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Research article

ON THE NOVEL INHIBITORY ACTION OF MUSHROOM EXTRACT OF *CORIOLUS VERSICOLOR* AND IT'S BIOACTIVITY AGAINST DRUG RESISTANT BACTERIA *SALMONELLA TYPHIMURIUM* (MTCC 3241)

K.U Zaidi*[1,] Richa Jain[2] and Sadaf Quereshi [3]

Molecular Biotechnology Laboratory Centre for Scientific Research and Development, People's University Peoples Group, Bhopal. M.P. India

ABSTRACT: The aim of this study was to investigate the effects of the mushroom extract of *Coriolus versicolor* on drug resistant strain of *S. typhimurium* experimental results revealed that hot water ethanol extract and Tris- HCl ethanol extract of *C. versicolor* were not only effective against drug resistant strain of *S.typhimurium* (MTCC 3214) but also against other pathogenic microorganisms such as *K. pneumoniae*, *S. typhi*, *E. coli*, *S. pyogenes* and *Aspergillus niger*. The extracts exerted their effects on the microorganisms by inhibiting their growth.The optimum temperature and pH conditions for mycelial growth of *C. versicolor* were found to be 28±1ºC and 5.98 pH respectively. Maximum mycelial growth was observed by employing starch and ammonium sulphate as carbon and nitrogen source and on supplementing the cultivation medium with 20% (v/v) culture filtrate of *C. versicolor*. The present investigation highlights optimization of culture conditions and determination of antimicrobial spectrum *C. versicolor* .This indicate that the extracts of *C. versicolor* is a novel inhibitory source against drug resistant bacteria *salmonella typhimurium*

Key words: *Coriolus versicolor*, bioactive compounds, polysaccharopeptides, *Salmonella typhimurium.*

INTRODUCTION

Salmonellosis is one of the most common and widely distributed foodborne diseases. It constitutes a major public health burden and represents a significant cost in many countries. Millions of human cases are reported worldwide every year and the disease results in thousands of deaths. Salmonellosis is caused by the bacteria drug resistant *Salmonella typhimurium*. Today, there are over 2500 known types, or serotypes, of *Salmonella*. *Coriolus versicolor* commonly known as wood decaying fungi is used as traditional Chinese and Japanese medicine for centuries. It occurs in the wooden temperate zone of Asia, Europe and North America (Cui and Chisti 2003) Being a natural source for the anticancer polysaccharides, it is considered to be a mushroom of high medicinal significance (Chu˙ et al 2002 ; Fisher and Yang 2002 ; Ooi and Liu,1999; Matsubara et al 2005) Besides this, the polysaccharides are also found to have antimicrobial, immunomodulating and antioxidant activity (Cheng and Leung 2008; Ulrike et al 2005) In today's scenario, the resistance in human beings to antimicrobial and antibiotics has resulted in a failure of the treatment to cure diseases. Elements responsible for creating resistance can be carried out on mobile elements and spread rapidly within human beings and animal pathogens. Mushroom extract can be a better alternative for the treatment of diseases caused by such drug resistant microorganisms. This investigation deals with the antimicrobial spectrum of the *C. versicolor* extracts and studies on the optimization of its cultural conditions, to get the maximum biomass yield for the extraction of its bioactive compounds.

MATERIALS AND METHODS

Microorganisms:

The strain of *Coriolus versicolor* was obtained from NCIM Pune (Accession no. 1076). A frozen stock of fungi was revived on PDA plates and slants for 7 days at 28±1˚C. To ensure the viability of the organism sub-culturing was performed at appropriate time intervals.

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Cultivation media for *C. versicolor*

Pure culture of *C. versicolor* was grown in submerged culture condition in triplicate sets in 500 mL Erlenmeyer flasks containing 100mL of Potato Dextrose Broth (HIMEDIA, India). Each flask was inoculated with mycelial disk (8mm diameter) of *Coriolus versicolor*, grown on Potato Dextrose Agar (HIMEDIA, India). The flasks were then incubated at 28 ±˚C for five weeks. The biomass obtained was used for extraction of various bioactive compounds and polysaccharopeptide.

Extraction of bioactive compounds from *C. versicolor*

For extraction of bioactive compounds from *C. versicolor* its biomass was collected by centrifugation (Eppendorf, Germany) at 3000 rev min^{-1} for 5 min at 4ºC. The mycelial pellet was then suspended in minimum quantity of normal saline and crushed in aseptic conditions followed by ultra-sonication (Lark, India) at 4ºC. After sonication the homogenized biomass was allowed to centrifuge at 8000 rev min^{-1} for 10 min at 4ºC, supernatant was collected and divided into two equal parts and processed for extraction of bioactive compounds using standard protocol.

(a) Hot water-ethanol extraction:

Multi-step hot water extraction of *C. versicolor* was used for recovery of polysaccharides, $1/3^{rd}$ volume of distilled water was added to supernatant and allowed to boil for 6 hours, twice, and cooled. The concentrate was subjected to precipitation with 95% ethanol at -20ºC for 24 h. The precipitate was separated by centrifugation at 8000 rev min^{-1} for 15 min at 4ºC and then stored at -20ºC as crude polysaccharide sample for further analysis.

(b) Tris HCl- Ethanol Extractions:

Equal volume of Tris-HCl (pH 8) was added to the supernatant and kept overnight at 4ºC followed by precipitation with equal volume of ethanol. The content was centrifuged at 8000 rev min^{-1} for 15 min at 4ºC. Pellet obtained was re-extracted using ethanol followed by centrifugation at 10,000 rev min^{-1} for 5 min at 4ºC. The pellet was stored at -20ºC.

Antimicrobial effect of extracts obtained from *C. versicolor*

Antimicrobial activity of extracts of *C. versicolor*, against pathogenic microorganisms was determined by agar well diffusion method. The pathogens used in the experiment were obtained from Microbial Type Culture Collection (MTCC), Chandigarh or were isolated and identified in C.S.R.D., People's Group, Bhopal, India.

Test Microorganisms

Human pathogenic bacteria and fungi - *Staphylococcus aureus* (MTCC- 96), *Klebsiella pneumoniae* (MTCC-4032), *Salmonella typhi* (MTCC-733) and *Pseudomonas aeruginosa* (MTCC-7083), *Escherichia coli*, *Salmonella typhimurium* (MTCC-3214), *Streptococcus pyogenes* (MTCC-1926), *Microsporum* sp., *Trichophyton rubrum* (MTCC-3272), *Candida albicans* (MTCC-227), *Aspergillus niger*.

Well Diffusion Method

Antimicrobial property of extracts of *C. versicolor* was evaluated by varying its concentrations. Bacterial and fungal cultures were inoculated in 4-5 mL Brain Heart Infusion Broth (Hi-Media) and incubated at 37±1°C for 24 h and 28±1°C for 3-5 days for bacteria and fungi, respectively. A known amount 3ml of these suspensions were separately added to Erlenmeyer flasks containing 100ml sterilized and cooled (40-45°C) nutrient agar medium (NAM) and yeast extract agar medium (YEM). Flasks were gently shaken to mix bacterial and fungal cells in the medium. Aliquots of 20 ml seeded medium were poured in each sterile Petri dish, then agar well was formed in the seeded medium with the help of a sterile cork borer having inner diameter of 5mm. 50µl of different concentrations of extracts *viz*., 100mg to 1000mg were poured into the wells. The plates were left for 30 minutes to allow the diffusion of the extract at room temperature. The zone of inhibition formed by different concentrations of extract was recorded after 24h incubation at 37±1°C for bacteria and 3-5 days at 28±1°C for fungi. Minimum inhibitory concentration (MIC) was determined in terms of zone of inhibition.

Effect of temperature on growth of *C. versicolor*

For studying temperature requirement of fungus, Potato Dextrose Broth was inoculated with mycelial disk of *C. versicolor*. Inoculated flasks were incubated for 10 days at the following temperatures viz. 5, 10, 15, 20, 25, 28, 32, 35 and 37˚C. After completion of incubation period the biomass was separated by centrifugation at 3000 rev min^{-1} for 5 min at 4ºC. Mycelial pellet was collected and dried at 110ºC for 24 h till constant weight. All the determinations were carried out in triplicate sets.

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Effect of hydrogen ion concentration (pH) on growth of *C. versicolor*

To study the effect of different pH on the growth of fungus, the pH of medium was adjusted at different pH levels viz. 4.0, 4.5, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.0 with the help of dilute NaOH and HCl. The pH was checked with the help of pH meter. The inoculated flasks were incubated for 10 days at temperature obtained optimum in the previous experiment. After completion of incubation period, growth was measured in terms of mycelial dry weight (mg 100 mL^{-1}) as described above.

Study of nutritional parameters for maximum biomass production of *C. versicolor*

(a) Carbon sources:

Effect of carbon sources on the mycelial growth of *C. versicolor* was studied using basal media containing (g L^{-1}) diammonium hydrogen phosphate, 5.00 ; dihydrogen potassium phosphate, 1.00 ; magnesium sulphate, 0.50 ; yeast extract, 0.10. The basal medium was supplemented with different carbon sources (1%w/v) i.e., lactose, dextrose, sucrose, starch, cellulose, glycerol, maltose, sodium acetate. Carbon sources were filter sterilized using 0.22µm nitrocellulose filter paper and then added to the medium at 45°C before pouring. The flasks were inoculated with *C. versicolor* (8 mm discs) and incubated at 28±1ºC for 10 days. After incubation, the biomass was processed in a similar manner as described earlier.

(b) Nitrogen sources:

Basal media supplemented with different nitrogen sources (ammonium acetate, ammonium bicarbonate, ammonium nitrate, ammonium sulphate, ammonium persulphate, ammonium phosphate and urea) was used to study the effect of various nitrogen sources on growth of *C. versicolor*. Sterilized medium was inoculated with agar discs (8 mm) of *C. versicolor* under aseptic conditions and incubated at 28ºC for 10 days. The dry weight of biomass was measured and evaluated for effect of nitrogen sources on growth of *C. versicolor*.

(c) Culture filtrates:

For studying the effect of culture filtrate of *C. versicolor* on growth of C. *versicolor*, culture filtrates were obtained from previous experiments. The filtrates were incorporated with Potato Dextrose Agar (PDA) (20 ml) at the concentration of 1%, 5%, 10%, 15% and 20% (v/v). Plate without complementary extract was treated as control. The sterilized media was poured in Petri plates and inoculated with agar disc (8mm) of *C.versicolor*. The inoculated plates were incubated at 28ºC and colony diameter was recorded after 5 days.

RESULTS

The hot water and Tris HCl ethanolic extracts from *Coriolus versicolor* mycelium were light to dark brown in appearance and were stable in hot water. They were found soluble in water but insoluble in methanol, pyridine, chloroform, benzene and hexane. On studying antimicrobial spectrum of extracts from *C. versicolor*, it was found that the extracts possessed broad spectrum antibacterial activity. The minimum inhibitory concentrations of extracts against various pathogens are given in Table-1.a and 1b. Among the test pathogenic fungi tested *C. versicolor* extracts were found effective against *Aspergillus niger.*

Effect of temperature on growth of *C. versicolor*

Growth of *C. versicolor* was observed between 10 to 32˚C (Figure.1). The optimal temperature for growth was found to be 28 ºC, whereas growth was absent at 35 and 37 ºC.

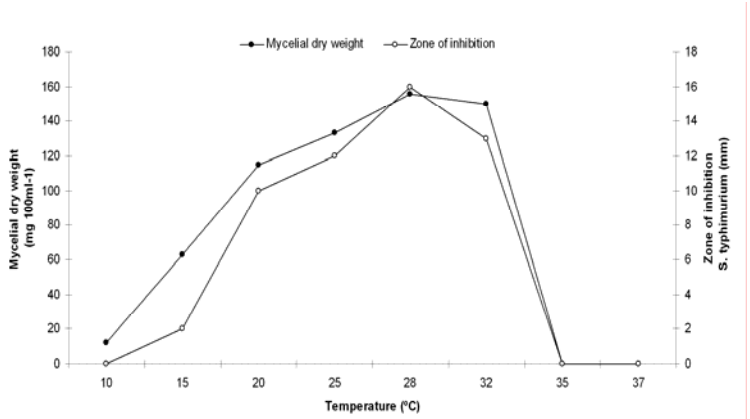

Figure: 1 Effect of different temperatures on the mycelial growth of *C. versicolor*

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Table-1a: MIC values of Hot water extract of *Coriolus versicolor* against pathogenic microorganisms

Test Pathogen	Zone of inhibition in different concentration of extract of *Coriolus versicolor* (mg/ml)									
	100	200	300	400	500	600	700	800	900	1000
S. aureus	-	-	-	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-	9	11	12
S. typhi	-	-	-	13	14	15	18	20	22	24
E. coli	-	-	-	-	-	-	8	10	12	13
S. typhimurium	-	-	5	10	11	13	16	18	20	24
S. pyogenes	-	-	-	-	-	-	-	-	-	3
Microsporum gypsum	-	-	-	-	-	-	-	-	-	6
Trichophyton rubrum	-	-	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-	-	-
Aspergillus niger	-	-	-	-	-	6	9	13	15	18

Table-1b: MIC values of Tris HCl extract of *Coriolus versicolor* against pathogenic microorganisms

Test Pathogen	Zone of inhibition in different concentration of extract of *Coriolus versicolor* (mg/ml)									
	100	200	300	400	500	600	700	800	900	1000
S. aureus	-	-	-	-	-	-	-	-	-	-
K. pneumoniae	-	-	-	-	-	-	-	7	9	10
S. typhi	-	-	-	12	15	18	20	23	26	29
E. coli	-	-	-	-	-	-	-	5	8	15
S. typhimurium	-	-	2	6	8	11	16	18	19	22
S. pyogenes	-	-	-	-	-	-	-	-	6	10
Microsporum gypsum	-	-	-	-	-	-	-	-	-	-
Trichophyton rubrum	-	-	-	-	-	-	-	-	-	-
Candida albicans	-	-	-	-	-	-	-	-	2	5
Aspergillus niger	-	-	-	-	-	3	7	9	11	17

Effect of hydrogen ion concentration (pH)

The pH of the medium was found to decrease after sterilization. The mycelial growth occurred in the range tested, being maximum at pH 5.98 (Figure.2).

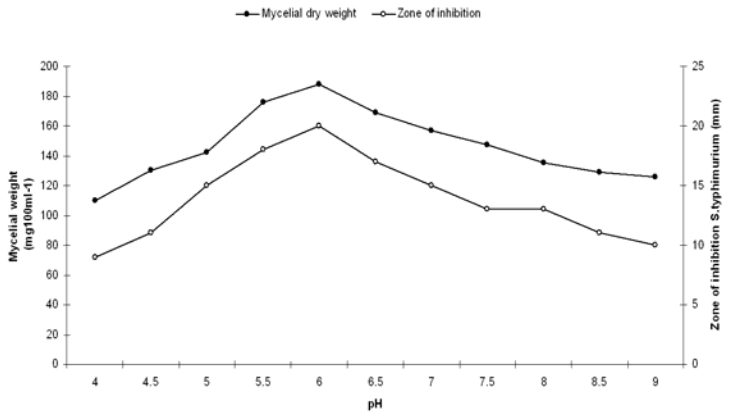

Figure: 2 Effect of different pH on growth of *C. versicolor*

Effect of carbon sources on growth of *C. versicolor*

Every fungus requires nutrient sources, of two basic types *i.e.* carbon and nitrogen, and mineral sources, for its proper growth. The effects of different carbon sources on the mycelial growth of *C. versicolor* were studied in submerged fermentation. *C. versicolor* was found to utilize different carbon sources (Figure.3).

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Maximum growth was observed when starch (168±2 mg 100 mL^{-1}) was used as a carbon source followed by glucose (112±2 mg 100 mL^{-1}) and cellulose (61±1 mg 100 mL^{-1}) while minimum growth was observed with sodium acetate (10.5±0.5 mg 100 mL^{-1}). On using other carbon sources mycelial growth was not found to be satisfactory.

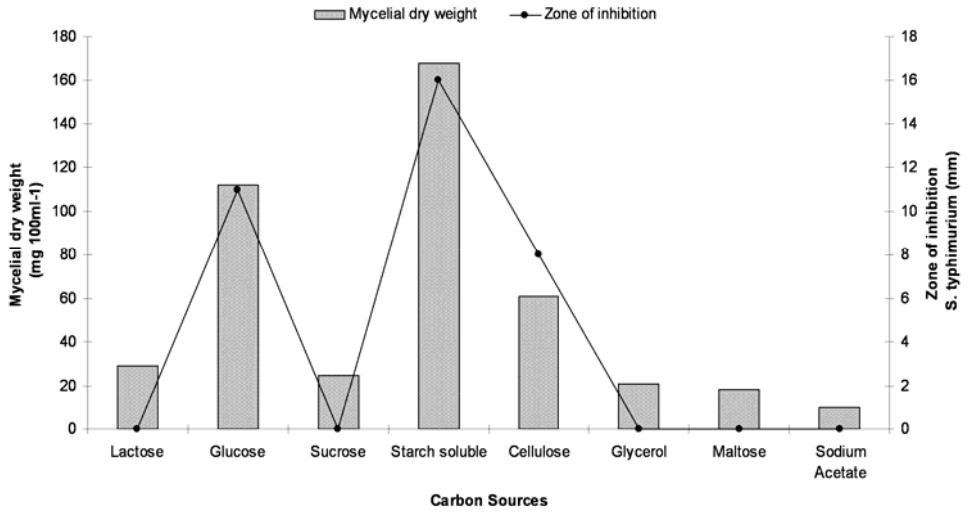

Figure: 3 Effect of different carbon sources on the growth of *C. versicolor*

Effect of Nitrogen sources on growth of *C. versicolor*

Growth of *C. versicolor* was investigated in presence of different nitrogen sources (Figure. 4). In the cultivation media, the carbon source supplemented was the one that obtained optimum in the previous studies. Maximum biomass production occurred when ammonium sulphate was used as nitrogen source i.e., (179±0.5 mg 100 mL^{-1}), while ammonium bicarbonate and ammonium persulphate inhibited the mycelial growth of *C. versicolor*.

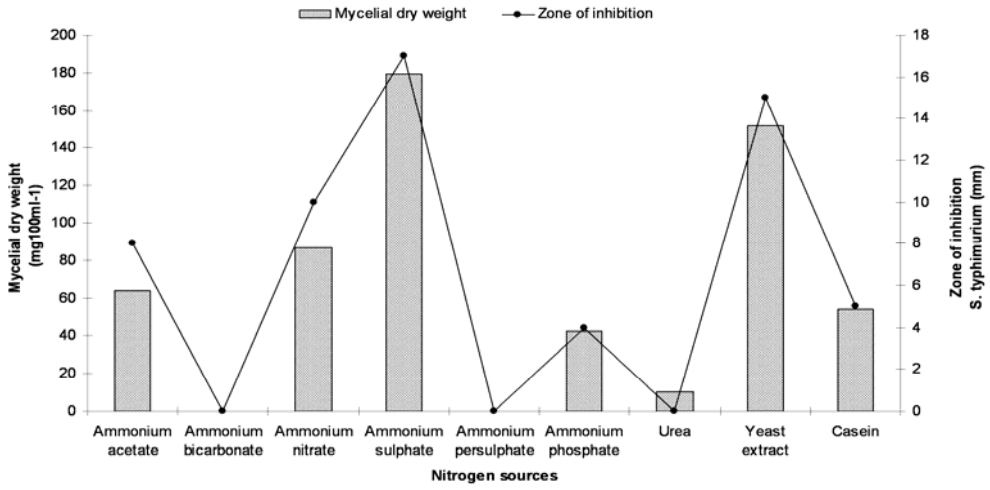

Figure: 4 Effect of different nitrogen sources on *C. versicolor*

Effect of culture filtrate of *C. versicolor* on mycelial growth

The results of effect of *C. versicolor* culture filtrate was noted in terms of colony diameter after 5 days of incubation at 28°C on PDA plates containing different concentrations of culture filtrates. Rapid increase in growth was observed in medium supplemented with culture filtrates as compared to control. The maximum diameter of growth that is 5.3±0.05 cm was observed in media containing 20% (v/v) concentration of culture filtrate (Table.2).

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Table: 2 Effect of different concentrations of culture filtrate on *C. versicolor*

Concentration of Culture filtrate of *Coriolus versicolor* (v/v)	Colony Diameter of *Coriolus versicolor* (cm) (Mean±SD)
1%	4.0±0.05
5%	4.6±0.05
10%	4.8±0.05
15%	5.1±0.05
20%	5.3±0.05
Control	2.3±0.05

DISCUSSION

In submerged culture *C. versicolor* was found to grow as mycelial pellet. The growth tends to be highly viscous because of the suspended filamentous biomass and the extracellular dissolved polymers. The bioactive compounds produced during fermentation can be extracted from either mycelial biomass or from biomass free culture broth. The pelleted growth is considered to be best for extraction of bioactive compounds. In *in vivo* animal studies, *C. versicolor* extract were observed to display a broad spectrum of antibacterial and antifungal activity against common pathogens such as *Escherichia coli*, *Candida albicans* and *Staphylococcus aureus* (Kevin et al 2002). Several scientists have reported that extract of *Coriolus versicolor* possessed antibacterial activity against *Klebsiella pneumoniae* CCM 2318, *Mycobacterium megmatis* CCM 2067 and *Bacillus subtilis* ATCC 6633 (Coban et al 2008). In our studies it was found that both hot water- ethanol extract and Tris HCl-ethanol extracts were effective against gram negative and gram positive bacteria. These results are in accordance to previous reports. Inhibition in growth of *S. typhimurium* a drug resistant bacteria was very prominent indicating that *C. versicolor* extracts could be employed for treatment of enteric infections caused by such organisms.

The temperature and pH conditions during fermentation directly affect the growth metabolism of organisms. The temperature optima for different strains of *C. versicolor* vary in a range 25-30ºC (Rau et al 2009 and Liao,1990). The results of optimization studies of *C. versicolor* showed that 28ºC was most suitable for growth.

The quality, quantity, morphology and nutrient consumption by mycelium are considered important criteria for determining the optimal condition for fungal growth (Yang and Liau,198; Knudson and Stack,1991. Liao, have reported that the maximum mycelial growth of *C. versicolor* was obtained using glucose and mannose as the carbon sources, while no growth was observed in arabinose, glycerol, inositol and ribose. Others have also reported glucose as most widely utilized carbon source by most of the fungi followed by fructose and mannose **(**Griffin,1994 and Tavares at al,2005). Although, it has been reported that glucose concentration is the most important factor in exopolysaccharide production and also in cell growth of *C. versicolor* our investigations revealed that natural starch obtained from potato can also be utilized for production of biomass of *C. Versicolor* (Tavares at al,2005) . Among the saccharides tested hexoses supported high level of mycelial growth(Chang et al,1978). Nitrogen is another important growth determining factor in any medium and it has been observed that no mycelial growth occured in its absence (Lin et al 2003). Role of nitrogen in growth of *C. versicolor* has been reported by (Darbyshire et al 1969). In general, organic nitrogen sources such as casein and peptone are considered to be better for growth of mushroom species as compared to inorganic nitrogen sources (Chang and Miles, 1989). Whereas Liao, has reported that maximum mycelial growth of *C. versicolor* was observed in the presence of ammonium chloride and no growth occurred in the presence of urea. In present studies, ammonium sulphate supported best growth while yeast extract (organic nitrogen) also supported good growth i.e. 152 mg 100 mL^{-1}. On supplementing *C. versicolor* culture filtrate in medium the growth increased but was not logarithmic. The incorporation of culture filtrates may provide extra-nutrient as well as growth factors that supported growth and thus resulted in increased colony diameter of *C. versicolor*.

C. versicolor has been valued through out the world as both food and medicine for thousands of years. Several commercial mushrooms extracts are reported effective against various diseases (Nakazato et, al 1994; Collins and Ng, 1997; Cui et al, 2007). Resistance to antibiotics is emerging in microorganism and multiple drug resistant organisms causing severe hazards in treatment of infectious diseases. Hence, mushroom derived antimicrobial substances have received considerable attention in recent years. It is evident from present analysis that *C. versicolor* extracts can be used to combat diseases caused by pathogenic microorganisms. Crude extracts possessed antagonistic activity against various pathogenic bacteria especially against *Salmonella typhimurium* and can be used to treat diseases caused by them. The extracts showed positive reactions with Benedict reagent, Fehling's reagent and Folins Phenol Ciocalteau reagent indicating presence of proteinaceous and saccharide components in it. Further purification and characterization of bioactive component of *C. versicolor* are in progress. Physiologically active compounds from *C. versicolor* differ in their structure, composition and activity. Thus these extracts represent a potential source and act as a strong barrier to restrict the growth of pathogens.

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