Phytochemical Analysis and Evaluation of Antibacterial Activity of Different Extracts of Soil-Isolated Fungus *Chaetomium cupreum*

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Abstract

Background: *Chaetomium cupreum* is soil-isolated pigment-producing fungus used as biocontrol agent. **Hypothesis:** The aim of the present study was to evaluate the bioactive constituents and antibacterial potential of *C. cupreum* extracts on bacteria such as *Staphylococcus aureus*, *Bacillus subtilis*, *Salmonella typhi*, and *Proteus vulgaris*. **Materials and Methods:** The qualitative and quantitative screening of secondary metabolites was done by standard procedures. The antibacterial activity of different extracts of *C. cupreum* was determined by agar well diffusion method and broth microdilution methods. **Results:** Among the different extracts of *C. cupreum*, ethyl acetate and n-butanol extracts exhibited antibacterial activity. In the n-butanol extract, the most susceptible organism was found to be *B. subtilis* with zone of inhibition 18.5 ± 0.7 mm, followed by *P. vulgaris* 15.5 ± 0.7 mm, *S. typhi* 12.5 ± 3.5 mm, and *S. aureus* 12.0 ± 2.8 mm at 0.5 mg/ml, whereas in the ethyl acetate extract, the most susceptible organism was found to be *P. vulgaris* with zone of inhibition 17.5 ± 0.7 mm followed by *B. subtilis* 17.0 ± 1.4 mm, *S. typhi* 14.0 ± 0.7 mm, and *S. aureus* 13.5 ± 0.7 mm at 0.5 mg/ml. **Conclusion:** The obtained results suggest that *C. cupreum* extracts contain compounds that possess antibacterial activity.

Keywords: Azaphilones, bacteremia, bacterial infections, biopigments, *Chaetomium cupreum*, metabolites, resistance

INTRODUCTION

Microorganisms have caused epidemic, endemic diseases, and deaths of hundreds of millions of humans as well as animal morbidity and mortality worldwide. Microorganisms have developed sophisticated defense mechanisms for their survival in the host cells and to overcome the host defense system such as cells of the immune system, lysozyme attack, cytokinines, complements, antibodies, and phagocytosis.^[1] Bacterial infection process attachment and penetration to epithelial cells, multiplication, synthesis of toxins and enzymes, and finally damages the whole cell.^[2] The *Staphylococcus aureus* is a most pathogenic organism of the genus *Staphylococcus*, and it causes community-acquired and nosocomial infections worldwide.^[3] The *S. aureus* is found in the digestive tracts, livestock, human skin and causes ophthalmic infections with skin infections and sepsis.^[4] It is reported that *S. aureus* causes more fatalities in the USA than AIDS because of antibiotic resistance.^[5] The skin and soft-tissue infections are caused

by methicillin-resistant *S. aureus* (MRSA) strain known as USA300.^[6] The presence of MRSA has also been established in cats, horses, and rabbits by researchers.^[7] *Bacillus subtilis* damages mammalian membrane by producing lecithinase enzyme. In humans, *B. subtilis* causes allergic reactions and food poisoning by the production of extracellular toxins.^[8] This bacterium causes endocarditis in infants, bacteremia in the patients with the impaired immune system.^[9] In hospitals, causes wound infection in hospitalized patients due to the spores production.^[9] *B. subtilis* also contaminates laboratory media.^[8]

The *Salmonella* species are Gram-negative bacteria that cause typhoid fever and enterocolitis, intestinal or diarrheal disease

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in different hosts.^[10] Typhoid fever is one of the global health problem caused by the *Salmonella enterica* serovar typhi.^[11] Typhoid fever transmitted by the ingestion of food, water, or animal products or close contact with an infected individual contaminated with *S. enterica* serovar typhi bacteria.^[12] The *Salmonella* bacteria infect different cells of the immune system and epithelial cells by surviving within the membrane-bound compartment *Salmonella*-containing vacuole for its virulence.^[13] The genus *Proteus* of *Enterobacteriaceae* family^[14] is a Gram-negative, rod-shaped bacteria, cause urinary tract infection (UTI) and wound infections humans and animals. The *Proteus* bacilli cause UTIs, and colonize in different organs of host such as the urethra, ureter bladder, and kidneys.^[15] *Proteus* transmitted by contaminated meat or food items is described as an opportunistic etiological agent of respiratory tract and wounds infections, eyes, ears, nose, burns, skin throat, and gastroenteritis.^[16] The increasing drug toxicity and multidrug resistant to bacteria are serious problems to the well-being of humankind worldwide.

Biopigments or microbial pigments are natural-colored substances produced by microorganisms, especially fungi and bacteria. Biopigments are produced from natural sources which can replace synthetic dyes.^[17] Microorganisms are produced variety of pigments by fermentation process which has higher pigment yields and lower residues compared to that of the plants and animals.^[18] Natural pigments are one of the research alternatives with potent inhibitory properties and minimal side effects. Fungi are also known to produce various pigments with different structures and biological activities. The *Chaetomium* species produce a class of secondary metabolites called azaphilones. Azaphilones are fungal pigments with pyrone-quinone structures and a highly oxygenated bicyclic core with a chiral quaternary center.^[19] Azaphilones possess antimicrobial, anticancer, antiviral, antioxidant, antifungal, and anti-inflammatory activities by the production of vinylogous gamma-pyridones.^[20] The purpose of the present study was to extract the secondary metabolites/biopigments from fungus *Chaetomium cupreum* and explore the antimicrobial activity.

MATERIALS AND METHODS

Isolation of *Chaetomium cupreum*

The *C. cupreum* was isolated from a litter soil sample collected from the GKV Campus, Bengaluru, Karnataka, India. The isolation of fungus *C. cupreum* was carried out from litter soil sample collected from the GKV campus, Bengaluru, Karnataka, India. The isolation was carried out by the serial dilution method on potato dextrose agar (PDA) medium. A volume of 50 mg of soil sample was dissolved in 10 ml of distilled water in a test tube. Then, 1 ml of soil sample was spread onto the PDA medium and incubated at room temperature. After incubation growth of number of different fungal colonies were observed, from which pigment-producing colony was transferred to a new PDA medium plates and incubated at room temperature at 28°C for 3–4 days.

Identification *Chaetomium cupreum*

The pure fungus culture was identified based on morphological and microscopic characteristics and named as *C. cupreum*-SS02.^[21] The morphological identity of *C. cupreum* was confirmed by National Fungal Culture Collection of India (NFCCI), by Agharkar Research Institute, Pune, Maharashtra, India. The molecular technique based on the molecular phylogenetics of the internal transcribed spacer (ITS) region gene sequencing, using universal primers, ITS-1 (TCCGTAGGTGAACCTGCGG) was the forward primer and ITS-4 TCCTCCGCTTATTGATATGC) was the reverse primer was performed.^[22] In GeneBank, homologous sequences search was performed using Basic Local Alignment Search Tool (BLAST) (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/blast>).^[23] The BLAST search for ITS sequence analysis available in GenBank showed 99% homology with other strains of *C. cupreum*. The sequence was deposited in National Centre for Biotechnology information (NCBI) GenBank with accession number KF668034. The culture was deposited in the NFCCI, with accession number NFCCI 3117. The isolated fungus was maintained on PDA plates and PDA slants at 4°C.

Submerged fermentation

For inoculum preparation, the fungus was grown at 25°C on a PDA plate for 7 days, then 5-mm mycelial discs were bored out from the periphery of the colony and transferred to 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) and incubated at 26°C ± 2°C on a rotary shaker at 120 rpm for 20 days to achieve the highest pigment production.^[24] Submerged fermentation produces high mycelial production and thus high is the pigment production in shorter time.^[25] The culture of edible mushrooms produces high pigment production through submerged fermentation.^[24] The *C. cupreum* showed the maximum pigment production in PDB medium. The PDB medium contains nutrients such as metal ions and other micronutrients useful for the enzymes to work effectively and increase the metabolite and pigment production.^[26]

Extraction of extracellular pigments/metabolites

The extraction of pigments was carried out according to the method.^[27] After 20 days of incubation, biomass was removed by filtration through Whatman No. 1 filter paper and the broth containing the extracellular pigment/metabolites was obtained. The cultural broth obtained was used for extraction of pigments/compounds by the liquid-liquid method in 500 ml of separating funnel using four different solvents from nonpolar to polar (chloroform, ethyl acetate, n-butanol, and methanol) in the ratio of 1:1. The 50 ml of filtered broth and 50 ml of solvent was taken in separating funnel and shaken well for 20 min and allowed to stand until the aqueous layer, and organic layers get separated. The organic layer was collected, filtered through Whatman No. 1 filter paper, and transferred to 250 ml of beaker. This process was repeated three times with the same broth and same solvent until no more pigment diffused into the solvent.

The whole broth was extracted by using similar procedure with chloroform, ethyl acetate, n-butanol, and methanol. Then, the organic layer was evaporated using vacuum rotary evaporator at 45°C. The crude dried extract was obtained and stored at 4°C for future use.

Estimation of pigment yield

The mycelium after 20 days of incubation at 28°C ± 2°C at room temperature was separated from broth by filtration through Whatman No. 1 filter paper. The separated mycelium was washed thrice with distilled water and then air-dried at room temperature. The dry weight (DW) of the mycelium and the crude extract produced was estimated. The pigment yield was expressed as mg/g mycelial DW in 1 L of PDB^[28] and calculated by the formula:

$$\text{Biomass estimation} = (\text{DW of crude extract/DW of mycelium}) \times 1000$$

Qualitative screening of secondary metabolites

The isolated four different solvent extracts of *C. cupreum* were screened for the presence of different secondary metabolites by standard procedure.^[29,30] The stock solution of 1.0 mg of fungal extract was diluted in 1.0 ml of the respective solvent.

Alkaloids

Dragendroff's test – A volume of 0.5 ml of fungal extract was mixed with 2 ml of hydrochloric acid (HCl) and 1.0 ml of Dragendroff's reagent. The formation of orange-red precipitate confirms the presence of alkaloids.

Mayor's test – A volume of 1.2 ml of the fungal extract was taken in a test tube, 0.2 ml of 1% aqueous HCl was added and kept in water bath and 0.1 ml Mayer's reagent (potassium mercuric iodide) were added. The appearance of yellow-colored precipitate indicates alkaloids.

Flavonoids

Alkaline reagent test – To a volume of 1.0 ml of the fungal extract, few drops of diluted sodium hydroxide (NaOH) were added. The appearance of yellow color formation which later becomes colorless on addition of a few drops of diluted acid and thus confirms the presence of flavonoids.

Shinoda test – To a volume of 4 ml of fungal extract, 1.5 ml methanol solution (50%) was added, then heated, and metal magnesium was added. Then, few drops of HCl were added, red color will be observed for flavonoids and orange color for flavones.

Carbohydrates

Molisch's test – A small quantity of the fungal extract was dissolved separately in 4 ml of distilled water and filtered, then few drops of alcoholic α-naphthol solution (1%) and 2 ml of conc. sulfuric acid was added. The appearance of brown ring at the junction of two liquids indicates the presence of carbohydrates.

Glycosides

Legal's test – A volume of fungal extract was hydrolyzed in

HCl solution in a water bath. Then, 1.0 ml of pyridine and few drops of sodium nitroprusside solution were added and few drops of NaOH were added. The appearance of pink-red color confirms the presence of glycosides.

Saponins

Foam test – To 1 ml fungal extract, 2 ml of distilled water was added and shaken well. The persistent foam formation indicates the presence of saponins.

Lead acetate test – A volume of 1 ml of the fungal extract was treated with 1% lead acetate solution. The appearance of white precipitate confirms the presence of saponins.

Tannins

Ferric chloride test – To a volume of 0.5 ml of fungal extract, 1.0 ml of water was added in a test tube and filtered. Then, few drops of 0.1% ferric chloride were added and observed for brownish-green or blue-black coloration.

Lead acetate test – To a volume of 5.0 ml of the fungal extract, a few drops of 1% lead acetate was added. The formation of yellow precipitate confirms the presence of tannins.

Potassium dichromate test – A 5.0 ml of the fungal extract was mixed with 1.0 ml aqueous potassium dichromate solution (10%). The yellowish-brown precipitate confirms the presence of tannins.

Phytosterol

Salkowski test – A volume of 1.0 ml of fungal extract was mixed with 1.0 ml of conc. sulfuric acid. The appearance of red color confirms the presence of sterols.

Phenolic

Folin–Ciocalteu test – To a volume of 1.0 ml of fungal extract, 1.0 ml of diluted Folin–Ciocalteu reagent (1:1) was added. The appearance of blue color confirms the presence of phenolic compounds.

Terpenoids

Salkowski's test – To a volume of 2.0 ml of fungal extract, a few drops of concentrated sulfuric acid were added. The formation of reddish-brown precipitate produced indicates the presence of terpenoids.

Coumarins

To a volume of 2 ml of fungal extract, 3 ml of 10% NaOH was added. The formation of yellow color indicates the presence of coumarins.

Azaphilones

To a volume of 2 ml of fungal extract was mixed with few drops of ammonia. The formation of red for purple color indicates the presence of azaphilones.

Quantitative estimation of secondary metabolites

Determination of total flavonoid content

The flavonoids content was determined spectrophotometrically according to the aluminum chloride (AlCl₃) method.^[31] Flavonoids give yellow color when treated with AlCl₃ reagent (1% w/v),

and this principle is used in flavonoid estimation. Fungal extract 0.5 ml was mixed with 0.15 ml of sodium nitrite (5%) and 2 ml distilled water and the reaction mixture was allowed to react for 6 min. After 6 min, 0.15 ml AlCl_3 (10% AlCl_3 w/v) solution and 2 ml of NaOH (4%) solution are added, then 5 ml distilled water was added. The solution was allowed to react for 15 min and absorbance was measured at 510 nm. The calibration curve was generated from rutin reference standard and results of total flavonoid content (TFC) are expressed as rutin equivalents (REs/g DW).

Antibacterial activity of solvent extracts from *Chaetomium cupreum*

Bacterial strains

The cultures used for antibacterial activity such as *S. aureus* (NCIM 2079), *Salmonella typhi* (NCIM 2051), *Proteus vulgaris* (2027), and *B. subtilis* (2063) were obtained from the National Chemical Laboratory, Pune, Maharashtra, India. All the tested bacteria were maintained on the Mueller Hinton agar (MHA) medium.

Agar well diffusion method

The well diffusion method was employed for the determination of antimicrobial activity^[32] with slight modifications. Briefly, 5 mm wells were made on preinoculated solidified agar medium (inoculum size: 100 μl of a microbial suspension containing 10^8 CFU/ml of bacteria, (McFarland's standard), wells were filled with 50 μl of n-butanol and ethyl acetate extracts dissolved in dimethyl sulfoxide (DMSO) (0.5 to 2 mg/ml) and the plates were incubated at 37°C for 24 h. DMSO was used as negative control (50 μl /ml) and streptomycin (25 μg /ml) as positive control. The zone of Inhibition diameters was measured in millimeters (mm) after the incubation period.

Determination of minimum inhibitory concentration and minimum bactericidal concentration by Broth microdilution method

The broth microdilution method was used (M27-A2, Clinic and Laboratory Standards Institute (CLSI))^[33] to determine the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs). Two hundred microliters of two-fold serially diluted n-butanol and ethyl acetate extracts in nutrient broth (1000 TO 0.95 μg /ml) were added to the wells of 96-well microtiter plate and inoculated with 50 μl of a bacterial suspension containing 10^8 CFU/ml and incubated at 37°C for 24 h. The antibiotic streptomycin was used as positive control and DMSO as a negative control. After incubation, the MICs and MBCs values were recorded. Fifty microliters of cultured broth was radically streaked onto the MHA medium and further incubated for respective time and temperature as mentioned above.

RESULTS

Isolation and identification of the fungus

Species of *Chaetomium* are capable of producing novel secondary metabolites. In the present study, *C. cupreum* is taken for testing its production for secondary metabolites. The isolated

fungus was identified as *C. cupreum* based on morphological and microscopic characteristics. The identification was further confirmed by NFCCI and Fungal Identification Service, Agharkar Research Institute, Pune, Maharashtra, India. The culture was deposited in the NFCCI, with accession number NFCCI 3117. BLAST search performed for the sequence of ITS analysis showed 99% homology with other strains of *C. cupreum* available in GenBank. The sequence was deposited in NCBI GenBank with accession number KF668034.

Cultural Morphology.

The *C. cupreum* exhibited typical white cottony colonies on the PDA. The diffusion of deep red color into the media was observed around the colony after 7–10 days of incubation on PDA plate, which suggested that *C. cupreum* produces extracellular, water-soluble pigments [Figure 1].

Extraction of secondary metabolites

The extraction of extracellular metabolites from *C. cupreum* was done by using cold extraction method [Figure 2]. The extraction revealed high pigment yield through submerged fermentation method from *C. cupreum*. The extraction was done by using four different solvents from nonpolar to polar in the consecutive manner (chloroform, ethyl acetate, n-butanol, and methanol).

Estimation of pigment yield

The dry yield of extracellular product produced by *C. cupreum* after submerged fermentation was estimated. The dry pigment yield for chloroform extract was 26.85 mg/g DW, ethyl acetate extract 120.06 mg/g DW, n-butanol extract 69.85 mg/g DW, and methanol extract 176.73 mg/g DW from 1 L of PDB after 20 days of incubation [Figure 3]. The maximum pigment yield was found in methanol extract, followed by ethyl acetate extract and n-butanol extract, whereas least yield was found in chloroform extract. The chloroform extract was yellow

Figure 1: Morphology of *Chaetomium cupreum* on potato dextrose agar medium. A-dorsal view, B-ventral view, C-Perithecia, D-Perithecia with ascospores by using lactophenol cotton blue stain

Figure 2: Extraction of pigments from *Chaetomium cupreum* by different solvents

Figure 3: Estimation of pigment yield of different solvent extracts of *Chaetomium cupreum*

in color, whereas ethyl acetate was bright red and n-butanol was sticky paste deep red in color and methanol extract was colorless paste [Figure 4].

Qualitative screening of secondary metabolites

The fermentation and extraction method showed different

types of secondary metabolites in *C. cupreum* extracts. The secondary metabolites such as flavonoids, carbohydrates, saponins, tannins, glycosides, phytosterol, phenolic terpenoids, azaphilones, and coumarins compounds were present, whereas alkaloids were absent in the *C. cupreum* extracts [Table 1]. In chloroform extract of *C. cupreum* different types of secondary metabolites such as, flavonoids, saponins, tannins, terpenoid, azaphilones, and coumarins were present, whereas carbohydrates and phenolic compounds were absent. In ethyl acetate extract, except alkaloids and terpenoid all other secondary metabolites such as flavonoids, carbohydrates, saponins, tannins, glycosides, phytosterol, phenolic, azaphilones, and coumarins were present. In n-butanol extract, secondary metabolites such as flavonoids, saponins, tannins, azaphilones, and coumarins compounds were present, whereas carbohydrates, alkaloids, and phenolic compounds were absent. In methanol extract, all secondary metabolites were present except alkaloids and phytosterol compounds. Thus, these results showed that the extraction method is greatly based on the solvent polarity. Terpenoids also possess antimicrobial, antifungal, antiviral,

anti-allergenic, antispasmodic, antihyperglycemic, and immunomodulatory properties. Saponins have precipitating and coagulating properties for red blood cells. The *C. cupreum* extracts also contain carbohydrates, glycosides, and coumarins which are known to be useful for the immune system by increasing body strength. The present study indicated that *C. cupreum* extracts are potential source of different secondary metabolites such as flavonoids, saponins, tannins, phytosterol, phenolic, azaphilones, coumarins, and terpenoid compounds. All the extracts contain flavonoids, terpenoids, and saponins.

Figure 4: (a) Rutin standard calibration curve. (b) Determination of total flavonoid content by aluminum chloride method. Experimental results are expressed as mean $\pm$ standard deviation ($n = 3$)

Quantitative estimation of secondary metabolites

Total flavonoid content

The TFC of *C. cupreum* was obtained from calibration curve of standard rutin ($y = 0.38 \times 1.828$; $r^2 = 0.986$) and expressed as milligrams RE/100 g DW of the extract [Figure 5a]. The TFC of different extract of *C. cupreum* is presented in Figure 5b. The results showed significant amount of flavonoid content present in all the four extracts of *C. cupreum*. In chloroform extract, the TFC was obtained with a value of 5.52 ± 0.00 mg of Rutin equivalent /grams dry weight, in ethyl acetate extract the flavonoid content with the value of 9.98 ± 2.87 mg RE/g DW, in n-butanol extract the flavonoid content with the value of 10.23 ± 2.87 mg RE/g DW, and in methanol extract the flavonoid content with the value of 5.61 ± 0.19 mg RE/g DW was obtained at 50 μ g/ml concentration. The results showed that n-butanol extract possesses higher flavonoid content than the ethyl acetate extract of *C. cupreum* at different concentrations.

The results obtained showed higher TFC in n-butanol extract (10.23%) followed by ethyl acetate extract (9.98%), methanol extract (5.61%), and chloroform extract (5.52%). The flavonoid content percentage increased with increase in concentration. This indicates that middle polar solvents are more suitable for flavonoid compound extraction than the polar solvents. The TFC of methanolic extracts of two endophytic fungi *Aspergillus* species (CPIL-1) and *Penicillium* species (CPIMR-2) in terms of quercetin equivalent, the flavonoid content was 130.50 ± 6.11 mg/g and 94.91 ± 4.48 mg/g DW, respectively, of fungal extract.^[34] Thus, from the present investigation, medicinal properties of the *C. cupreum* can be identified based on the phytochemicals present in it.

Antibacterial activity

Antibacterial activity of ethyl acetate extract

The effect of ethyl acetate extract of *C. cupreum* on the antibacterial activity is presented in Figure 5a and b. In

Table 1: Qualitative screening of secondary metabolite of *C. cupreum* extracts

Secondary metabolites	Tests	Chloroform extract	Ethyl acetate extract	n-butanol extract	Methanol extract
Alkaloids	Dragendroffs	-	-	-	-
	Mayers	-	-	-	-
Test for flavonoids	Alkaline reagent	+	+	+	+
Test for carbohydrates	Molischs	-	+	-	+
Test for glycosides	Legals	-	+	+	+
Test for saponins	Foam	+	+	+	+
	Lead acetate	+	+	+	+
Test for tannins	Ferric chloride	-	+	+	+
	Lead acetate	+	+	+	+
	Potassium dichromate	+	+	+	+
Test for phytosterol	Salkowski	-	+	-	-
Test for phenolic compounds	Follin ciocalteau	-	+	-	+
Test for terpenoids	Salkowki	+	-	+	+
Coumarins	NaOH	+	+	+	+
Azaphilones	Ammonia	+	+	+	+

(-Absent, +Present)

Figure 5: (a) Antibacterial activity of ethyl acetate extract of *Chaetomium cupreum* by agar well diffusion method. Values are represented as mean $\pm$ standard deviation ($n = 3$). Significance difference between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are significantly different at $P < 0.05$. (b) The zone of inhibition diameter at different concentration of the ethyl acetate extract of the *Chaetomium cupreum*. The zone of inhibition was measured against (A) *Staphylococcus aureus*, (B) *Salmonella typhi*, (C) *Proteus vulgaris*, (D) *Bacillus subtilis*. (Positive control (PC) – Tetracycline, Negative control (NC) - DMSO)

ethyl acetate extract of *C. cupreum*, the most susceptible organism was found to be *P. vulgaris* with zone of inhibition of 17.50 ± 0.70 mm in diameter followed by *B. subtilis* 17.00 ± 1.40 mm, *S. typhi* 14.00 ± 140 mm, and *S. aureus* 13.50 ± 0.70 mm at 0.5 mg/ml, whereas at higher concentration, the zone of inhibition of *B. subtilis* was 21.50 ± 0.70 mm in diameter, followed by *P. vulgaris* 20.50 ± 0.70 , *S. aureus* 18.00 ± 0.95 mm, and *S. typhi* 17.00 ± 0.70 mm at 2.0 mg/ml.

Antibacterial activity of n-butanol extract

The effect of antibacterial activity of n-butanol extract of *C. cupreum* is presented in Figure 6a and b. In n-butanol extract of *C. cupreum*, the most susceptible bacteria was found to be *B. subtilis* with zone of inhibition of 18.50 ± 0.70 mm in diameter, followed by *P. vulgaris* 15.50 ± 0.70 mm and *S. typhi* 12.15 ± 0.90 mm, whereas *S. aureus* was the least sensitive with zone of inhibition of 12.00 ± 2.98 mm in diameter at 0.5 mg/ml. Similarly, at higher concentration of n-butanol extract, *B. subtilis* was most sensitive with zone of inhibition of 21.50 ± 0.93 mm in diameter followed by *P. vulgaris* 20.00 ± 0.90 mm and *S. typhi* 17.50 ± 0.85 mm, whereas *S. aureus* was with zone of inhibition 15.00 ± 0.40 mm

Figure 6: (a) Antibacterial activity of n-butanol extract of *Chaetomium cupreum* by agar well diffusion method. Values are represented as mean $\pm$ standard deviation ($n = 3$). Significance difference between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are significantly different at $P < 0.05$. (b) The zone of inhibition diameter at different concentration of the n-butanol extract of the *C. cupreum*. The zone of inhibition were measured against (A) *Staphylococcus aureus*, (B) *Salmonella typhi*, (C) *Proteus vulgaris*, (D) *Bacillus subtilis*. (Positive control (PC) - Tetracycline, Negative control (NC) - DMSO)

in diameter at 2.0 mg/ml. In the butanol extract, it was observed that all the four bacteria exhibited antibacterial activity with maximum zone of inhibition ranging from 12.00 ± 0.98 to 18.50 ± 0.70 mm in diameter at 0.5 mg/ml and 15.00 ± 0.40 – 21.50 ± 0.93 mm in diameter at 2.0 mg/ml. The comparison of the standard compound with *C. cupreum* extracts in antimicrobial activity is represented in Table 2.

Minimum inhibitory concentrations

The MIC values of n-butanol and ethyl acetate extract of *C. cupreum* are presented in Table 3. The MICs values of n-butanol extract of *C. cupreum* exhibited by each organism is 0.62 mg/ml against both *S. aureus* and *P. vulgaris*, 0.31 mg/ml against *B. subtilis* and 0.25 mg/ml against *S. typhi*, respectively. Whereas, the MIC values of ethyl acetate extract exhibited by different organisms are 0.015 mg/ml against *S. typhi*, 0.031 mg/ml against *P. vulgaris*, 0.062 mg/ml against *S. aureus*, and 0.125 mg/ml against *B. subtilis*. The MIC values of standard compound observed are 0.031 μ g/ml for *P. vulgaris* and *S. aureus*, 0.062 μ g/ml for *B. subtilis*, and 0.125 μ g/ml for *S. typhi*. The antimicrobial activity of dichloromethane of *Chaetomium atrobrunneum* with zone of inhibition 25 mm against

Table 2: The comparison of standard compound tetracycline with *C. cupreum* extracts in antimicrobial activity

	Antibacterial activity			
	<i>Proteus vulgaris</i>	<i>Bacillus subtilis</i>	<i>Salmonella typhi</i>	<i>Staphylococcus aureus</i>
Ethyl acetate extract (0.5 mg/ml)	17.50±0.70	17.00±1.40	14.00±1.40	13.50±0.70
n-Butanol extract (0.5 mg/ml)	15.50±0.70	18.50±0.70	12.15±0.90	12.00±2.98
Tetracycline (25 µl/ml)	24.00±0.70	23.50±0.70	18.00±0.87	16.00±0.90

Table 3: MIC and MBC of n-butanol and ethyl acetate extracts of *C. cupreum*

Name of organism	n-Butanol		Ethyl acetate		Tetracycline	
	Concentration (µg/ml)					
	MBC	MIC	MBC	MIC	MBC	MIC
<i>Staphylococcus aureus</i>	1.00	0.062	1.00	0.062	0.031	0.015
<i>Salmonella typhi</i>	0.5	0.25	0.062	0.015	0.125	0.007
<i>Proteus vulgaris</i>	0.25	0.062	0.25	0.031	0.031	0.015
<i>Bacillus subtilis</i>	0.062	0.031	0.5	0.125	0.062	0.007

Escherichia coli and 13 mm against *S. aureus* at 0.04 g/10 ml and ethyl acetate extract with zone of inhibition 23 mm against *E. coli* and 13 mm against *S. aureus* at 0.03 g/10 ml.^[35]

Whereas in the present study, in ethyl acetate extract of *C. cupreum*, the most susceptible organism was to found be *P. vulgaris*, whereas *S. aureus* was the least sensitive at 0.5 mg/ml [Figure 6a]. Furthermore, in n-butanol extract of *C. cupreum*, the most susceptible organism was *B. subtilis* followed by *P. vulgaris* at 0.5 mg/ml [Figure 6a]. The antibacterial effect of tetracycline standard was also studied, and it was found that the most susceptible organism was *P. vulgaris* with zone of inhibition diameter 24.00 ± 0.70 mm in diameter, followed by *B. subtilis* 23.50 ± 0.70 mm, *S. typhi* 18.00 ± 0.87 mm, and *S. aureus* 16.00 ± 0.90 mm at 25 µg/ml. In the present study, ethyl acetate and n-butanol extracts of *C. cupreum* showed moderate antibacterial activity. Whereas, chloroform and methanol extracts have not exhibited any antibacterial activity.

Minimum bactericidal concentrations

The MBCs of n-butanol and ethyl acetate extract of *C. cupreum* are shown in Table 3. The MBC values of n-butanol extract of *C. cupreum* exhibited by each organism is 0.062 mg/ml against *B. subtilis* 0.25 mg/ml against *P. vulgaris* 0.5 mg/ml against *S. typhi* and 1 mg/ml against *S. aureus*. Whereas, the ethyl acetate extract shows the MBC value of 0.062 against *S. typhi*, 0.25 mg/ml *P. vulgaris*, 0.5 mg/ml against *B. subtilis*, and 1 mg/ml against *S. aureus* respectively. The MBC values of standard compound observed for each organism are 0.007 µg/ml for *B. subtilis* and *S. typhi* and 0.015 µg/ml for *P. vulgaris* and *S. aureus* [Table 3].

Discussion

The *C. cupreum* was isolated from soil sample and identified on the basis of morphological, cultural, and molecular

characteristics. The *C. cupreum* produces red-colored, extracellular, and water-soluble pigments on PDA and PDB. In biomass estimation, maximum dry yield was found in methanol extract followed by ethyl acetate extract, whereas in chloroform, less dry yield was found. In the present study, chloroform and methanol extracts of *C. cupreum* did not show antibacterial activity, which indicates that the extracts do not contain active compounds which could be responsible for antimicrobial activities. Whereas, ethyl acetate and butanol extracts showed antibacterial activity indicating the presence of broad-spectrum antibacterial substances in the *C. cupreum* which can be effectively applied against human pathogenic bacteria. These results show that these pigment extracts are exhibiting higher antimicrobial activities as compared to methanolic extracts from mushroom samples, which have exhibited MIC value in the range of 2.5–10 mg/ml,^[35] and they are also exhibiting better antimicrobial activity as even compared to the dichloromethane and ethyl acetate extract of the *C. atrobrunneum* against *E. coli* and *S. aureus*.^[36] The MIC and MBC values of our extracts are comparable with the values that are reported by certain endophytic fungal extracts.^[37] Further work is needed toward the evaluation of their antimicrobial potential against a wider range of microorganisms and finally the identification and characterization of the active compounds responsible for this activity that will provide new starting material for the development of novel antibiotics.

Conclusion

The *C. cupreum*, a well-known soil fungus, was used to study the antibacterial activities by using its different solvents extracts. The *C. cupreum* was isolated from litter soil and grown in PDB. The obtained solvent extracts were subjected for testing antimicrobial activity. The butanol and ethyl acetate crude extracts showed significant antibacterial activities, and the results are comparable to standard antibiotic compound, respectively. The obtained results clearly confirmed that both the n-butanol and ethyl acetate extracts of the *C. cupreum* contain potential bioactive compounds that can be used in pharmaceutical purposes after toxicological and preclinical evaluations.

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Conflicts of interest

There are no conflicts of interest.

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