eluted first and moved further up the TLC plate. Hence, the more polar the compound, shows lower R_f (Retention factor) value, and the less polar the compound, show larger the R_f value. Thus, it can be concluded that the mushroom extract of *P. opuntiae* studied here has both polar and nonpolar compounds [22]. This could also justify the difference in the extraction efficiency and antimicrobial activity between the two solvent extracts. Though methanol and ethanol exhibit closer polarity index, there is however a significant difference between these two solvents. As a result, bioactive compounds with a very specific affinity towards ethanol are extracted with ethanol, unlike methanol. According to Bubueanu (2017 [34]) the *Pleurotus ostreatus* extract was characterized by two major spots, one was ferulic acid ($R_f \sim 0.9$ and ~ 0.55) and other was coumarins ($R_f \sim 0.1, 0.6$ and 0.8). The presence of ergosterol (T3) with four major spots ($R_f \sim 0.3-0.9$) was also found in different solvent systems. Thus, the present study on *P. opuntiae* elicits similar findings with major spots corresponding to

the same Rf values as stated above. It was also found that in *P. opuntiae* extract, the highest percentage area (76.75% and 49.37%) covered by the Rf value 0.3, matched to the Rf value of ergosterol as mentioned above. This can further be justified by the higher extraction efficiency of ethanol, as ergosterol shows relatively lower polarity and hence soluble to a greater extent in ethanol compared to methanol. So, these findings suggest strong evidence-based similarities for presence of ergosterol, ferulic acid and coumarins along with other compounds respectively. Further analysis by digital chromatographic techniques like HPTLC posted strong scientific evidence validating the TLC results. The differentiation in HPTLC profile is an important and powerful procedure that has often been used for detection of compounds [35]. Huizhen Li [36] in his HPTLC study of *Pleurotus ostreatus* recorded Rf = 0.34 & Rf = 0.31 and found it to be corresponding with saponin which is also same with our HPTLC results as the maximum area at wavelength 366 nm is being Rf = 0.34, which is 49.37%, and the maximum area at wavelength 540 nm is being Rf = 0.34 which is 14.13%. This result is also being supported by the mycochemical screening test for the presence of saponins in the extract. As a result, the presence of saponin is confirmed in higher amounts in *P. opuntiae* extract [36].

As per the definition of antimicrobial coating from the literature, it should be some synthetic resin or epoxide or a nano-emulsion that may release certain chemicals to inhibit the growth of pathogens; hence, to prove this release of antimicrobial chemicals from the extract we used a TLC plate to show the separated antimicrobial compounds. We used mushroom extract for natural antimicrobial coating applications. The silica has been used as the antibacterial surface design with the incorporation or adhesion of mushroom extracts due to their properties of being non-toxic, eco-friendly, cheap, easily available and not containing heavy metals, presenting support to silica-based surfaces by natural adjuvants to promote its activity. Some papers had reported that the natural extracts need to be used in an optimized amount to have the best performance for the coating. The natural additives included biopolymers like chitosan, gluten, gelatin, etc. Mushroom extracts on the other hand presented itself as “self-sustained edible coatings”. Different biopolymers, such as proteins, lipids, polysaccharides or their combinations, have been used as carriers to produce edible coatings with antimicrobial properties. Since it is well reported that mushroom have large amount of polysaccharide (glucans, chitin, mannan, galactans, xylans, etc.) the purpose of edible coating is well fulfilled [37].

The main coating techniques in food packaging are spraying, dipping or spreading, which have all been carried out by us at different stages of experimentation. In this context, we propose that *P. opuntiae*, an edible mushroom, can act as an edible antimicrobial coating in future. Antimicrobial edible coatings are ready to provide an effective alternative in active packaging materials to improve the safety of processed food products for commercial purposes [38,39]. The mushroom antimicrobial coating can be used in two ways: one by immobilizing it on a packaging film or the other relatively easy method by spreading a layer of extract on the food material. The mushroom releases its active compounds by forming a matrix and releasing active mycochemicals presenting a layer or coat over the food. Our results have shown that mushroom extracts exhibit stronger antibacterial properties against several pathogens. They could be incorporated into an edible film and coating formulation as antimicrobial agents, which will lead to improved food safety [40].

It is well known from the literature that basidiomycete comprises numerous bioactive compounds that facilitate a range of functional properties, acting individually and/or synergistically. *A. blazei* includes several other compounds, such as ergosterol, other forms of fatty acids, polysaccharides and alkaline compounds, which can also play an important role in synergistic antimicrobial activity [41]. Sudirman et al. [42] purified the antimicrobial compound fraction of Rf value 0.73 from *Lentinus cladopus* LC4, which is also similar with the results of our TLC but the structure of that compound is still unknown [42]. The developed HPTLC method gave strong fingerprints for species authentication through direct band comparison and can confirm the existence of bioactive compounds. Thus, the present study provides sufficient information about the therapeutic efficacy of the

medication and in the identification, standardization and quality assurance of the specific natural product. In conclusion, the findings of the qualitative assessment of the HPTLC fingerprint images should aid in the detection and quality assurance of the medication and ensure therapeutic efficacy [43].

Our evidence-based study provides facts about the use of mushroom as a functional food. It contains different components (bioactives) which might be acting “synergistically” in an in-vivo physiological system to produce different medicinal attributes which may not be “individually” present in desired compounds if they are isolated and tested “in-vitro”. Since mushrooms, and specifically *Pleurotus opuntiae*, are widely used as edible food supplement in the Indian sub-continent and the Asian diaspora worldwide, this study is a preliminary attempt to indicate the potential antimicrobial activity of the mushroom, identifying it as a super-food with tremendous medicinal properties [3].

This research is one of the few studies identifying the antimicrobial action of the fungal extracts derived from their fruiting body. Our findings also support the possible application of fungal metabolites in pharmaceutical products and the pharmaceutical industry [26]. With an increasing number of bacteria developing resistance to commercial antibiotics or MDR, extracts and derivatives from mushrooms hold a great promise for novel medicines in modern times [2,44,45]. In the light of the current global pandemic, more multidisciplinary methods, such as phyto-micro/nanotechnology-enabled strategies, may become front-runners in the battle against infectious diseases and global initiative for the prevention and treatment of these infections [3,45]. Lastly, we need to scrutinize the recently evolving diverse cellular machinery of microbial flora [46–48] and resistant viruses by extensive research into natural-based therapeutics to improve preventative foresight.

5. Conclusions

The results from our study present an insight on the prospective use of the mycopharmaceuticals in the management and treatment of infectious diseases. The data obtained focuses on the importance of primary screening strategies as a putative step by presenting new avenues for clinical as well as pharmaceutical research and development. The best total antibacterial activity (TAA) in the present study was shown by ethanolic extract of *P. opuntiae* against *P. mirabilis*. Our study findings support mushroom as a potential source of new chemical entities (NCEs) which in turn presents a possible new class of antibiotics; yet the conformational and structural prediction of the active compounds remain unexplored. With this, the conventional antibiotics can now be replaced with newer and effective natural antimicrobials developed from mushroom extracts to treat several deadly and multi-drug resistant infectious diseases. Additionally, this provides further scope in directly utilizing the extracts by exploiting the possible synergetic effects of the bioactives by eliminating the need to individual component synthesis and characterization. The mushroom used in our study was found to have a great degree of antimicrobial effect against the selected pathogens. However, more extensive analytical studies are needed in future to appropriately use these mushroom antimicrobials in medicine as an active pharmaceutical ingredient (API). Furthermore, in future these mushrooms which are edible could be used in the form of nano-encapsulating edible coatings for food and drug safety. Thus, this functional food extract could act tremendously in naturally curbing off infections and providing a holistic approach to therapeutics.

Supplementary Materials: The following are available online at <https://www.mdpi.com/article/10.3390/coatings11040484/s1>, Figure S1: (A) Shows antimicrobial activity of *P. opuntiae* (Ethanol extract) against pathogens; (B) Shows antimicrobial activity of *P. opuntiae* (Methanol extract) against pathogens, Figure S2: Shows TLC profile of ethanol and methanol extracts of *P. opuntiae* in 100% Chloroform solvent system, Figure S3: TLC and ImageJ program chromatograms of (A) Ethanol extract fraction and (B) methanol fraction of *P. opuntiae* in 100% chloroform which shows only 2 spots respectively (Total 4 spots), Figure S4: Shows the bioautography result of *Proteus vulgaris* (before spraying and after spraying INT) and *Shigella flexneri* (after spraying INT) in silica gel coated plate, Table S1: Standardization of different solvent systems for TLC to separate compounds in form of total no. of spots in ethanol and methanol extracts of *P. opuntiae*.

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