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Biochemical and Antimicrobial Properties of *Calocera viscosa* Collected from Western Ghats Region of Karnataka

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Abstract

Different organic solvent extracts of *Calocera viscosa* (Pers.) Fr. (Dacrymycetaceae), prepared in increasing order of polarity were screened for their antimicrobial activity against Gram -ve, Gram +ve, plant and human pathogenic bacteria (ten) and human and plant pathogenic fungi (twenty) using agar well diffusion assay. The pattern of inhibition was found to vary with the solvent used for extraction and the microorganisms tested. However, most of the organic solvent extracts viz., petroleum ether, chloroform and methanol showed significant antimicrobial activity (ranging between 3.3 to 32 mm) against the tested microbes. Most of the extracts showed a higher activity against the Gram -ve and plant pathogenic bacteria as compared to the Gram +ve and human pathogenic bacteria. Same time the extracts showed in higher activity against human pathogenic fungi as compared to the plant pathogenic fungi. Petroleum ether, chloroform and methanol extracts were found to exhibit inhibition of a wider range of bacterial strains; however, the highest zone of inhibition (14 mm) was recorded by the methanol extract against *S. typhi*, *E. coli* and *X. campestris* followed by the petroleum ether and chloroform extract against *S. aureus*, *P. syringae* and *P. aeuroginosa* at 100% concentration. Petroleum ether and methanol extracts were found to be maximum zone of inhibition range of fungal strains; however, the highest zone of inhibition (14 mm) was showed by the petroleum ether extract against *C. albicans* and *F. solani*, followed by methanol extract against *C. indicum* and *A. flavus* at 100 % concentration. In contrast, the human fungal strains like, *C. merdarium* and *T. rubrum* also plant fungal strains like, *C. damatium* and *C. lindemuthianum* are completely absent in all the three solvent extracts. The increasing of extracts concentration whereas we observed the increasing diameter of zone of inhibition. Preliminary biochemical analysis of the extracts showed the presence of alkaloids, flavonoids and phenols in all extracts. Glycosides and sterols presence in chloroform and methanol extracts. Saponins presence both petroleum ether and methanol, whereas tannins and triterpenoids presence in chloroform and methanol extracts respectively. The results of the present study show that *Calocera viscosa* fruiting body extracts possess bioactive compounds having significant antimicrobial activity, making it a potential natural source of new antimicrobial agents.

Keywords: *Calocera viscosa*, Fruiting bodies, Biochemical analysis, Human and plant pathogenic microorganisms, Wild mushrooms, Western Ghats.

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Contents

1. Introduction

Despite tremendous progress in human medicine, infectious diseases caused by bacteria, fungi, viruses and parasites are still a major threat to public health. Their impact is particularly large in developing countries due to relative unavailability of medicines and the emergence of widespread drug resistance [1]. During the last few decades, the various problems associated with an irrational use of orthodox medicines, development of drug resistance as well as the appearance of undesirable side effects of certain antibiotics [2], has necessitated a renewed interest in nature as source of effective and safer alternative in the management of human infections [3-5].

There is no gainsaying that plants remain the greatest source of bioactive compounds which can be developed and used as drugs [6]. Mushrooms have been used as a part of regular diet for their nutritional and medicinal values mostly by the ethnic group of Asian people from time immemorial. They contain minerals, vitamins and nutritive compounds, proteins, polysaccharide and have a low fat content [7]. Their therapeutic interest in promoting human health is known for thousands of years. Modern scientific investigations showed that mushrooms have immense potentiality against a wide range of human ailments such as cardioprotective [8], hepatoprotective [9, 10], chemo-preventive [11, 12], immunomodulatory [13] and also strong free radical scavenging activity [14-17, 11].

In recent years, multiple drug resistance in human pathogenic microorganisms has developed due to indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. This situation has forced scientists to search for new antimicrobial substances from various sources as novel antimicrobial chemotherapeutic agents [18]. Because of these burning problems, the scientific community, in search of new therapeutic alternatives, has studied many kinds of mushrooms, which are a nutritionally functional food, and a source of physiologically beneficial and nontoxic medicines [19]. Several compounds extracted from mushrooms revealed antifungal and anti-bacterial activity [13, 20]. Antimicrobial drugs have long been used for prophylactic and therapeutic purposes. Resistance of microorganisms to antibiotics has created an immense clinical problem in the treatment of infectious diseases [21]. Hence, the current investigation was undertaken to biochemical and antimicrobial properties of *Calocera viscosa* collected from Western Ghats region of Karnataka for their antimicrobial activities.

2. Materials and Methods

Fungal material

The *Calocera viscosa* (Fig-I) were collected from Western Ghats region (13˚51'56.30"N, 75˚03'12.50"E) which is located in Haniya, Hosanagar taluk, Shimoga district, Karnataka, India, during the month of June to August 2013. The *Calocera viscosa* of mushroom was picked from the litter and decaying soil surface, with help of forceps and then they were cleaned and air dried in an oven at 40˚ C for 48h. dried mushroom samples were powdered mechanically for further use. The specimens were identified with the help of standard literatures [7, 22-24]. The voucher specimens have been deposited in the laboratory of Mycology and Department of Applied Botany, Jnana Sahyadri, Kuvempu University, Shankaraghatta, Shimoga, Karnataka.

Chemicals and reagents

All chemicals and reagents used in the present study were purchased from reliable firms like Hi-Media Laboratories Pvt. Ltd and were of analytical grade.

Extraction

Fresh mushrooms were randomly collected of 150g and air-dried in an oven at 40˚C for 48h. Dried powdered mushroom sample was extracted by stirring with 200 ml of petroleum ether, chloroform and methanol at 30˚C for 24h at 150rpm and filtering through Whatman No. 4 filter paper. The total extract was then rotary evaporated to dryness at 40˚C and redissolved in respective solvents to a concentration of 10 mg/ml and stored at-20˚C for further use [16].For the entire analysis, compounds of extract were dissolved in dimethyl sulphoxide (DMSO). The yield of extracts obtained from pet ether was 4.65gm, followed by chloroform (7.56gm) and methanol (24.35gm) in Table-I. The highest % of extracts was obtained from methanol of *Calocera viscosa* (Pie chart-I). Each extract was transferred to glass vials and kept at 4˚C before use.

Preliminary biochemical analysis of *Calocera viscosa* extracts

To assess the chemical composition of the various extracts qualitatively, a preliminary biochemical analysis was conducted according to the standard methods [25, 26]. Using these methods, the presence of several biochemicals's including alkaloids, flavonoids, cardiac glycosides, phenols, saponins, sterols, tannins and triterpenoids, and was evaluated (Table-II). These compounds are bioactive and have been reported to exhibit antibacterial activities [27-29].

Test for alkaloids (Wagner's test): Each extract was stirred with 5ml of 1.5% aqueous HCl and filtered. The filtrates were then used for testing the presence of alkaloids. Formation of a brown-coloured flocculent precipitate upon addition of a few drops of Wagner's reagent (1.27g of iodine and 2g of potassium iodide dissolved in 100ml of water) to the filtrate, indicated the presence of alkaloids in the extract.

Test for flavonoids (Ferric chloride test): About 0.5g of each extract was boiled with 5ml of distilled water and then filtered. To 2ml of this filtrate, a few drops of 10% ferric chloride solution were added. A green-blue or violet colouration indicated the presence of a phenolic hydroxyl group.

Test for cardiac glycosides (Keller-Killiani test):

To 0.5g of the extract diluted to 5ml in water, 2ml of glacial acetic acid containing one drop of ferric chloride solution was added. This was underlayed with 1ml of concentrated sulphuric acid. A brown ring at the interface indicated the presence of a deoxysugar characteristic of cardenolides. A violet ring may appear below the brown ring, while in the acetic acid layer a greenish ring may form just above the brown ring and gradually spread throughout this layer.

Test for phenols: Test solution and few drops of 5 % glacial acetic acid and 5 % sodium nitrate added. Observation of muddy yellow, olive, niger brown or deep chocolate coloured precipitate. It is indicates the presence of phenols.

Test for Saponins: One gram of each extract was boiled with 5ml of distilled water and filtered. To the filtrate, about 3ml of distilled water was further added and shaken vigorously for about 5 minutes. Frothing which persisted on warming was taken as an evidence for the presence of saponins.

Test for sterols (Salkowski reaction): A few milligrams of the plant extract were dissolved in 2ml chloroform and then 2ml of conc. H_2SO_4 was added from the sides of the test tube. The test tube was shaken for a few minutes. Red colour development in the chloroform layer indicated the presence of sterols.

Test for tannins (Ferric chloride reagent test): The test sample of each extract was taken separately in water, warmed and filtered. To a small volume of this filtrate, a few drops of 5 % w/v solution of ferric chloride prepared in 90 % alcohol were added. Appearance of a dark green or deep blue colour indicated the presence of tannins.

Test for triterpenoids (Salkowski test): To 0.5g of each extract, 2ml of chloroform was added, followed by a further addition of 3ml of concentrated H_2SO_4 to form a layer. A reddish brown colouration of the interface indicated the presence of triterpenoids.

Microorganisms

All the test microorganisms were obtained from the culture collection of the Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, Chandigarh, India and American Type Culture Collection (ATCC). The viability of the organisms was maintained by regular transfer into freshly prepared on nutrient agar (Hi-Media) and Potato dextrose agar (PDA) at 28˚C and stored at 4˚C until used. A total of ten bacterial (3 Gram -ve, 2 Gram +ve, 2 human and 3 plants) and twenty fungal (11 human and 9 plants) strains were used for the evaluation of the antimicrobial activity (Table-III, IV, V, VI, VIII and IX).

Bacterial test organisms

Gram -ve: (Table-III).

1. *Escherichia coli* (MTCC-1559)
2. *Klebsiella pneumonia* (MTCC-7028)
3. *Pseudomonas aeuroginosa* (MTCC-1934)

Gram +ve: (Table-IV).

1. *Staphylococcus aureus* (MTCC-902)
2. *Streptomyces pneumonia* (MTCC-4734)

Human pathogens: (Table-V).

1. *Salmonella parathyphi* (MTCC-1088)
2. *Salmonella typhi* (MTCC-968)

Plant pathogens: (Table-VI).

1. *Agrobacterium tumefaciens* (MTCC-431)
2. *Pseudomonas syringae* (MTCC-1604)
3. *Xanthomonas campestris* (MTCC-2286)

Fungal test organisms

Human pathogens: (Table-VIII).

1. *Candida albicans* (ATCC-10231)

2. *Candida krusei* (ATCC-6258)
3. *Chrysosporium indicum* (MTCC-4266)
4. *Chrysosporium keratinophilum* (MTCC-1367)
5. *Chrysosporium merdarium* (ATCC-900628)
6. *Chrysosporium zonatum* (ATCC-845981)
7. *Epidermophyton floccosum* (MTCC-613)
8. *Microsporum gypseum* (MTCC-2157)
9. *Trichophyton equinum* (ATCC-6275)
10. *Trichophyton kanei* (MTCC-2091)
11. *Trichophyton rubrum* (MTCC-1538)

Plant pathogens: (Table-IX).

1. *Alternaria alternate* (MTCC-7202)
2. *Alternaria solani* (ATCC-26934)
3. *Alternaria tomentosa* (ATCC-16404)
4. *Aspergillus flavus* (ATCC-9170)
5. *Colletotrichum capsici* (MTCC-2071)
6. *Colletotrichum dematium* (ATCC-60192)
7. *Colletotrichum lindemuthianum* (ATCC-90028)
8. *Fusarium oxysporum* (MTCC-2485)
9. *Fusarium solani* (MTCC-2935)

Preparation and its sterilization

For the agar well-diffusion method of antimicrobial susceptibility were tested on solid (Agar-agar) media in petriplates. For the fungal assay, PDA (39gm/L), were used for developing surface colony growth. The suspension culture, for fungal cell growth was done by preparing 2.4% (w/v) PDB (Potato dextrose broth) was taken for evaluation. All the media prepared were then sterilized by autoclaving the media at (121˚C) for 20 minutes [30].

Antibacterial activity by Agar-well diffusion method

The bacteria were grown in Muller-Hinton media (Hi-Media Pvt. Ltd., Mumbai, India) at 37˚C and maintained on nutrient agar slants at 4˚C and stored at-20˚C. Inoculum of test organisms was prepared by growing pure isolate in nutrient broth at 37˚C for overnight. The overnight broth cultures was sub-cultured in fresh nutrient broth and grown for 3hrs to obtain log phase culture. The agar plates were prepared by pour plate method using 20ml M-H medium. The sterile M-H agar medium is cooled to 45˚C and mixed thoroughly with 1ml of growth culture of concerned test organism (1 x 108 cells) and then poured into the sterile petridishes and allowed to solidify. Wells of 6 mm size were made with sterile cork borer and test extracts were added. Extracts were added at different concentrations 12.5%, 25%, 50% and 100%. The diameter of zones of inhibition was measured in mm using Hi-Media zone reader [31].At the end of the incubation period, inhibition zones formed on the medium were evaluated by measuring the diameters of the zones of inhibition in millimeter. Inhibitory activity of the DMSO was also tested as negative control. Studies were performed in triplicate. Standard antibiotic such as Ampicillin (30µg), Streptomycin (30µg), Tetracycline (30µg) and Ciprofloxacin (30µg) were used as positive control (Table-VII).

Antifungal activity by Agar-well diffusion method

Calocera viscosa extracts were tested for antifungal activity by agar-well diffusion technique [30], with a little modification. The fungal spore suspension was prepared by the addition of a loopful of fungal spores in a 5ml of sterile distilled water and 1ml Tween 20. Then fungal spore suspension was spread evenly on the petriplate containing 20ml of solidified potato dextrose agar. Four wells were punched at the corner by using sterile cork borer of 6mm diameter. The different solvent extracts of *Calocera viscosa* were loaded to the four wells by using 100µl micropipette in 4 different concentrations i.e., 12.5mg/ml, 25mg/ml, 50mg/ml and100mg/ml respectively. Clotrimazole, Fluconazole, Mancozeb and Captan are used as a positive control and DMSO is used as a negative control (Table-X). All the plates were incubated at 23±2˚C fungal growth was determined by measuring the diameter of zone of inhibition after 5 days of incubation. The test was done in triplicates to arrive concordant result. The agar plates were incubated at 37˚C for 24hrs.

Table I. Total yield of mushrooms extracts obtained various organic solvents (150gm in 200ml)

Mushroom species	Organic solvents	Yield of extract in gm
Calocera viscosa	Petroleum ether	4.65
	Chloroform	7.56
	Methanol	24.35

Table II. Preliminary biochemical analysis of *Calocera viscosa* (Pers.) Fr.

Secondary metabolites	Petroleum ether extract	Chloroform extract	Methanol extract
Alkaloids	+	+	+
Flavonoides	+	+	+
Glycosides	-	+	+
Phenols	+	+	+
Saponins	+	-	+
Sterols	-	+	+
Tannins	-	+	-
Triterpenoids	-	-	+

Note: '+' is Present, '-'is Absent

Table III. Antibacterial activities of *Calocera viscosa* extract against Gram -ve bacteria

Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Escherichia coli	7	8	10	13	5	6	9	13	6	7	8	14
Klebsiella pneumoniae	6	8	10	12	8	9	11	12	5	6	8	12
Pseudomonas aeuroginosa	7	9	11	14	8	10	12	14	6	8	12	13

Note: "-"--- No Activity

Table IV. Antibacterial activities of *Calocera viscosa* extract against Gram +ve bacteria

Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Staphylococcus aureus	7	9	12	14	6	8	10	12	5	7	8	10
Streptomyces pneumoniae	6	8	11	13	4	6	9	11	6	8	10	12

Note: "-"---No Activity

Table V. Antibacterial activity of different extracts of *Calocera viscosa* against human pathogenic bacteria

Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Salmonella parathyphi	6	8	10	12	4	6	8	10	7	8	10	12
Salmonella typhi	5	7	9	12	6	8	10	13	7	10	12	14

Note: "-"---No Activity

Table VI. Antibacterial activity of different extracts of *Calocera viscosa* against plant pathogenic bacteria

Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Agrobacterium tumefaciens	6	7	10	12	7	9	10	12	5	7	9	13
Pseudomonas syringae	5	6	8	10	8	9	10	14	6	7	8	10
Xanthomonas campestris	6	8	12	14	7	8	9	12	7	10	12	14

Note: "-"---No Activity

Table VII. Antibacterial activity of standard drug and control against Gram -ve, Gram +ve, human and plant pathogenic bacteria

Test organism	Standard				Control
	Ampicillin	Streptomycin	Ciprofloxacin	Tetracycline	DMSO
Gram -ve pathogens					
Escherichia coli	27	x	x	x	-
Klebsiella pneumoniae	32	x	x	x	-
Pseudomonas aeuroginosa	28	x	x	x	-
Gram +ve pathogens					
Staphylococcus aureus	x	20	x	x	-
Streptomyces pneumoniae	x	24	x	x	-
Human pathogens					
Salmonella parathyphi	x	x	25.3	x	-
Salmonella typhi	x	x	32	x	-
Plant pathogens					
Agrobacterium tumefaciens	x	x	x	24	-
Pseudomonas syringae	x	x	x	28	-
Xanthomonas campestris	x	x	x	22	-

Note: 'x'-Not applicable, '–'- No activity

Table VIII. Antifungal activities of *Calocera viscosa* extract against human pathogenic fungi by agar well diffusion method

Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Candida albicans	8	9.3	12	14	-	-	11	13	7	8	11.3	12
Candida krusei	6	7	9	12	-	-	-	-	8	9	10	13
Chrysosporium indicum	6	8	10	12	6	7	12	14	7	9	11	14
Chrysosporium keratinophilum	7	8	10	11	-	-	-	-	6	7	8	10
Chrysosporium merdarium	-	-	-	-	-	-	-	-	-	-	-	-
Chrysosporium zonatum	-	-	-	-	5	7	8	12	6	8	10	12
Epidermophyton floccosum	-	-	-	-	6	7	10	14	-	-	-	-
Microsporum gypseum	6	8	9	11	-	-	-	-	5	9	11	13
Trichophyton equinum	-	-	9	12	-	-	-	11	-	-	-	-
Trichophyton kanei	5	5.6	7.3	10.3	4.3	4.6	7	9	6	8	9	12
Trichophyton rubrum	-	-	-	-	-	-	-	-	-	-	-	-

Note: "-"---No Activity

Table IX. Antifungal activities of *Calocera viscosa* extract against plant pathogenic fungi by agar well diffusion method

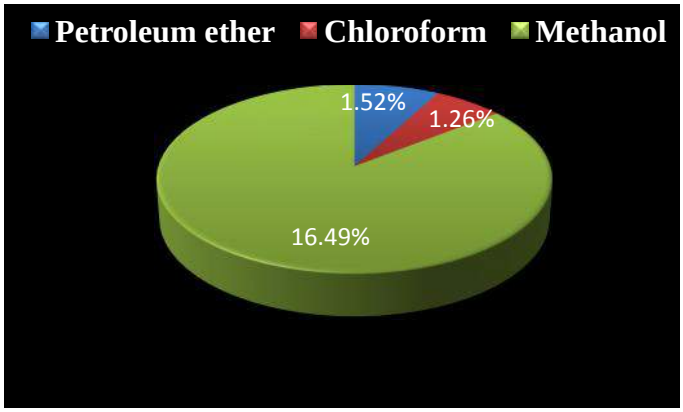
Pathogens	Zone of inhibition(mm)											
	Petroleum ether extract				Chloroform extract				Methanol extract			
	Concentration (mg/ml)											
	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %	12.5 %	25 %	50 %	100 %
Alternaria alternate	3.3	4.6	6	8	4	6	9	10.3	6	7	9	12
Alternaria solani	4	6	6.6	10	5.6	7	8	12	4.3	6	8	13
Alternaria tomentosa	-	-	-	-	-	-	-	-	-	-	-	-
Aspergillus flavus	3.3	4	5	6	4.3	5.6	7	14	8	9	12	14
Colletotrichum capsici	6	8	9	13	5	8	10	12	6	7	8	10
Colletotrichum dematium	-	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum lindemuthianum	-	-	-	-	-	-	-	-	-	-	-	-
Fusarium oxysporum	5	6.3	8	9	-	-	8	10	4	7	8	13
Fusarium solani	6.6	9	12	14	5	6.3	9	12	5.6	8	10	12

Note: "-"---No Activity

Table X. Antifungal activity of standard drug and control against human and plant pathogenic fungi

Test organism	Standard				Control
	Clotrimazole	Fluconazole	Mancozeb	Captan	DMSO
Human pathogens					
Candida albicans	26	27	x	x	-
Candida krusei	30	32	x	x	-
Chrysosporium indicum	28	29	x	x	-
Chrysosporium keratinophilum	30	26	x	x	-
Chrysosporium merdarium	24	22	x	x	-
Chrysosporium zonatum	24.3	26	x	x	-
Epidermophyton floccosum	30	31	x	x	-
Microsporum gypseum	28	32	x	x	-
Trichophyton equinum	26.3	25.3	x	x	-
Trichophyton kanei	24.6	28	x	x	-
Trichophyton rubrum	29	27	x	x	-
Plant pathogens					
Alternaria alternate	x	x	24	25	-
Alternaria solani	x	x	32	30	-
Alternaria tomentosa	x	x	25	24	-
Aspergillus flavus	x	x	26.3	26	-
Colletotrichum capsici	x	x	27	23	-
Colletotrichum dematium	x	x	25	26	-
Colletotrichum lindemuthianum	x	x	26	28	-
Fusarium oxysporum	x	x	30	29	-
Fusarium solani	x	x	28	30	-

Note: 'x'-Not applicable, '–'- No activity



Pie chart I: Showing total yield in % from different solvents extracts of *Calocera viscosa* (Pers.) Fr.

Figure I: Natural habitat of *Calocera viscosa* (Pers.) Fr.

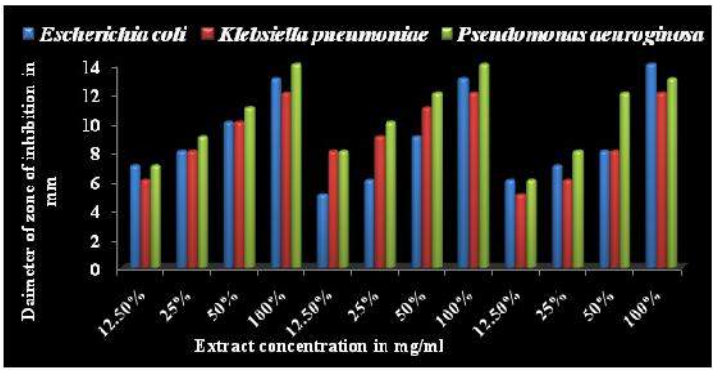


Figure II: Comparative zones of inhibition of different solvent extract of *Calocera viscosa* against Gram -ve bacteria

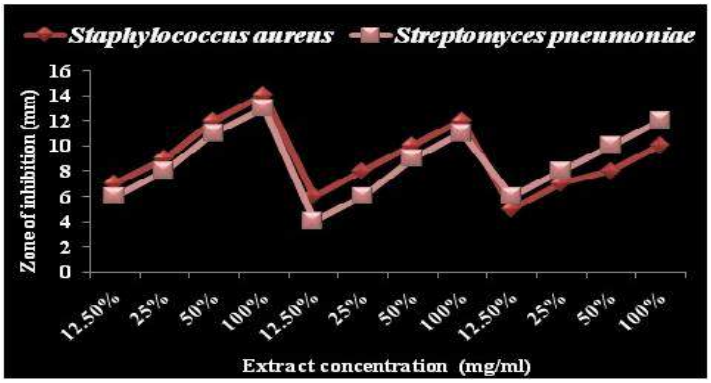


Figure III: Comparative zones of inhibition of different solvent extract of *Calocera viscosa* against Gram +ve bacteria

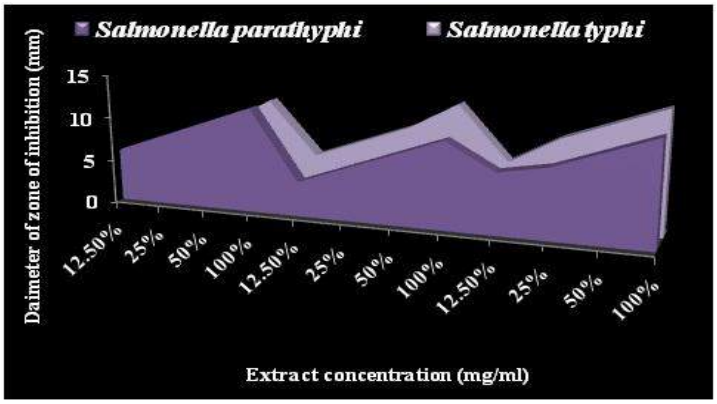


Figure IV: Comparative zones of inhibition of different solvent extract of *Calocera viscosa* against human pathogenic bacteria

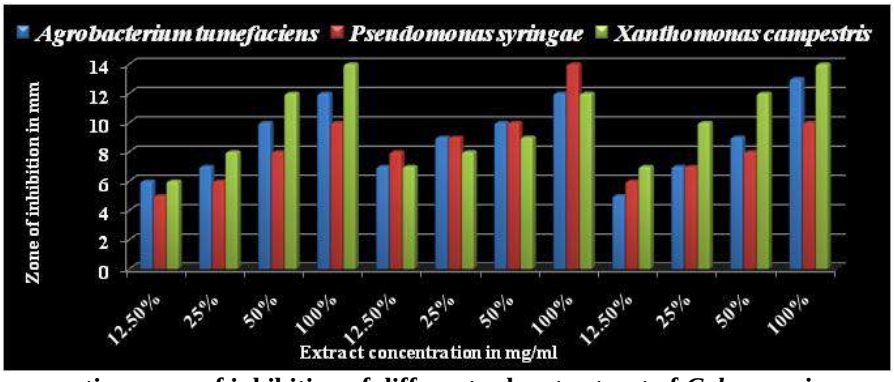


Figure V. Comparative zones of inhibition of different solvent extract of *Calocera viscosa* against plant pathogenic bacteria

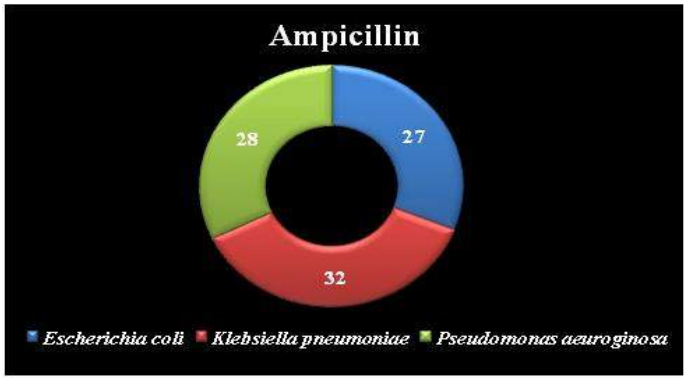


Figure VI. Showing antibacterial activity of standard drugs against Gram -ve pathogens

Figure VII. Showing antibacterial activity of standard drugs against Gram +ve pathogens

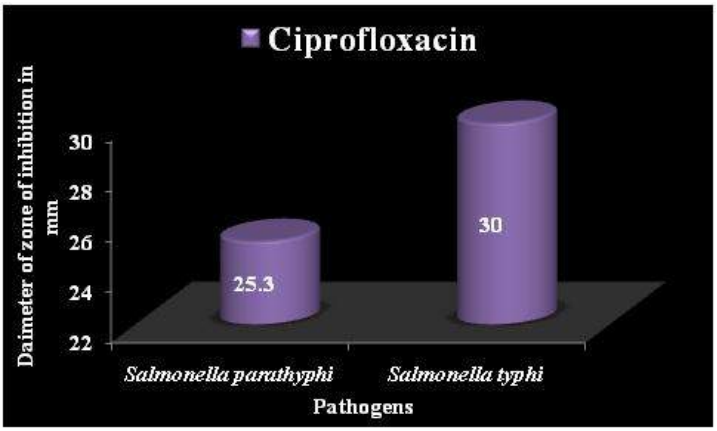


Figure VIII. Showing antibacterial activity of standard drugs against human pathogens

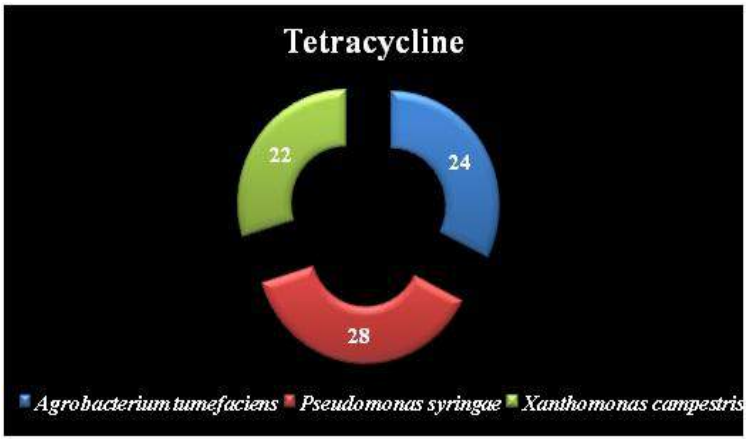


Figure IX. Showing antibacterial activity of standard drugs against plant pathogens

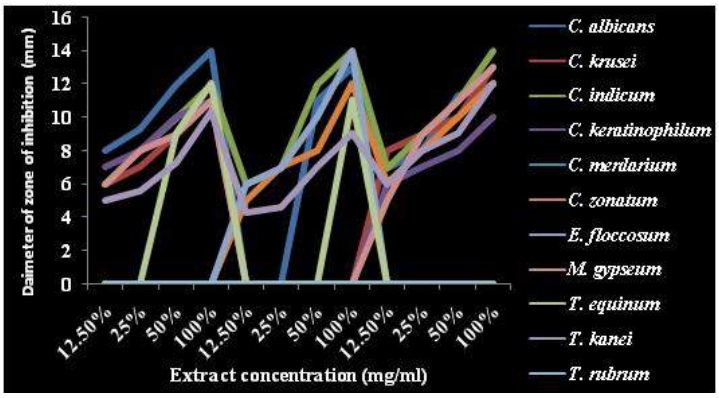


Figure X. Mean zones of inhibition of different solvent extract of *Calocera viscosa* against human Pathogenic fungi

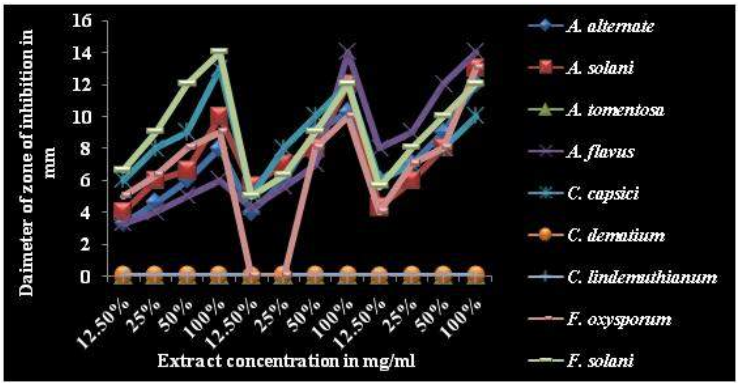


Figure XI. Mean zones of inhibition of different solvent extract of *Calocera viscosa* against plant pathogenic fungi

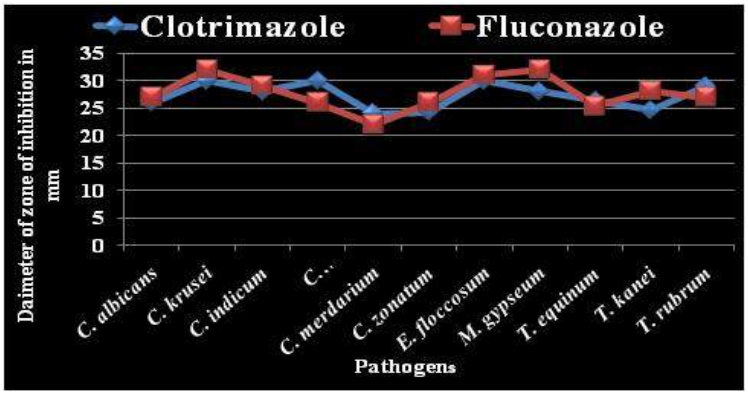


Figure XII. Showing antifungal activity of standard drugs against human pathogens

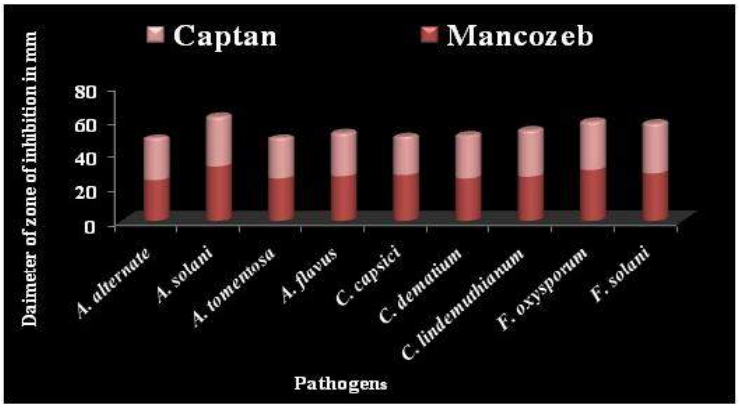


Figure XIII. Showing antifungal activity of standard drugs against plant pathogens

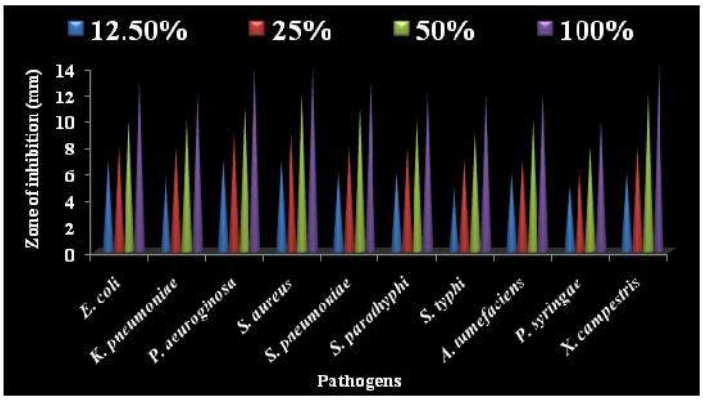


Figure XIV: Antibacterial activity of Petroleum ether extracts of *Calocera viscosa* against various strains

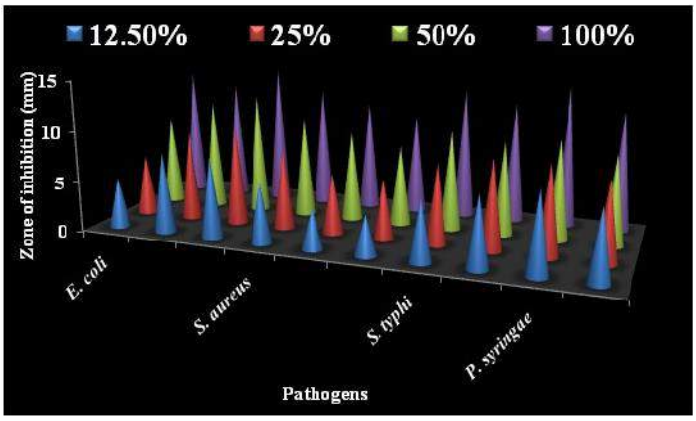


Figure XV. Antibacterial activity of Chloroform extracts of *Calocera viscosa* against various strains

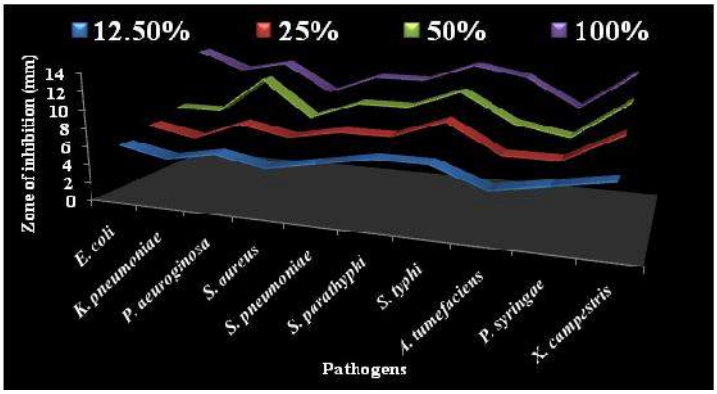


Figure XVI. Antibacterial activity of Methanol extracts of *Calocera viscosa* against various strains

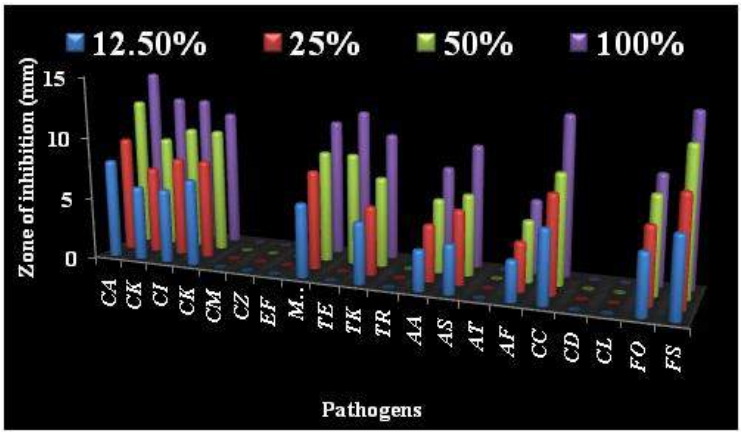


Figure XVII. Antifungal activity of Petroleum ether extracts of *Calocera viscosa* against various strains

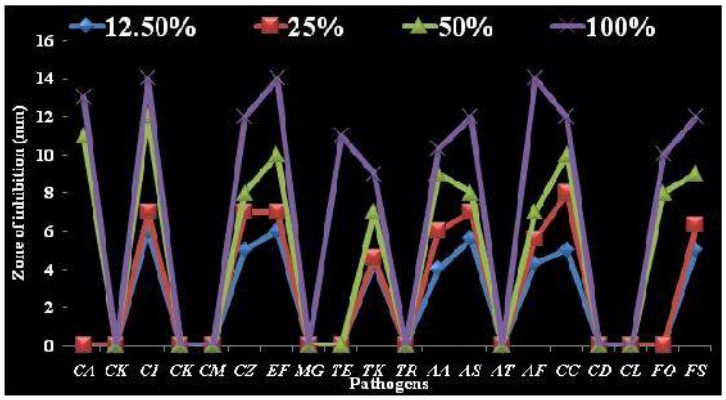


Figure XVIII. Antifungal activity of Chloroform extracts of *Calocera viscosa* against various strains

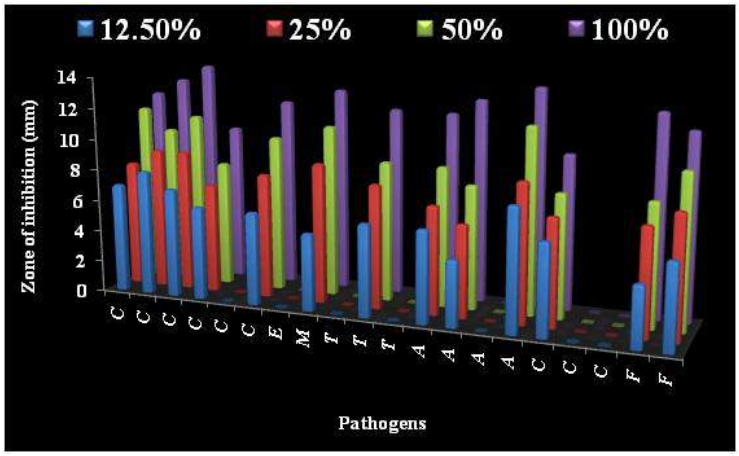


Figure XIX. Antifungal activity of Methanol extracts of *Calocera viscosa* against various strains

3. Results and Discussion

Preliminary biochemical analysis

With an aim to identify the chemical nature of compounds responsible for the antimicrobial response, the present study involved a preliminary biochemical analysis of the crude extracts, the results of which have been depicted (Table-II). Preliminary biochemical analysis of fruiting body extracts showed the presence of alkaloids, flavonoids and phenols in all the three extracts, viz., petroleum ether, chloroform and methanol. However, glycosides and sterols were found to be present in two extracts with the exception of the petroleum ether extract. Saponins and triterpenoids were found to be in all the tested extracts except chloroform; while tannins were found to be present only in chloroform extracts, whereas absent in petroleum ether and methanol, respectively.The relative antimicrobial activity of mushroom extracts may not be easily correlated with any individual component but with a mixture of compounds present in these extracts. There are reports showing that alkaloids are responsible for the antifungal activity [32]. It has also been suggested that the antimicrobial activity is mainly due to the presence of alkaloids, tritrepenoids and other natural polyphenolic compounds or due to free hydroxyl groups. Moreover, secondary metabolites such as tannins and other compounds of phenolic nature are also classified as antimicrobial compounds. Therefore, the presence of this biochemical (alkaloids, tannins, triterpenoids and sterols) could justify to some extent the observed antimicrobial activity in the current study.

Antimicrobial activity

The results of the antimicrobial response shown by different extracts and the standard antibiotics are summarized (Figures-II to XIX). All the extracts prepared exhibited variable degree of antimicrobial activity against all the tested microorganisms. The different solvent extracts of the basidiocarps of *Calocera viscosa* wild mushrooms were tested against three Gram-negative (*E. coli, K. pneumonia* and *P. aeuroginosa*) Table-III, two Gram-positive (*S. aureus*and *S. pneumoniae*) Table-IV, two human pathogenic (*S. parathyphi* and *S. typhi*) Table-V and three plant pathogenic (*A. tumefaciens, P. syringae* and *X. campestris*) Table-VI, bacteria and eleven human pathogenic (*C. albicans, C. krusei, C. indicum, C. keratinophilum, C. merdarium, C. zonatum, E. floccosum, M. gypseum, T. equinum, T. kanei*and *T. rubrum*) Table-VIII and plant pathogenic (*A. alternate, A. solani, A. tomentosa, A.flavus, C. capsici, C. dematium, C. lindemuthianum, F. oxysporum*and *F. solani*)Table-IX, fungi by the agar-well diffusion method. All the extracts showed different degree of antimicrobial activity at concentrations of 12. 5 %, 25 %, 50 % and 100 %

against the Gram -ve (Fig-II), Gram +ve (Fig-III), human pathogenic (Fig-IV), and plant pathogenic (Fig-V) bacteria and human pathogenic (Fig-X) and plant pathogenic (Fig-XI) fungi. Among all the bacteria's, *S. pneumoniae* showed highest sensitivity (13-14mm) towards mushrooms extracts (*Calocera viscosa*) followed by *P. aeuroginosa, A. tumefaciens, E. coli, S. typhi, P. syringae* and *X. campestris*, whereas in fungi's *C. krusei* showed highest sensitivity (12-14 mm), followed by *C. indicum, T. equinum. C. zonatum, T. kanei, M. gypseum, A. alternate, A. solani, C. capsici, F. oxysporum, C. albicans, A. flavus* and *F. solani*at 100 % concentration in all the three extracts of *Calocera viscosa.*

Among the three organic solvent extracts, petroleum ether and methanol fractions showed inhibition against a broader range of bacterial strains; however, the highest activity was recorded by the methanol extract against *E. coli* (14mm zone of inhibition), followed by *S. typhi* and *X. campestris* (Fig-XVI). The fungal strains were found to be more susceptible to acetone and methanol fractions. Petroleum ether and methanol extracts exhibited a strong antimicrobial activity against all bacterial (Gram -ve, Gram+ve, human and plant pathogenic) strains tested (Fig-II, III, IV and V). The Chloroform extract showed lower antibacterial activity against all bacterial strains tested (Fig-XV), except *P. aeuroginosa*, *P. syringae* and *S. typhi*, exhibiting a zone of inhibition in the range of 4-10mm against all Gram -ve, Gram +ve, human and plant pathogenic bacteria. Petroleum ether extracts showed a response similar to that of methanol extracts forming an inhibition zone in the range of 5-14mm against all type of bacteria (Fig-XIV). Contrary to petroleum ether, chloroform and methanol extracts, prepared in increasing order of solvent polarity formed a higher inhibition zone (4-14mm).

All the three extracts also showed a good antimicrobial response against all human and plant pathogenic fungal strains tested (Fig-XVII, XVIII and XIX), exhibiting a zone of inhibition in the range of 3.3-14mm, except *C. merdarium, T. rubrum, A. tomentosa, C. dematium* and *C. lindemuthianum* (Fig-X and XI). The extracts prepared in increasing order of solvent polarity exhibited a higher response than the lower concentrations of the extracts. Petroleum ether and methanol extracts exhibited a higher antimicrobial response as compared to the chloroform extracts. Maximum activity was exhibited by the petroleum ether extracts followed by the methanol and chloroform extracts. On the whole, most of the extracts showed a higher response against the various fungi (10-14mm zone of inhibition), similar to the different bacterial strains (6-14mm zone of inhibition) tested, highlighting the potential of this mushroom as an excellent source of antimicrobial agent.

The effectiveness of the extracts against different microorganisms was compared with the antimicrobial response shown by the antibiotics, viz., ampicillin (Fig-VI), streptomycin (Fig-VII), ciprofloxacin (Fig-VIII), tetracycline (Fig-IX), clotrimazole, fluconazole (Fig-XII), mancozeb and captan (Fig-XII), used as standard drugs (Table-VII and X). All the three organic solvent extracts showed a higher activity against most of the microorganisms when compared with streptomycin. However, as compared to the response exhibited by streptomycin and tetracycline, only the petroleum ether and methanol extracts prepared in increasing order of solvent polarity were found to show a comparative activity against only two microorganisms, viz., *S. aureus* and *X. campestris*. Among the four antifungal drugs tested, fluconazole was found to be least effective. Clotrimazole, mancozeb and captan exhibited almost a comparable response; however, a slightly higher response was shown by mancozeb. The antifungal response exhibited by the petroleum ether and methanol extracts against some of the fungi was also nearer to that of the other two standard drugs (fluconazole and captan). Thus, the results in the present study show that *C. viscosa* extracts exhibited significant activity against most of the tested microorganisms which was comparable to that of the standard drugs. The antimicrobial activity of different solvent extracts of mushroom is changeable and has a lower antimicrobial activity as to comparison of antibiotics viz., Ampicillin, Streptomycin, Ciprofloxacin, Tetracycline, Clotrimazole, Fleuconazole, Mancozeb and Captan (Table-VII and X).

The scientific community, while searching for new therapeutic alternatives, has studied many kinds of mushrooms and has found various therapeutic activities such as anticarcinogenic, anti-inflammatory, immuno-suppressor and antibiotic, among others [30]. The effects of different mushroom extracts on pathogens and microorganisms are studied by a very large number of researchers in different parts of the world [33-41]. We have already reported potent anticancer, hepatoprotective activities of other edible mushrooms [42, 43]. The results of the present study strengthened the outcomes of earlier works done by others that showed mushrooms produced a great variety of antimicrobial agents. For instance, it is known that the extract from fruit bodies of several *Lactarius* sp. [44, 45]; *Fomitopsis* sp. [46]; *Boletus* sp. [47]; *Cortinarius* sp. [48]; *Ganoderma lucidum*, *Navesporus floccosa* and *Phellinus rimosus* [49]; *Pleurotus tuber-regium* [50]; *Amanita caesarae*, *Armillaria mellea*, *Chroogom phusrutilus*, *Clavaria delphustruncates*, *Clitocybe geotropa*, *Ganoderma* sp., *Ganoderma carnosum*, *Hydnum repandum*, *Hygrophorus agathosmus*, *Lenzites betulina*, *Leucoagaricus pudicus*, *Paxillusin volutus*, *Polyporus arcularius*, *Rhizopogon roseo*, *Sarcodonim bricatus*, *Suillus collitinus*, *Trametes versicolor*, *Tricholoma auratum*, *Tricholoma fracticum* [51]; *Lactarius deliciosus*, *Sarcodon imbricatus* and *Tricholoma portentosum* [52]; *Russula delica* [53]; *Pleurotus eryngii*var. *ferulae* [54]; *Infundibulicybe geotropa*, *Lactarius controversus*, *Lactarius delicious* and *Phellinus*

hartigii [55]; *Lactarius indigo* [56] and *Stereum ostrea* [57] contain a wide range of antimicrobial activity. We have also reported earlier that *L. squarrosulus* has a potential free radical scavenging activity [58]. The result of the former and current study may suggest that the basidiocarp of *Calocera viscosa* is a source of pharmacologically active substances having diverse therapeutic applications. Bio-assay guided isolation of active principle is currently underway to characterize the antimicrobial compound of these investigations.

4. Conclusion

The present study has revealed that, the biochemical and antimicrobial properties by agar-well diffusion method of the wild mushroom under study and suggest that the different extracts of *Calocera viscosa* showed inhibitory effect against all the tested microbial strains but had more antimicrobial activity against Gram negative, Gram positive, human pathogenic and plant pathogenic bacteria and human pathogenic fungi as compared to plant pathogenic fungi. The zone of inhibition study also suggests that the extracts had shown antimicrobial activity in a concentration dependent manner against the test microorganisms and was comparable with the standard drugs. Further, from the antimicrobial study it was observed that the order of activity was in the sequence of petroleum ether extract >chloroform extract >methanol extract. The results presented in this project are only based on different extract and did not specify any defined antibacterial substances. In present study the use of different solvent extract are capable to extract the various biochemical compound from *Calocera viscosa* show various beneficial effects which was supported by a variety of literature. From previous studies in addition to the current it could be concluded that *C. viscosa* contain a potential metabolite and can be bioactive contents of the mushroom is the promising natural antimicrobial agents that can be harnessed as potential antimicrobial agents. Further, extensive studies are recommended for the mushroom to actually identify the bioactive components responsible for their antimicrobial activities.

5. Acknowledgement

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