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# ANTIMICROBIAL ACTIVITY OF SECONDARY METABOLITES FROM *CHAETOMIUM CUPREUM*-SS02 ISOLATED FROM SOIL

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## ABSTRACT

The antimicrobial efficacy of methanol, ethyl acetate, chloroform and butanol extracts of fungus *Chaetomium cupreum* was determined by employing well diffusion and broth micro-dilution techniques. *Chaetomium* species are the largest genus of saprophytic ascomycetous fungus, which belongs to the class Sordariomyetes and family *Chaetomiaceae*. Ethyl acetate and butanol extracts exhibited antibacterial activity against four bacteria, *Staphylococcus aureus, Salmonella typhi*, *Proteus vulgaris, Bacillus subtilis* tested. In the butanol extract, all the four bacteria exhibited antibacterial activity with zone of inhibition ranging from 12.0 to 18.5mm at 0.5mg/ml concentration. In the butanol extract, the most susceptible organism was found to be *B. subtilis* followed *P. Vulgaris,S. typhi* and *S. aureus* Whereas in ethyl acetate extract, all the four bacteria exhibited antibacterial activity with zone of inhibition ranging from 13.5 to 17.0 mm at 0.5mg/ml concentration. The most susceptible organism was to found be *P. Vulgaris* followed by *B. subtilis*, *S. typhi* and *S. aureus*

**KEYWORDS**: ***Chaetomium cupreum*, Azaphilones, epipolythiodioxopiperazines, metabolites.**

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# INTRODUCTION

Fungi are ubiquitous in nature, which are known to produce a variety of metabolites belonging to numerous groups of chemical compounds, including organic acids, polyynes, polyketides (such as quinones, anthraquinones, xanthones, etc.), mono– to triterpenes (including volatiles and steroids), bioactive polysaccharides, lipopolysaccharides, proteins, N– and S–containing compounds [1,2].The genus *Chaetomium* fungi is considered to be a rich source of novel and bioactive secondary metabolites of great importance. *Chaetomium* is with the largest number of species among saprophytic ascomycetes, which belongs to the *Chaetomiaceae* family. Since Kunze first established this genus in 1817, more than 350 *Chaetomium* species have been described[3, 4, 5]. This fungal species is widely distributed in different biotopes, such as soils, marine, animal dung, hair, textiles, plant seeds and some other substrates rich in cellulose. *Chaetomium* species are often used to produce cellulose in industry, and are also used as biocontrol agents in agriculture[6]. Untill now, a variety of more than 200 secondary metabolites belonging to diverse structural types such as chaetoglobosins, epipolythiodioxopiperazines, azaphilones, xanthones, anthraquinones, chromones, depsidones, terpenoids, chaetomin, chaetoglobosins, cochliodinol sterigmatocystin, O-methylsterigmatocystin, chaetochromin, chaetocin and mollicellin G and steroids have been discovered[7]. Most of these fungal metabolites exhibited antitumor, cytotoxic, antimalarial, enzyme inhibitory, antibiotic, as natural dyes and other activities. Chaetomium species produce an interesting set of coloured secondary metabolites, called azaphilones. Azaphilones can be defined as: "Structurally diverse class of fungal secondary metabolites (polyketide derivatives), namely pigments with pyrone-quinone structures containing a highly oxygenated bicyclical core and a chiral quaternary center[8, 9]. Their name arose as a result of their affinity for ammonia. They react with amino group present in proteins, amino acids and nucleic acids to form red or purple *vinylogous*ɣ-*pyridones* due to the exchange of pyrane oxygen for nitrogen and thus form water-soluble pigments[10]. Azaphilones exhibit a wide range of interesting biological activities such as antimicrobial, antifungal, antiviral, antioxidant, cytotoxic, nematicidal and anti-inflammatory activities. The potent biological activities of azaphilones may be related to their production of vinylogous ɣ-pyridones[11, 12].Investigations on *Chaetomium* species have been previously reported in numerous types of compounds such as benzoquinone derivatives [13], tetra-S-methyl derivatives [14],azaphilones[15, 16, 17],bis-azaphilones[17], indo-3-yl-[13] cytochalasans and chaetogobosinanalogs[18, 19, 20, 21, 22, 23, 24], anthraquinone-chromanone[24], globosumones[25], chaetochalasin A [26], depsidones [26, 27]and longirostrerones A-D [28]. *Chaetomium cupreum* is an abundantly found soil fungus exhibiting antagonism against numerous fungal phytopathogens[29]. Ketomium, a commercial product, has been developed from this species, and is being widely used as broad spectrum bio- fungicide for the disease control in various crops [30]. It is also known to biodegrade catechin, a well-known recalcitrant compound[31]. The purpose of the present study was to extract the secondary metabolites from fungus *Chaetomium cupreum* and explore and then the antimicrobial activity of the metabolites produced by this fungus.

# MATERIALS AND METHODS

### *1.Isolation and Identification of fungus.*

*Chaetomium cupreum* was isolated from a litter sample collected from the GKVK campus, Bangalore. The isolation was carried out by the serial dilution method[32] on Potato Dextrose Agar (PDA) medium. The isolated fungus was identified as *Chaetomium cupreum*SS02 based on colony morphological and microscopic characteristics. The morphological identity was confirmed by National Fungal Culture Collection Of India NFCCI, Agharkar Research Institute, Pune, India. To confirm the species, sequence analysis of the ITS region using universal primers (Forward primer, ITS 1 – TCCGTAGGTGAACCTGCGG and Reverse primer, ITS 4Z-TCCTCCGCTTATTGATATGC) was performed. Nucleotide Blast to the obtained sequence was performed in NCBI (www.ncbi.nlm.nih.gov/) using blast suite. 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 *Chaetomium cupreum* available in Gen bank. The sequence was deposited in NCBI Genbank with accession Number KF668034.*Chaetomium cupreum*SS-02 culture, isolated and identified in the lab was used in the studies. The stock culture was maintained on a PDA slant at 4 ºC.

### *2.Fermentation and Extraction*

For inoculum preparation, the fungus was grown at 25°C on a PDA plate for 5 days, then 5 mm mycelial discs were bored out from the periphery of the colony and transferred to 100 ml of Potato Dextrose Broth and incubated at 26 ± 2°C on a rotary shaker at 120 rpm for 20 to 24 days. After incubation, biomass was removed by filtration and the broth containing the extra cellular pigment was obtained. This broth was then filtered using Whatman filter paper No. 1 to remove the residual mycelium. The broth obtained was used for extraction of the pigments using various organic solvents, depending on their polarity. Solvents like methanol, ethyl acetate, chloroform and butanol were used to extract the pigments. The solvent phase was collected, filtered and removed by evaporation under vacuum, using rotary evaporator. The crude dried extract was obtained and stored at 4c˚ for further use.

### *3. Antibacterial activity of solvent extracts from Chaetomium cupreum*

### *3.1. Bacterial strains*

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

### *3.2. Agar diffusion method*

The well diffusion method was employed for the determination of antimicrobial activity according to the method [33], with slight modifications. Briefly, 5mm wells were made on pre-inoculated 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 butanol and ethyl acetate extracts dissolved in DMSO (2 to 0.5 mg/ml) and the plates were incubated at 37°C for 24hrs. DMSO served as a negative control and Streptomycin (25µg/ml) served as positive control. The zone of Inhibition diameters was measured in millimetres (mm) after the incubation period.

### *3.3. Determination of MIC and MBC by Broth micro dilution method*

The broth microdilution method was used (as described in M27-A2, CLSI)[34] to determine the Minimum Inhibitory Concentrations (MICs) and Minimum bactericidal Concentrations (MBCs). 200 µl of two-fold serially diluted butanol and ethyl acetate extracts in nutrient broth (NB) (100 to 0.95 µg/ml) were added to the wells of a sterile 96-well microtiter plate and inoculated with 50 µl of a bacterial suspension containing $10^8$ cfu/ml, and incubated at 37 °C for 24hrs.The antibiotic streptomycin was used as positive control and DMSO served as a negative control. After incubation, the Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs) values were recorded. Minimum Inhibitory Concentrations (MICs) is defined as the lowest concentration at which no visible microbial growth is observed. Fifty microlitre of cultured broth was radially streaked onto the MHA medium and further incubated for respective time and temperature. The complete absence of growth on the agar surface at the lowest concentration is defined as the MBC.

## RESULTS AND DISCUSSION

### *1.Fermentation and Extraction*

Diffusion of deep red coloured pigments into the media was observed around the colony on PDA plate and suggested that it is water soluble metabolite.The different quantity of 204mg of methanol extract, 139 mg of ethyl acetate extract, 31.0 mg of chloroform extract, 80.8 mg of butanol extract was obtained per gram of mycelium biomass dry weight.

### *2.Antibacterial activity of the solvent extracts*

The extraction of metabolites was done from broth by using differents solvents such as chloroform, ethyl acetate, methanol and butanol. The results revealed that among the four different solvent extracts, ethyl acetate and butanol extract showed antibacterial activity whereas chloroform and methanol extract did not show antibacterial activity.The antibacterial activity of ethyl acetate and butanol extracts of *Chaetomium cupreum* was determined by employing well diffusion and broth micro-dilution techniques. Both ethyl acetate and butanol extracts showed a concentration-dependent antibacterial activity against all the four bacteria, *Staphylococcus aureus, Salmonella typhi*, *Proteus vulgaris, Bacillus subtilis*. The negative control DMSO did not inhibit any tested microorganisms. In the butanol extract, all the four bacteria exhibited antibacterial activity with maximum inhibition zone with diameter of 12.0 to 18.5mm at 0.5 mg/ml concentration. The most susceptible organism was found to be *B. subtilis* with zone of inhibition18.5 mm, followed by *P. Vulgaris*15.5 mm *S. typhi* 12.5 mm and *S. aureus* 12.0 mm at 0.5mg/ml concentration (Table.1; Figure). In the ethyl acetate extract, all the four bacteria exhibited antibacterial activity with maximum inhibition zone with diameter of 13.5 to 17.0 mm at 0.5 mg/ml concentration. The most susceptible organism was to found be *P. Vulgaris* with zone of inhibition 17.5 mm followed by *B. subtilis*17.0 mm, *S. typhi* 14 mm and *S. aureus* 13.5 mm at 0.5mg/ml concentration (Table. 2; Figure 2).

### *3.Determination of MIC and MBC*

Both Minimum Inhibitory Concentrations (MICs) and Minimum Bactericidal Concentrations (MBCs)of butanol and ethyl acetate extracts against all the four bacteria is presented in Table 3. The butanol extract is exhibiting the MIC value of 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*. Whereas the butanol extract shows the MBC value of 0.062 mg/ml against *B.subtilis* 0.25 mg/ml against *P. vulgaris* 0.5 mg/ml against *S. typhi* and and 1mg/ml against *S.aureus*.The ethyl acetate extract is exhibiting the MIC value of 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* Whereas the ethyl acetate extract shows the MBC value of and 0.062 against *S. typhi,*0.25 mg/ml *P. Vulgaris*, 0.5 mg/ml against *B.subtilis* and 1mg/ml against *S.aureus*.

## DISCUSSION

These results show that these pigment extracts are exhibiting higher antimicrobial activities as compared to methonalic extracts from mushroom samples, which have exhibited MIC value in the range of 2.5 to 10mg/ml[35] and they are also exhibiting better antimicrobial activity as even compared to the dichloro methane and ethyl acetate extract of the *chaetomium atrobrunneum* conducted aganist *E.coli* and *S. aureus*[36].The MIC and MBCvalues of our extracts are comparable with the values that are reported by certain endophytic fungal extracts [37].In the present study, all tested bacteria were resistant towards chloroform and methanol crude extracts of *Chaetomium cupreum* which indicates that the extracts do not contain active compounds that 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 *Chaetomium cupreum* which can be effectively applied against human pathogenic bacteria.

**Table 1**
***Antimicrobial activity of butanol extract of Chaetomium cupreum***

| Name of organism | Concentrations (mg/ml) | | | | Streptomycin (100µg/ml) |
|---|---|---|---|---|---|
| | 0.5 | 1.0 | 1.5 | 2.0 | |
| | Zones of inhibition in mm | | | | |
| *Staphylococcus aureus* | 12.0±2.8 | 13.5±1.4 | 14.0±0.7 | 15.0±0.4 | 16±1.41 |
| *Salmonella typhi* | 12.5±3.5 | 13.5±0.7 | 15.5±0.5 | 17.5±3.5 | 18 ±1.41 |
| *Proteus vulgaris* | 15.5±0.7 | 16.0±0.2 | 17.5±0.6 | 20.0±4.2 | 24.5±0.7 |
| *Bacillus subtilis* | 18.5±0.7 | 19.0±0.7 | 20.0±1.7 | 21.5±1.1 | 23.5±2.21 |

**Table 2**
***Antimicrobial activity of ethyl acetate extract of Chaetomium cupreum***

| Name of organism | Concentrations (mg/ml) | | | | Streptomycin (100µg/ml) |
|---|---|---|---|---|---|
| | 0.5 | 1.0 | 1.5 | 2.0 | |
| | Zones of inhibition in mm | | | | |
| *Staphylococcus aureus* | 13.5±0.7 | 15.5±0.7 | 17.0±0.7 | 18.0±4.2 | 19.5±2.1 |
| *Salmonella typhi* | 14±1.4 | 14.5±0.7 | 15.5±0.7 | 17.0±0.7 | 17.5 ±0.7 |
| *Proteus vulgaris* | 17.5±0.7 | 18.1±0.4 | 19.2±0.5 | 20.5±0.7 | 25.5±0.7 |
| *Bacillus subtilis* | 17.0±1.4 | 18.0±4.2 | 19.0±0.6 | 21.5±0.7 | 25±0.70 |

**Table 3**
***MIC and MBC (in mgs) of butanol and ethyl acetate extracts of Chaetomium cupreum.***

| Name of organism | Butanol | | Ethyl acetate | | Streptomycin | |
|---|---|---|---|---|---|---|
| | MBC | MIC | MBC | MIC | MBC | MIC |
| | Concentration in mg/ml | | | | | |
| *Staphylococcus aureus* | 1.00 | 0.062 | 1.00 | 0.062 | 0.031 | 0.015 |
| *Salmonella typhi* | 0.5 | 0.25 | 0.062 | 0.015 | 0.125 | 0.007 |
| *Proteus vulgaris* | 0.25 | 0.062 | 0.25 | 0.031 | 0.031 | 0.015 |
| *Bacillus subtilis* | 0.062 | 0.031 | 0.5 | 0.125 | 0.062 | 0.007 |

**Figure 1**
***Antimicrobial activity of butanol extract of Chaetomium cupreum treated to (A) Staphylococcus aureus, (B) Salmonella typhi, (C) Proteus vulgaris, (D) Bacillus subtilis. NC is negative control and PC is positive control.***

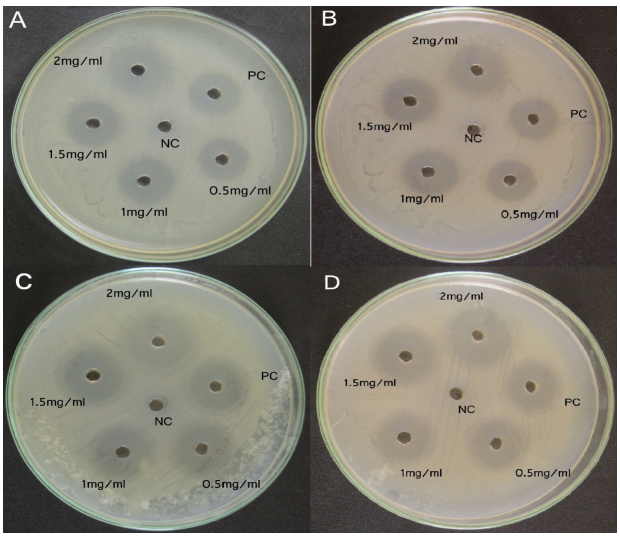
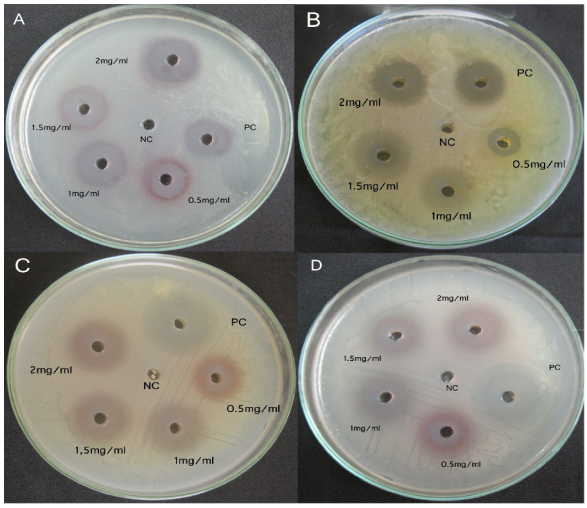


**Figure 2**

***Antimicrobial activity of ethyl acetate extract of Chaetomium cupreum treated to (A) Staphylococcus aureus, (B) Salmonella typhi, (C) Proteus vulgaris, (D) Bacillus subtilis. NC is negative control and PC is positive control.***




## CONCLUSION

*Chaetomium cupreum*, a well known soil fungi was used to study the antibacterial activities by using its different solvents extracts. The *Chaetomium 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 activites and the results are comparable to standard antibiotic compound, respectively. The obtained results clearly confirmed that both the butanol and ethyl acetate extracts of the *Chaetomium cupreum* contain potential bioactive compounds that can be used in pharmacological purposes after toxicological and preclinical evaluations. 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.

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