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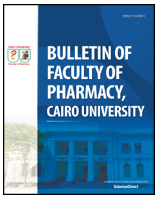

Original Article

Evaluation of antioxidant properties of different extracts of *Chaetomium cupreum* SS02

Nazir Ahmad Wani[a], Sharmila Tirumale[a,*]

[a] *Department of Microbiology and Biotechnology, Jnanabharathi Campus, Bangalore University, Bengaluru-560056, Karnataka, India*

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ABSTRACT

To evaluate the antioxidant properties of different extracts (chloroform, ethyl acetate, n-butanol and methanol extracts) of fungus *C. cupreum* SS02. The antioxidant properties were evaluated by using five antioxidant methods, metal chelating assay, 2,2-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid (ABTS•), hydroxyl radical (HO•) scavenging assay, superoxide anion ($O_2^{\cdot}$) radical scavenging assay, and nitric oxide ($NO^{\cdot}$) scavenging assay. Methanol extract of *C. cupreum* showed highest metal chelating activity (63.61 ± 0.07 mg EDTAE/g DW), followed by chloroform extract (57.25 ± 0.12 mg E DTAE/g DW) and n-butanol extract (27.33 ± 0.07 mg E DTAE/g DW) whereas ethyl acetate extract showed least metal chelating activity (18.35 ± 0.07 mg E DTAE/g DW) at 50 µg/ml. In ABTS assay, n-butanol extract showed highest ABTS inhibition activity (19.91 ± 0.14 µmol RE/g DW), followed by ethyl acetate extract (13.07 ± 0.59 µmol RE/g DW) and chloroform extract (13.07 ± 0.82 µmol RE/g DW) whereas methanol extract showed the lowest activity (11.65 ± 0.16 µmol RE/g DW at 50 µg/ml). In hydroxyl radical scavenging assay, n-butanol extract showed highest scavenging activity (31.95 ± 0.21 mg RE/g DW), followed by ethyl acetate extract (28.19 ± 0.21 mg RE/g DW), methanol extract (22.93 ± 0.37 mg RE/g DW) and chloroform extract (18.04 ± 0.21 mg RE/g DW) at 50 µg/ml. In superoxide anion scavenging assay, chloroform extract showed highest scavenging activity (56.44 ± 0.03 mg RE/g DW) followed by ethyl acetate extract (49.88 ± 0.09 mg RE/g DW), n-butanol extract (19.49 ± 0.09 mg RE/g DW) and methanol extract (7.75 ± 0.06 mg RE/g DW) at 50 µg/ml. The highest inhibition of nitric oxide radical was observed in chloroform extract of *C. cupreum* (11.23 ± 0.11%) followed by ethyl acetate extract (7.62 ± 0.06%), n-butanol extract (4.72 ± 0.90%) and methanol extract (3.20 ± 0.06%) at 50 µg/ml. The results showed that different extracts of *C. cupreum* have significant antioxidant activity due to the presence of different phytochemicals. Thus, it is suggested that *C. cupreum* extracts should be further studied for their antioxidant properties for food and pharmaceutical applications.

1. Introduction

The free radicals are molecules containing one or more unpaired electrons in the atomic or molecular orbital's [1]. It is because of this unpaired electron which gives a high degree of reactivity to the free radical. The free radicals are products of a normal cellular mechanism. These are divided into two types, reactive oxygen species (ROS) and reactive nitrogen species (RNS). The ROS includes oxygen radicals and other oxidants which are converted into radicals whereas NOS includes nitric oxide and nitrogen dioxide radicals and nonradicals such as N_2O_3, ONOO- and nitrous acid. The reactive oxygen species (ROS) and reactive nitrogen species have both deleterious and beneficial effects on the living system [2]. The excess of ROS and NOS causes serious biological damage which is termed oxidative stress and nitrosative stress respectively [3–5]. The oxidative stress results from the overproduction of ROS and NOS on one side and deficiency of enzymatic and non-enzymatic antioxidants on another side. This results in the disturbance in the equilibrium balance of prooxidant and antioxidant in the cell. This disturbance causes deleterious damage to cell lipids, membranes, proteins, DNA and inhibiting their normal function. Oxidative stress has been associated with various diseases such as carcinogenesis, aging, atherosclerosis, neurodegenerative diseases (Alzheimer's disease and Parkinson's disease), rheumatoid arthritis, cancer, diabetes mellitus and hypertension [6]. Thus it is important for a cell to maintain a constant balance between the beneficial and harmful effects of free radicals and this process is called redox regulation. The redox

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* Corresponding author.
E-mail address: sharmilabub@gmail.com (S. Tirumale).

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regulation protects the organisms from oxidative stress and maintains a mechanism called redox homeostasis [7]. Metals on the other hand such as cobalt, iron, chromium, and copper can undergo redox reactions and produce reactive species such as nitric oxide and superoxide anion radical which are harmful to the cells [8]. The iron-induced oxidative stress is found to involve in human colorectal cancer [5]. The asbestos contains about 30% (weight) of iron and is found to increase the risk of asbestosis, the second major cause of lung cancer [9].

Most of the free radicals are derived from oxygen [10]. The molecular oxygen (dioxygen) is itself is a radical and an addition of one electron to dioxygen forms superoxide anion radical (O2•−) [10]. The production of superoxide occurs in mitochondria of a cell [11]. The superoxide generated both in vivo and in foods can undergo several reactions, including dismutation to give H_2O_2. The hydroxyl radical (•OH) is produced from reduction of oxygen and is the most reactive free radical [12]. The nitric oxide (NO•) is a radical with one unpaired electron in the orbital. The NO• is generated in the cells by nitric oxide synthases which metabolize arginine to citrulline with the formation of NO• [13]. NO• reacts with oxygen and water to form nitrate and nitrite anions. The nitrosative stress condition is associated with increased production of reactive nitrogen species [14,15]. This condition results when NO• production exceeds the organism's ability to neutralize and eliminate them. The nitrosative stress causes nitrosylation reactions in the cell and alters protein structure and function.

Fungi are ubiquitous in nature which are known to produce different types of secondary metabolites belonging to numerous groups of chemical compounds, including organic acids, polyenes and phenolic compounds (quinones, anthraquinones, xanthones), mono to triterpenes (including volatiles and steroids), bioactive polysaccharides, lipopolysaccharides, proteins, and N, S containing compounds [16]. *Chaetomium* is a fungus established by Kunze in 1817, of the family Chaetomiaceae, division Ascomycota. *Chaetomium* is one of the largest genera of saprophytic ascomycetes which comprises of > 300 species worldwide [17,18]. Chaetomium species produce an interesting class of secondary metabolites, called as azaphilones. The most remarkable and valuable properties of azaphilones include their natural origin, yellow-red spectra, thermostability (in comparison with other natural red pigments) and water solubility. The azaphilone compounds produced by different *Chaetomium* species display various biological activities such as antioxidant, nematicidal, antimicrobial, antifungal, anticancer and inflammatory activities [19]. However, there are no studies on the antioxidant properties of *C. cupreum* extract. Hence, this study was conducted to investigate the antioxidant properties of extracellular water-soluble metabolites from *C. cupreum* SS02.

2. Materials and methods

2.1. Chemicals and reagents

Ethyl acetate, n-butanol, methanol, sulphuric acid, hydrochloric acid, acetonitrile were purchased from Merck (Mumbai, India). Whatman No. 1 filter paper, potato dextrose agar, potato dextrose broth, ferric chloride, iron sulfate, potassium persulfate, ABTS reagent, EDTA, rutin, naphthylethylenediamine dihydrochloride, potato dextrose agar, potato dextrose broth were purchased from Merck (Mumbai, India).

2.2. Isolation of C. Cupreum

The isolation of fungus *C. cupreum* was carried out from litter soil sample collected from the Gandhi Krishi Vignana Kendra (GKVK) campus, Bangalore, Karnataka, India. For inoculation preparation, in 10 ml of distilled water 50 mg of soil sample was added. Then, 1 ml test sample was transferred to freshly prepared PDA medium and incubated at 28 °C. Further isolation was carried out by diluting the soil sample till pure colony of fungus was obtained on potato dextrose agar (PDA) medium plates. After incubation, the growth of different types of fungal colonies was observed, from which pigment producing colonies were sub-cultured and incubated at 28 °C [20].

2.3. Identification of C. Cupreum

The fungus *C. cupreum* was isolated from a litter soil sample collected from the GKVK campus, Bangalore, Karnataka, India. The isolation was carried out by the serial dilution method on potato dextrose agar (PDA) medium. The strain with pigment production was selecting and identified as *Chaetomium cupreum*-SS02 based on morphological and microscopic characteristics [21]. The morphological identity was confirmed by NFCCI, Agharkar Research Institute, Pune, India. For species identification based on the molecular phylogenetics of the internal transcribed spacer (ITS) -5.8S region of rDNA using universal primers; ITS-1 (TCCGTAGGTGAACCTGCGG) was the forward primer and ITS-4 TCCTCCGCTTATTGATATGC) was the reverse primer was performed [22]. Search for homologous sequences in Gen Bank was performed using BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/ blast) [23]. 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 Gen Bank with accession Number KF668034. The *Chaetomium cupreum* culture was deposited in the National Fungal Culture Collection of India (NFCCI) Agharkar Research Institute, Pune, India, with accession Number NFCCI 3117. The isolated fungus was maintained on potato dextrose agar (PDA) plates and potato dextrose agar slants at 4 °C.

2.4. Fungus maintenance and cultural media

The isolated fungus was maintained on potato dextrose agar (PDA) plates and potato dextrose agar slants at 4 °C. In submerged fermentation, potato dextrose broth (PDB) medium was used for high metabolite production.

2.5. Inoculum preparation and submerged fermentation (SmF) conditions

For inoculum preparation, the fungus was grown at 28 ± 2 °C on a PDA plate for 7 days, than 5 mm fungal discs were transferred to 250 ml Erlenmeyer conical flasks containing 100 ml autoclaved PDB medium and incubated at 28 ± 2 °C on a rotary shaker at 120 rpm for 20 days to achieve the highest pigment production [24]. The fungus grows maximum when a temperature is maintained at 28 ± 2 °C. For maximum growth of fungi, dextrose acts as the carbohydrate source. The optimum pH was maintained at 7.0 for high pigment production.

2.6. Extraction of extracellular metabolites

The extraction of metabolites was carried out according to the liquid-liquid method [25]. After 20 days of incubation, biomass was removed by filtration through Whatman No. 1 filter paper and the broth containing the extracellular pigment or metabolites was obtained. The cultural broth obtained was used for extraction of pigments or metabolites by the liquid-liquid method in 500 ml of separating funnel using four different solvents from non-polar 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 separated. The organic layer was collected, filtered through Whatman No. 1 filter paper and transferred to 250 ml of a 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 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.

2.7. Antioxidant activity

The five different methods such as metal chelating, ABTS, nitric oxide, hydroxyl radical assays were used for evaluating antioxidant activities of different extracts of *C. cupreum*. Stock solutions of each fraction of *C. cupreum* were prepared separately in methanol at 5 mg/ml concentration from dry weight. These stock solutions were diluted in methanol to provide six different concentrations, 50, 100, 200, 300, 400 and 500 µg/ml. The EDTA and rutin standard stock solutions were also prepared in 5 mg/ml and were diluted in methanol to make different concentrations.

2.8. Determination of metal chelating activity

Ferrozine can form complexes with ferrous iron yielding a red colour. However, in the presence of chelating agents, there is disruption of the formation of the complex which leads to a decrease in the red colour. The metal chelating activity of *C. cupreum* extracts were carried out by a standard procedure [26]. The reaction mixture consists of 100 µl of fungal extract of different concentrations, 50 µl of 2 mM $FeCl_2{\cdot}4H_2O$ (0.002 g $FeCl_2$ in 10 ml distilled water) and 200 µl of 5 mM ferrozine (0.02462 g Ferrozine in 10 ml distilled water) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the solution (ferrous ion) for the formation of a red ferrous ion ferrozine complex was measured spectrophotometrically at 562 nm by using a UV–Visible Spectrophotometer. The lower absorbance at 562 nm indicates a higher chelating power. The chelating activity of extracts on Fe^{2+} was compared with that of ethylenediaminetetra acetic acid (EDTA) at a level of 50 µg/ml. The EDTA was used as reference standard. All the measurements were taken in triplicate and expressed as mean value ± standard deviation. The % metal chelating was calculated by given equation:

$$\% \text{ Inhibition} = A_0 - A_1/A_0 \times 100$$

where A_0 is absorbance of control and A_1 is absorbance of fungal extract.

2.9. Determination of free radical scavenging activity by 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$))

This assay is based on the ability of antioxidants to scavenge the 2, 2′-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid ($ABTS^{\bullet+}$) radical cation produced by reacting ABTS solution with a strong oxidizing agent such as potassium permanganate or potassium persulfate [27]. According to this procedure, the radical cation was prepared by mixing 10 ml of 7.4 mM ABTS stock solution (0.0389 g of ABTS in 10 ml of distilled water) with 10 ml of 2.45 mM potassium persulfate (0.0648 g potassium persulfate in 10 ml of distilled water) to produce free radicals persulfate in the dark for 12–16 h at room temperature. The $ABTS^{+}$ solution was diluted with ethanol (0.90 ml ABTS, 25 ml of ethanol) to obtain an the absorbance of 0.700 ± 0.02 at 734 nm spectrometrically. The reaction mixture consists of 0.1 ml of fungal extract and 0.9 ml of the $ABTS^{\bullet}+$ solution were shaken thoroughly and allowed to react for 30 min and absorbance was recorded at 734 nm. Results are expressed as µmol rutin equivalents (RE)/g dry weight.

$$\text{Inhibition} = A_0 - A_1/A_0 \times 100$$

where A_0 is absorbance of control (without sample) and A_1 is the absorbance of the fungal material

2.10. Determination of hydroxyl radical scavenging activity

The scavenging activity of *C. cupreum* extracts was determined by hydroxyl radical scavenging assay [28]. A volume of 100 µl of fungal extract of different concentration were added with 1.0 ml of iron-EDTA solution (0.13% ferrous ammonium sulphate and 0.26% EDTA), 0.5 ml of EDTA solution (0.018%), 1.0 ml of dimethyl sulphoxide (DMSO) (0.85% v/v in 0.1 M phosphate buffer, pH 7.4) and 0.5 ml of ascorbic acid (0.22%). The reaction sample was incubated in water bath incubated for 15 min at 80–90 °C and terminated by adding 1.0 ml of trichloroacetic acid (17.5% w/v, ice cold). A volume of 3 ml of Nash reagent ((75.0 g of ammonium acetate, 3.0 ml of glacial acetic acid, and 2 ml of acetyl acetone) was added to each tube and further incubated for 15 min at room temperature and absorbance was read at 412 nm. The reaction mixture containing all the reagents except sample was used as a control. Rutin was used as a standard compound. The scavenging % was calculated by the given formula:

$$HRSA(\%) = A_0 - A_1/A_0 \times 100$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of the fungal extract.

2.11. Determination of superoxide anion ($O_2^{\bullet}$) radical scavenging activity

Superoxide anion radical scavenging activity of different extracts of *C cupreum* was determined by the nitroblue tetrazolium reduction method [29]. The reaction mixture consists of 100 µl of *C. cupreum* extracts of different concentrations, 1.0 ml of nitroblue tetrazolium (NBT) regent (5 mM NBT in 50 mM phosphate buffer, pH 7.4), 1.0 ml of NADH solution (5 mM NADH in 50 mM phosphate buffer, pH 7.4) and 1.0 ml of PMS (5 mM PMS in 50 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25 °C for 5 min and intensity of the colour formed was measured using spectrophotometer at 560 nm against blank samples. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was added, and for the positive control sample was replaced with rutin. The decrease in absorbance at 560 nm by treatment with different extracts indicates increased superoxide scavenging activity in the reaction mixture. The abilities to scavenge the superoxide anion radical were calculated using the following equation,

$$\text{Superoxide radical scavenging activity}(\%) = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the sample extract.

2.12. Determination of nitric oxide ($NO^{\bullet}$) radical scavenging

This method is based on the inhibition of nitric oxide radical generated from sodium nitroprusside in phosphate buffer saline and determined by the Griess reaction [30]. A volume of 0.5 ml of fungal extracts of *C. cupreum* with different concentrations was added to 2.0 ml of 10 mM sodium nitroprusside PBS (0.7425 g/250 ml PBS, pH 7.4) and incubated for 150 min at room temperature. A volume of 0.5 ml of the Griess reagent (1% sulphanilamide, 2% *ortho*-phosphoric acid and 0.1% naphthylethylenediamine dihydrochloride) was added and the mixture was incubated at room temperature at 25 °C for 5 min. The scavengers of NO compete with oxygen, leading to the production a light pink to deep purple colored chromophore in the reaction mixture which is measured at 546 nm. The positive and negative controls were subjected to the same procedures as the sample, except that for the negative control, only the solvent was added, and for the positive control sample was replaced with rutin. Rutin was used as a standard. The decrease in absorbance was calculated by the equation:

$$\% \text{ Inhibition} = A_0 - A_1/A_0 \times 100$$

where A_0 is the absorbance of the control (without sample) and A_1 is the absorbance of the fungal extract

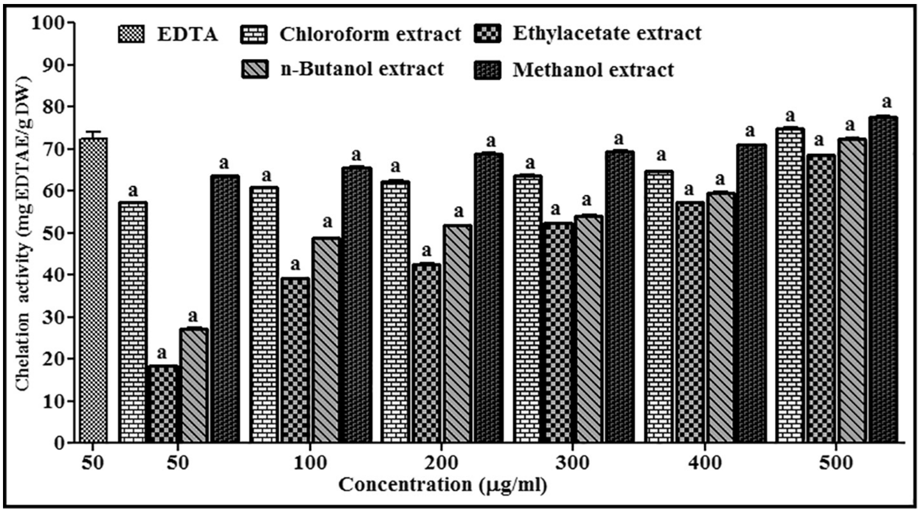

Fig. 1. Effect of different extracts of *C. cupreum* on metal chelating activity. Reference standard: EDTA. Bars in each column with common letters indicating significant difference ($p < 0.05$) according to Bonferroni Posttests. Experimental results are mean ± SD (n = 3).

2.13. Statistical analysis

The data was analyzed for statistical significance using analysis of variance (ANOVA), at 95% confidence level with Graph Pad Prism 6 software (Graph Pad Software, Inc., USA). The $p < 0.05$ was considered as significant. The Pearson's correlation test was used for correlation. The experimental results are expressed as mean ± SD of three independent measurements.

3. Results

3.1. Effect of ferrous ion chelating assay

The metal chelating activity of different extracts of *C. cupreum* is presented in Fig. 1. Among the four different extracts of *C. cupreum* studied for metal chelating activity, methanol extract showed higher metal chelating activity (63.61 ± 0.07 mg E DTAE/g DW), followed by chloroform extract (57.25 ± 0.12 mg E DTAE/g DW) and n-butanol extract (27.33 ± 0.07 mg E DTAE/g DW) whereas ethyl acetate extract showed least metal chelating activity (18.35 ± 0.07 mg E DTAE/g DW) at 50 μg/ml concentration respectively. Whereas metal chelating activity of EDTA standard was 72.50 mg E DTAE/g DW 50 μg/ml. Whereas chelating effect at higher concentration ranged from 74.96 ± 0.01 to 77.58 ± 0.43 mg EDTAE/g DW at 50 μg/ml in all the four extracts of *C. cupreum*. The results indicates all the four extracts have significant metal chelating activity and it was also found that chelating activity increases with the increase in concentration.

3.2. Effect of $ABTS^{\bullet +}$ radical scavenging activity

The ABTS inhibition percentage of different extracts of *C. cupreum* and their comparison with rutin standard is presented in Fig. 2. In ABTS assay rutin was used as reference standard and results were expressed as μmol rutin equivalents (RE)/g dry weight (DW). Among the four different extracts of *C. cupreum* studied for ABTS inhibition activity, n-butanol extract showed higher ABTS inhibition activity with the value of 19.91 ± 0.14 μmol RE/g DW, followed by ethyl acetate extract with the ABTS inhibition value of 13.07 ± 0.59 μmol RE/g DW and chloroform extract with the ABTS inhibition value of 13.07 ± 0.82 μmol RE/g DW whereas methanol extract showed the lowest ABTS inhibition activity with the value of 11.65 ± 0.16 μmol RE/g DW at 50 μg/ml concentration respectively. Whereas rutin standard showed higher ABTS value of 56.80 ± 2.10 μmol RE/g DW at 50 μg/ml concentration respectively. Whereas at higher concentration, n-butanol extract showed ABTS inhibition activity with the value of 53.53 ± 0.58 μmol RE/g DW, followed by ethyl acetate extract with the ABTS inhibition value of 44.98 ± 0.24 μmol RE/g DW and chloroform extract with the ABTS inhibition value of 40.57 ± 0.82 μmol RE/g DW whereas methanol extract showed the lowest ABTS inhibition activity with the value of 36.15 ± 0.17 μmol RE/g DW at 500 μg/ml concentration respectively. The results showed that *C. cupreum* extracts exhibit strong ABTS inhibition activity.

3.3. Effect of hydroxyl radical scavenging activity

The hydroxyl radical scavenging activity of different extracts of *C. cupreum* and their comparison with rutin standard is presented in Fig. 3. The results showed higher hydroxyl radical scavenging activity in n-butanol extract with the value of 31.95 ± 0.21 mg RE/g DW, followed by ethyl acetate extract with the value of 28.19 ± 0.21 mg RE/g DW and methanol extract with the value of 22.93 ± 0.37 mg RE/g DW whereas chloroform extract showed least scavenging activity with the value of 18.04 ± 0.21 mg RE/g DW at 50 μg/ml concentration respectively. The rutin standard showed scavenging activity of 70.21 ± 1.50 mg RE/g DW at 50 μg/ml concentration respectively. Whereas at higher concentration at 500 μg/ml, the scavenging activity of n-butanol extract was 83.45 ± 0.26 mg RE/g DW, followed by ethyl acetate with value of 70.67 ± 0.21 mg RE/g DW, chloroform extract with value of 69.17 ± 2.24 mg RE/g DW and methanol extract with value of 61.65 ± 0.2 mg RE/g DW respectively.

3.4. Effect of superoxide anion scavenging activity

The superoxide radical scavenging activity of different extracts of *C. cupreum* and their comparison with rutin standard is presented in Fig. 4. The highest superoxide anion scavenging activity was shown by chloroform extract (56.44 ± 0.03 mg RE/g DW) followed by ethyl acetate extract (49.88 ± 0.09 mg RE/g DW) and n-butanol extract (19.49 ± 0.09 mg RE/g DW) whereas methanol extract showed lowest scavenging activity (7.75 ± 0.06 mg RE/g DW) at 50 μg/ml concentration respectively. The superoxide anion scavenging activity of rutin standard was 85.30 ± 1.50 mg RE/g DW at 50 μg/ml concentration respectively. Similarly, at 500 μg/ml concentration, the highest superoxide anion scavenging activity was shown by chloroform extract (74.81 ± 0.14 mg RE/g DW) followed by ethyl acetate extract (72.43 ± 0.42 mg RE/g DW) and n-butanol extract (67.13 ± 0.25 mg RE/g DW) whereas methanol extract showed lowest scavenging activity (52.67 ± 0.96 mg RE/g DW) at 50 μg/ml concentration respectively

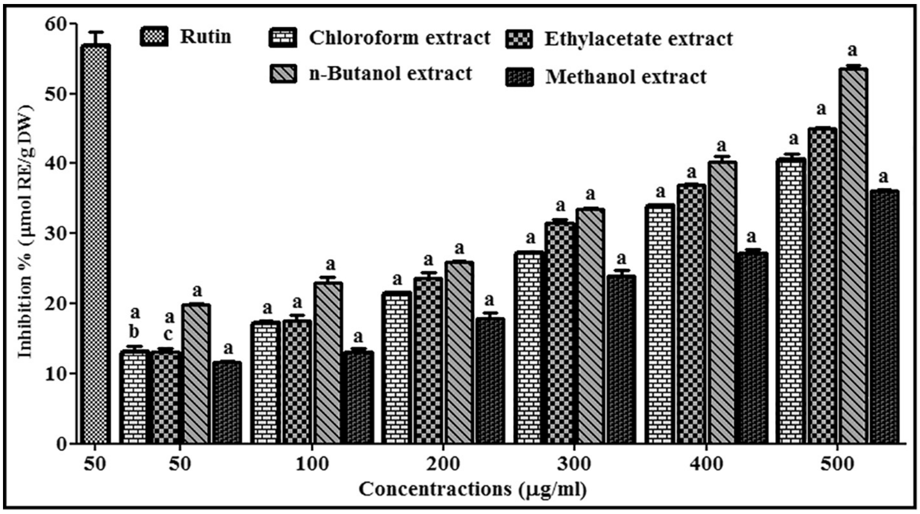

Fig. 2. Effect of different extracts of *C. cupreum* on ABTS inhibition. Reference standard: rutin. Bars in each column with common letter indicating significant difference ($p < 0.05$) according to Bonferroni Posttests. Experimental results are mean ± SD (n = 3).

3.5. Effect of nitric oxide radical scavenging assay

The nitric oxide radical scavenging activity of different extracts of *C. cupreum* and their comparison with rutin standard is presented in Fig. 5. The highest nitric oxide radical inhibition percentage was observed in ethyl acetate extract with the inhibition value of 17.62 ± 0.06%, followed by chloroform extract with the inhibition value of 11.23 ± 0.11%, n-butanol extract with the inhibition value of 4.72 ± 0.90% and methanol extract with the inhibition value of 3.20 ± 0.06% at 50 µg/ml concentration respectively. Whereas the inhibition of nitric oxide of rutin standard was 75.87 ± 1.50 at 50 µg/ml concentration respectively. Similarly at higher concentration at 500 µg/ml, the higher nitric oxide inhibition percentage was observed in ethyl acetate extract with the inhibition value of 70.88 ± 0.06%, followed by chloroform extract with the inhibition value of 56.58 ± 0.06%, n-butanol extract with the inhibition value of 28.09 ± 0.06% and methanol extract with the inhibition value of 14.25 ± 0.06% respectively.

4. Discussion

Free iron can form oxygen-derived free radicals during Fenton reaction which can results in various health problems [31]. So, the chelating of free iron by chelating agents or antioxidants from natural source can prevent the formation of these free radicals [32,33]. This method is based on the capacity to decolorize the iron-ferrozine complex. Ferrozine is a substance which can reacts with iron to form a coloured complex. The antioxidants compounds involve in the chelation of prooxidant metals. The transition metals act as catalysts for various free radical reactions by promoting oxidation. The redox-active transition metals (iron, copper, cobalt, chromium, vanadium, cadmium, nickel) transfer single electrons during redox reactions. The chelation of these metals by the chelating compounds decreases their prooxidant effect by reducing their redox potentials and stabilizing the oxidised form of the metal. The iron can stimulate lipid peroxidation by decomposition of lipid hydroperoxides into peroxyl and alkoxyl radicals which are harmful for the cell [34]. The antioxidant activity involves the chelation of metals thereby preventing catalysis of hydroperoxide decomposition. The chelating compounds inhibit the metal hydroperoxide complex formation which results in decrease in colour formation [35]. The measurement of colour reduction indicates the chelating power of the fungal extract. A decrease in absorbance indicates a higher chelating power of the extract. The results presented in Fig. 1 shows that highest metal chelating activity was found in methanol extract

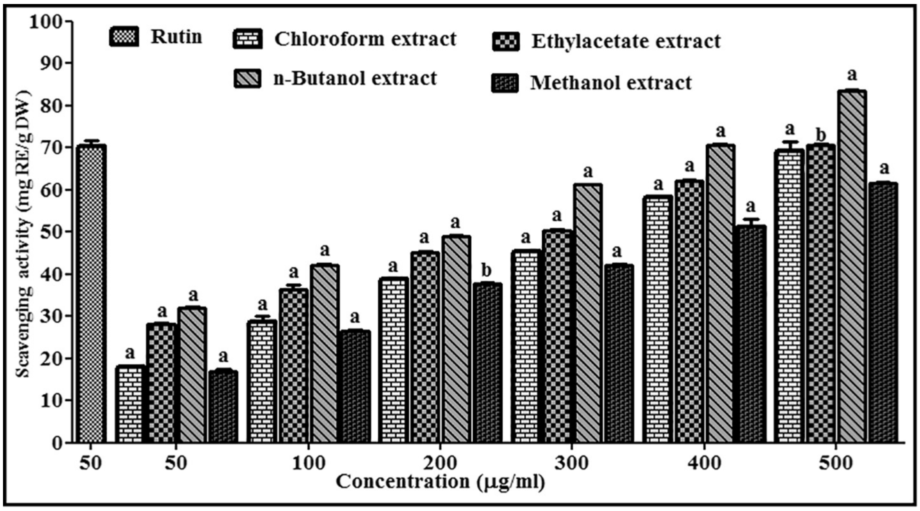

Fig. 3. Effect of different extracts of *C. cupreum* on scavenging activity by hydroxyl radical scavenging assay. Reference standard: rutin. Bars in each column with common letters indicating significant difference ($p < 0.05$) according to Bonferroni Posttests. Experimental results are mean ± SD (n = 3).

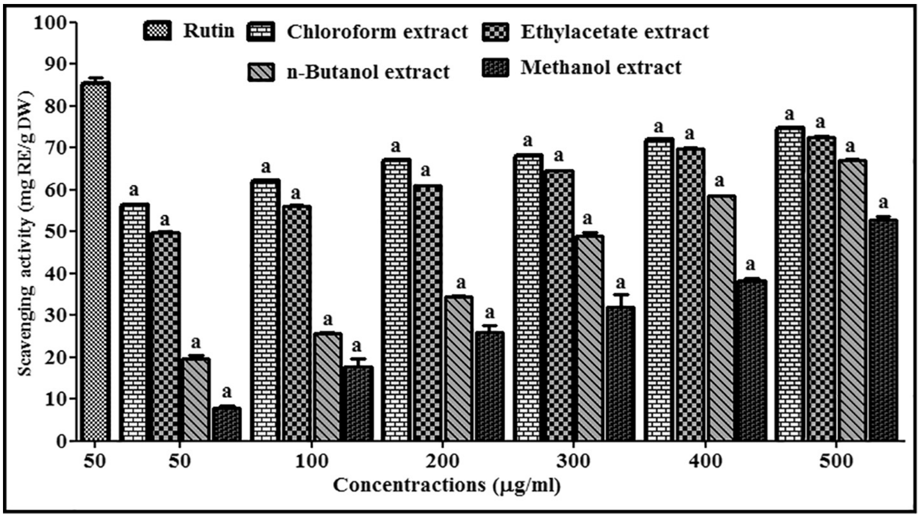

Fig. 4. Effect of different extracts of *C. cupreum* on scavenging activity by superoxide radical scavenging assay. Reference standard: rutin. Bars in each column with common letters indicating significant difference ($p < 0.05$) according to Bonferroni Posttests. Experimental results are mean ± SD (n = 3).

(63.61%) followed by chloroform extract (57.25%), n-butanol extract (27.33%) whereas ethyl acetate extract showed lowest metal chelating (18.35%) 50 µg/ml Similarly, the metal chelating activity of EDTA standard was 72.50% at the same concentration. There was significant difference observed for metal chelating activity between different extracts of *C. cupreum* ($p < 0.05$) according to Bonferroni's posttests. Also, a strong correlation was observed between chloroform extract and methanol extract ($r = 0.98$, $p > 0.05$) of *C. cupreum*. The metal chelating activity of *C. cupreum* extracts and standard EDTA decreased in the order of EDTA > methanol extract > chloroform extract > n-butanol extract > ethyl acetate extract at 50 µg/ml respectively. It was also found that all the four different extracts were capable of chelating Fe^{2+} ions. The results showed that there is decrease in colour formation with increase in concentration.

The ABTS free radical is formed by oxidation of ABTS with potassium persulfate which is reduced by hydrogen donating capacity of the fungal extract [36]. Similarly, in the presence of a hydrogen donating antioxidant compound or the fungal extract, the nitrogen atom quenches the hydrogen atom and thus decolorizes the solution. The hydrogen-donating ability of different extracts of *C. cupreum* was evaluated by measuring the decrease in the absorbance spectrum in the blue-green ABTS radical reaction. In Fig. 2 the highest ABTS inhibition percentage was found in the n-butanol extract (19.91%) of *C. cupreum* followed by ethyl acetate extract (13.07%), chloroform extract (13.07%) and methanol extract (11.65%). Whereas the ABTS inhibition percentage was less in all the four extracts of *C. cupreum* as compared to the rutin standard (56.80%) at the same concentration. There was a significant difference observed for metal chelating activity between different extracts of *C. cupreum* ($p < 0.05$) according to Bonferroni's posttests. In Fig. 2 a strong correlation was observed between chloroform and methanol extracts ($r = 0.99$, $p < 0.05$) of *C. cupreum*. ABTS is one of the most used assays for the evaluation of the antioxidant ability of natural products by scavenging the ABTS radical in the solution. The ABTS radical is soluble in both organic solvents and water thus lipophilic and hydrophilic compounds are analyzed. The ABTS inhibition activity of rutin and different extracts of *C. cupreum* decreased in the order of rutin > n-butanol extract > ethyl acetate extract > chloroform extract > methanol extract at 50 µg/ml respectively.

The hydroxyl radical (HO•) is formed due to the combination of Fe (II) and hydrogen peroxide, in the Fenton reaction [37]. The high concentration of HO• is also formed from its precursors. Thus, a high concentration of antioxidants is required to compete with hydroxyl radical, and its precursors such as H_2O_2, O_2 and HOCl. Also, the antioxidants can either scavenge or block the precursor formation for their

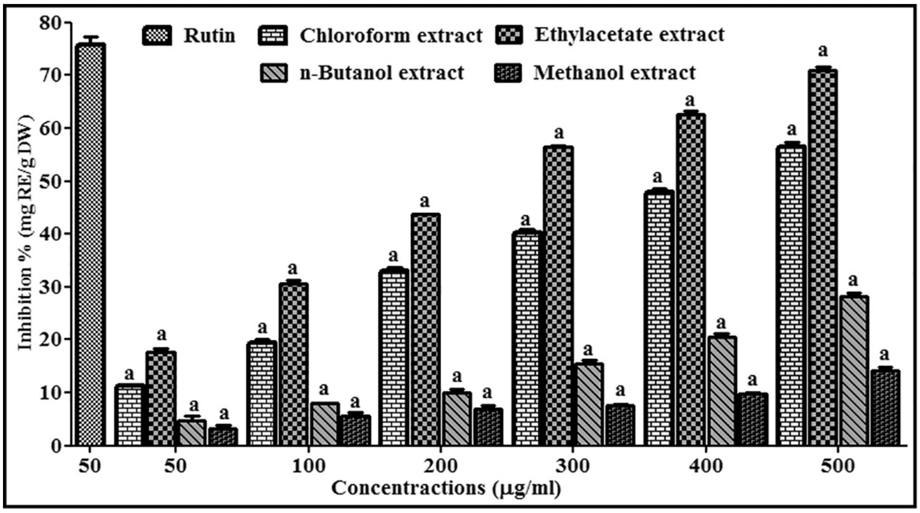

Fig. 5. Effect of different extracts of *C. cupreum* on nitric oxide inhibition. Reference standard: rutin. Bars in each column with common letters indicating significant difference ($p < 0.05$) according to Bonferroni Posttests. Experimental results are mean ± SD (n = 3).

effective antioxidant activity [38]. In Figure 3 the scavenging effect of *C. cupreum* extracts on hydroxyl radical was 18.04% by chloroform extract, 28.19% by ethyl acetate extract, 31.95% by n-butanol extract and 16.93% by methanol extract whereas 70.21% by rutin standard at 50 µg/ml concentration respectively. The hydroxyl radical scavenging activity of *C. cupreum* extracts and standard rutin decreased in the order of rutin > n-butanol extract > ethyl acetate extract > chloroform extract > methanol extract. There was a significant difference observed for metal chelating activity between different extracts of *C. cupreum* ($p < 0.05$) according to Bonferroni's posttests. Also, a strong correlation was observed between chloroform and methanol extracts (r = 0.99, $p < 0.05$) of *C. cupreum*.

The superoxide anion radicals are produced endogenously by xanthine oxidase (flavoenzymes), which liberate xanthine from hypoxanthine and convert them to uric acid ischemia reperfusion. The superoxide radical anion (O2•−) is produced due to the transfer of one electron to oxygen. This condition arises due to various metabolic processes or due to oxygen activation by irradiation. The O2•− scavenging activity can also be measured by using XOD, hypoxanthine or xanthine to generate superoxide anion radical at pH 7.4 [39,37]. The O2•− is also generated in a non-enzymatic reaction in which phenazine methosulfate (PMS) and nicotinamide adenine dinucleotide (NADH) reduce nitroblue tetrazolium (NBT) into a blue colored Formosan which is measured spectrometrically at 560 nm. The scavenging activity of rutin standard was 85.30% at 50 µg/ml. On the other hand the superoxide scavenging activity *C. cupreum* extracts was 56.44% by chloroform extract, 49.88 by ethyl acetate extract, 19.49% by n-butanol extract and 7.75% by methanol extract at 50 µg/ml concentration respectively. The superoxide radical scavenging activity of *C. cupreum* extracts and standard rutin decreased in the order of rutin > chloroform extract > ethyl acetate extract > n-butanol extract > methanol extract. There was a significant difference observed for metal chelating activity between different extracts of *C. cupreum* ($p < 0.05$) according to Bonferroni's posttests. In Fig. 4 a strong correlation was observed between chloroform and ethyl acetate extracts of *C. cupreum* but both extracts were not significantly different (r = 0.99, $p > 0.05$). The flavonoids are effective antioxidants mainly because they scavenge superoxide anion radicals [40]. This interaction of superoxide anion radicals with antioxidants compounds present in the different extracts of *C. cupreum* results in the decrease of absorbance which indicates the consumption of superoxide anion radicals in the reaction mixture.

Nitric oxide (NO) is a potent diffusible free radical various plays biological functions such as antimicrobial, antitumor, smooth muscle relaxation, neuronal messenger, inhibition of platelet aggregation and regulation of cell-mediated toxicity [41]. The increased nitrostatic stress condition is harmful to the cell function and chronic production of NO is responsible for septic shock, juvenile diabetes, multiple [42]. The peroxynitrate anion is harmful to the cell activity which is formed when nitric oxide reacts with superoxide radical [43]. Nitric oxide (NO) is a very unstable species because of its unpaired electron and is highly reactive to proteins and other molecules. The nitric oxide generated from sodium nitroprusside reacts with oxygen to produce stable nitrate and in the presence of antioxidant compounds, nitrite formation is inhibited by directly competing with oxygen in the reaction with nitric oxide which is estimated by using Griess reagent at 546 nm. In Fig. 5 the rutin standard showed 75.87% NO inhibition. The maximum NO inhibition was found in ethyl acetate (17.62%) followed by chloroform extract (11.23%), n-butanol extract, (4.72%) and methanol extract (3.20%) at 50 µg/ml. The results showed that NO inhibition by *C. cupreum* extracts was concentration dependent. Similarly, at 500 µg/ml concentration NO inhibition was found increased by 56.58% by chloroform extract, 70.88% by ethyl acetate extract, 28.09% by n-butanol extract and 14.25% by methanol extract respectively. There was significant difference observed for metal chelating activity between different extracts of *C. cupreum* ($p < 0.05$) according to Bonferroni's posttests. In Fig. 5 a significant correlation was observed between chloroform and ethyl acetate extracts of *C. cupreum* (r = 0.99, $p < 0.05$). The results presented in the Fig. 5 showed nitric oxide inhibition by different extracts of *C. cupreum* and it was found that the non-polar solvents (chloroform and ethyl acetate) have higher nitric oxide inhibition percentage than the polar solvents (n-butanol and methanol). The suppression of nitric oxide due to the scavenging activity of different extracts of *C. cupreum* was observed from the decrease in absorbance in the reaction mixture. The NO inhibition activity of rutin and different extracts of *C. cupreum* decreased in the order of rutin > ethyl acetate extract > chloroform extract > n-butanol extract > methanol extract at 50 µg/ml respectively.

Overall, there was a strong correlation between the scavenging activity among different extracts of *C. cupreum* as determined by five different antioxidant methods, metal chelating assay, ABTS assay, hydroxyl radical (HO•) scavenging assays, superoxide anion ($O_2^{\bullet}$) radical scavenging assay and nitric oxide ($NO^{\bullet}$) scavenging assay. The antioxidant activity of different extracts of *C. cupreum* showed significant correlation among different methods such as metal chelating vs ABTS method (r = 0.95, $p < 0.05$), metal chelating vs hydroxyl radical assay (r = 0.97, $p = 0.05$), metal chelating vs superoxide radical method (r = 0.97, $p < 0.05$), and metal chelating vs nitric oxide (r = 0.96, $p < 0.05$). Also ABTS method vs hydroxyl radical method (r = 0.99, $p < 0.05$), ABTS method vs superoxide radical method (r = 0.98, $p < 0.05$), ABTS method vs nitric oxide (r = 0.98, $p < 0.05$), hydroxyl radical vs superoxide radical method (r = 0.99, $p < 0.05$), hydroxyl radical vs nitric oxide (r = 0.99, $p < 0.05$) and nitric oxide (r = 0.99, $p < 0.05$). The phytochemicals contain hydroxyl groups which donate electrons or atoms to the oxygen and nitrogen free radicals or repair the antioxidant enzymes of a cell. If more phytochemical compounds are present in *C. cupreum* extract reduction will increase and absorbance will also increase due to change in color [44]. The results have shows that *C. cupreum* extracts are good free radical scavengers and thus can be useful in the treatment of various diseases which are caused by free radicals. The result shows that *C. cupreum* extracts possess significant antioxidant and antibacterial activities due to the presence of different types of phytochemicals. They break the free radical chain by sacrificing their own electrons or atoms to free radicals and without becoming free radicals by themselves. The antioxidants are the natural way of defending body cells against free radical attack. Human cells manufacture different types of antioxidant enzymes in order to control and destroy the free radical chain reactions. There are various types of antioxidants such as vitamin C, carotenes, vitamin E, and lipoic acid with antioxidant properties. In the antioxidant activity, the degree of color change either increase or decrease the absorbance at a particular wavelength is correlated with the antioxidant concentration in the reaction mixture. The ABTS and metal chelating activity are characterized by decrease in colour formation in the solution with the increase in the concentration of the antioxidant compound or extract. The antioxidant activity of all the four different extracts of *C. cupreum* was concentration dependent and antioxidant activity increased with the increase in concentration of fungal extracts. The formation of free radicals can be inhibited or reduced by reducing the H_2O_2 and hydroperoxides by chelation and scavenging activity [45].

Generally, different types of phytochemicals present in the biomaterial are important in antioxidant activity. The phytochemical analysis revealed different types of phytochemicals such as flavonoids, tannins, terpenoids, coumarins etc were found in all extracts of *C. cupreum*. Among different types of phytochemicals, flavonoid compounds were present with a high concentration in all extracts of *C. cupreum*. The flavonoids (polyphenolic compounds) have the capacity to chelate metals such as copper and iron which are potential producers of free radicals.

5. Conclusion

The evaluation of total antioxidant capacity (TAC) by using different antioxidant methods is a novel instrument to investigate the relationship between the antioxidant properties and oxidative stress. This study was focussed on the evaluation of the antioxidant potential of different extracts of *C. cupreum* by using five different in-vitro antioxidant methods. The study revealed that different extracts of *C. cupreum* have significant scavenging activity by using five different antioxidant methods in a dose-dependent manner. Thus *C. cupreum* can be a new source of natural antioxidants useful for industrial applications. These findings suggest further studies on the purification and characterization of the phytochemicals responsible for the antioxidant activities.

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Author Contributions

NAW carried out all the assays, analysis, and interpretation of results and wrote the initial manuscript. ST was responsible for the idea of research and interpretation of the results and edited the manuscript.

Ethics approval and consent to participate

Not applicable to this submission.

Conflict of interest

The authors declare no conflict of interest

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