Original Article

Evaluation of *In vitro* Antioxidant Potential of Active Metabolite Constituents of Different Extracts of *Chaetomium cupreum*-SS02 by Spectrophotometric Method

Nazir Ahmad Wani, Waseem Iqbal Khanday[1], Sharmila Tirumale

Department of Microbiology and Biotechnology, Jnanabharathi Campus, Bangalore University, Bengaluru, Karnataka, [1]PG and Research Centre in Biotechnology, MGR College, Hosur, Tamil Nadu, India

Abstract

Objective: The main objective of the study was to evaluate the antioxidant activities of *Chaetomium cupreum* extracts. **Materials and Methods:** The total flavonoid content was determined by using aluminum chloride method, whereas antioxidant activity (AA) was evaluated by ferric reducing antioxidant power assay, potassium ferricyanide reducing power assay, 2,2-diphenyl-1-picyl-hydrazyl method, β-carotene bleaching assay, cupric ion reducing antioxidant capacity assay, lipid peroxidation inhibition assay by thiobarbituric acid (TBA)-reactive substance method, and inhibition of hydrogen peroxide-induced erythrocyte hemolysis assay. **Results:** The ferric reducing AA of *C. cupreum* extracts at the concentration of 50 µg/mL was higher in ethyl acetate extract (6.11%) followed by chloroform extract (3.96%), n-butanol extract (2.44%), and methanol extract (2.02%) mg RE/g dry weight. The potassium ferricyanide reducing activity of *C. cupreum* extracts at the concentration of 50 µg/mL was higher in ethyl acetate extract (15.90%) followed by chloroform (9.50%), n-butanol (4.93%), and methanol extract (2.92%). The 2, 2-diphenyl-1-picryl-hydrazyl activity of *C. cupreum* extracts at 50 µg/mL was higher in ethyl acetate extract (36.13%) followed by n-butanol extract (24.17%), chloroform extract (15.04%), and methanol extract (4.71%). The β-carotene bleaching activity of *C. cupreum* extracts at 50 µg/mL after 1 h of incubation was higher in ethyl acetate extract at 12.88%, followed by chloroform extract (9.82%), n-butanol extract (5.63%), and methanol extract (3.76%). The cupric ion reducing AA (CUPRAC) of *C. cupreum* extracts at 50 µg/mL was highest in the methanol extract (18.62%) followed by ethyl acetate extract (9.72%), n-butanol extract (7.18%), and chloroform extract (2.46%) mg ACE/g dry weight. With regard to TBA reactive substance activity of *C. cupreum* extracts at 50 µg/mL, n-butanol extract showed the highest lipid peroxidation inhibition (55.39%) followed by chloroform extract (50.51%), ethyl acetate extract (46.27%), and methanol extract (43.60%). With regard to the hydrogen peroxide-induced hemolysis inhibition activity of *C. cupreum* extracts at the concentration of 500 µg/mL, ethyl acetate extract showed the highest inhibition (30.53%) followed by chloroform extract (26.42%) and n-butanol extract (9.16%). **Conclusion:** The results of the present study showed that *C. cupreum* extracts poses significant antioxidant potential.

Keywords: Antihemolysis, antioxidants, flavonoid, lipid peroxidation, oxidants

Introduction

The normal cells of humans maintain a low level of reactive oxygen species (ROS), but reducing equivalents maintain a constant level. This balance between ROS and reducing equivalents determines their redox status in the cells. It has been observed that oxidative stress is the first step in the formation of cellular malignancy and progression of cancer.[1,2] The ROS occurs due to the result of electron reductions of oxygen. Some free radicals have one or more unpaired electrons in their outer molecular orbitals such as nitric oxide (NO) radical and superoxide radical (O_2), hydroperoxyl radical, and hydroxyl radical (OH), whereas others which have unpaired electrons but are highly reactive can be converted to free radicals such as NO, hydrogen peroxide (H_2O_2), ozone (O_3), and trioxidane (HOOOH).[3] ROS are formed either

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Address for correspondence: Dr. Sharmila Tirmuale, Department of Microbiology and Biotechnology, Jnanabharathi Campus, Bangalore University, Bengaluru - 560 056, Karnataka, India. E-mail: sharmilabub@gmail.com

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by exogenous sources (such as pollutants, tobacco smoke, iron salts, and radiation) or by endogenous cellular sources (such as mitochondria and NADPH-dependent oxidase complexes through multiple mechanisms such as electron reduction of oxygen in the NADPH-dependent oxidases, P-450-dependent monooxygenases, xanthine oxidase, lipoxygenases, and cyclooxygenases). The ROS attack various biomolecules such as DNA, RNA, lipids, membranes, proteins, and enzymes and thus cause various diseases such as cancer, carcinogenesis, atherosclerosis, rheumatoid arthritis, neurodegenerative diseases (Alzheimer's and Parkinson's diseases), and hypertension.[4] Synthetic compounds which are presently used as antioxidants such as butyrate hydroxyanisole, butyrate hydroquinone, propyl gallate, and butyrate hydroxytoluene (BHT) are harmful for liver and are carcinogenic.[5] Some studies have shown that synthetic antioxidants cause a genotoxic effect[6] and various chronic diseases.[7]

Various filamenrous fungi produce enzymes, antibiotics, pigments, and additives, which are used in food, cosmetic, and pharmaceutical industries.[8] The products isolated from animal or plant source are instable and have low solubility.[9] Due to toxicity issues associated with synthetic compounds, most industries are now depending on microorganisms as an alternative source of natural compounds.[9,10] The red, yellow, and orange pigments are produced by various species of fungi including *Monascus*, *Asperigillus*, *Penicillium*, *Chaetomium*, and *Fusarium*.[11] Among these fungal organisms, only few members have been explored for food applications.

The different species of *Chaetomium* liberate a type of pigment known as azaphilones. Azaphilones are pyrone quinone ring pigments with oxygenated bicyclic core connected with a chiral quaternary center.[12] Azaphilone can react with the amino group of nucleic acids and proteins for the exchange of pyrane oxygen for nitrogen to attain colored and water-soluble properties.[13] The azaphilones have shown antioxidant, antimicrobial, antifungal, and anticancer activities.[14] The main objective of the present study was to evaluate the antioxidant activities of *Chaetomium cupreum* extracts by using different methods.

Materials and Methods

Chemicals and reagents

lactophenol cotton blue, chloroform, n-butanol, ethyl acetate and methanol, hydrochloric acid, Folin–Ciocalteu's reagent, ferric chloride, potassium dichromate, ferrous sulfate ($FeSO_4$), thiobarbituric acid (TBA), linoleic acid, b-carotene, sodium dodecyl sulfate, acetic acid, trichloroacetic acid (TCA), ascorbic acid, chloroform, ammonium acetate, cupric chloride, neocuproine, phosphate buffer, hydrogen peroxide, lead acetate, sodium carbonate, sodium nitrite, sodium hydroxide, aluminum chloride, iron sulfate, trolox, rutin, gallic acid, potassium ferric cyanide, potato dextrose agar, and potato dextrose broth were purchased from Merck (Mumbai, Maharashtra, India).

Procurement of fungal culture

The fungal culture *C. cupreum* was procured from the National Fungal Culture Collection of India (NFCCI), Agharkar Research Institute, Pune, India, with accession number NFCCI 3117.

Maintenance of culture

The fungus *C. cupreum* was cultured on potato dextrose agar and incubated for 7 days at 28°C ± 2°C at room temperature. After 7 days of incubation, morphological studies were carried out by microscopic observation by studying fungus mycelium, hyphae, and pigmentation on the potato dextrose agar (PDA) medium. The fungus was maintained on PDA plates and slants at 4°C.

Submerged fermentation

The fungus was cultured on potato dextrose agar at 28°C for 7 days. After fungus growth, 5-mm mycelial discs were bored out from the periphery of the plate and transferred to 250-mL Erlenmeyer conical flask containing 100 mL of potato dextrose broth. Then, the inoculated flasks were incubated at room temperature on a rotary shaker at 120 rpm for 20 days.[15]

Extraction of secondary metabolites/pigments

The extraction of secondary metabolites/pigments from *C. cupreum* broth was carried out by liquid–liquid method as described by Nazir and Sharmila.[16] After 20 days of incubation, the broth was filtered to remove mycelium and extraction was done by using a 500-mL separating funnel in the ratio of 1:1. A volume of 50 mL of fungal broth and 50 mL of solvent was measured in a separating funnel and manually shaken for 20 min. After shaking, the separating funnel was allowed to stand so that two layers will separate properly. The solvent layer was collected in a 250-mL beaker. The same process was repeated three times until no more metabolite diffuses into the solvent. The whole fungal broth was extracted in the similar manner by using four different solvents (chloroform, n-butanol, ethyl acetate, and methanol). The solvent layer was collected and evaporated by using a vacuum rotary evaporator at 45°C. The dried extract power obtained was stored at 4°C for future use.

Determination of total flavonoid content

The flavonoid content was determined spectrophotometrically according to the aluminum chloride ($AlCl_3$) method.[17] The flavonoid estimation is based on the principle of yellow color formation when flavonoid sample is treated with aluminum chloride reagent (1%). A volume of 0.5-mL fungal extract was mixed with 0.15 mL of sodium nitrite (5% $NaNO_2$) and 2 mL of distilled water, and the reaction mixture was allowed to react for 6 min. After 6 min, 0.15 mL-aluminum chloride (10% $AlCl_3$ w/v) solution and 2 mL of sodium hydroxide (4% NaOH) solution were added, and then 5-mL distilled water was used. The solution was allowed to react for 15 min and the absorbance was measured at 510 nm. The calibration curve was generated from rutin reference standard and the results of total flavonoid content (TFC) were expressed as rutin equivalents (RE/100 g dry weight).

Determination of antioxidant activity by ferric reducing antioxidant power assay

The antioxidant capacity of *C. cupreum* extracts was measured by ferric reducing antioxidant power (FRAP) assay.[18] The preparation of FRAP reagent included 20 mM 2,4,6-tripyridyl-S-triazine (TPTZ) (0.014 mL was dissolved in 10 mL of distilled water to form 40 mM hydrochloric acid solution, and then 0.031 g of TPTZ was added to 5 mL of 40 mM hydrochloric acid), 20 mM ferric chloride (0.016 g of ferric chloride was dissolved in 5 mL of distilled water), and 300 mM acetate buffer (2.46 g of sodium acetate was dissolved in 70 mL of distilled water, pH was adjusted 3.6 by adding 1 M hydrochloric acid). The FRAP reagent was made by mixing 2.5 mL of TPTZ, 2.5 mL of ferric chloride reagent, and 25 mL of acetate buffer (2.5:2.5:25 mL). A volume of 100 µL of fungal extract of various concentration was mixed with 800 µL of FRAP reagent. Then, 100 µL of distilled water was added and the reaction mixture was incubated in a water bath for 30 min at 37°C. The same procedure was followed for standard rutin. The control included all reagents except the test sample which was replaced by dimethyl sulfoxide. After incubation, the absorbance was measured at 593 nm. The observed values were compared with the stock solution at 0.5 mL of iron sulfate (1 mg/mL, $FeSO_4$). The calibration curve was generated from rutin standard and the results were expressed as rutin equivalents (RE/g dry weight).

Determination of antioxidant activity by potassium ferricyanide reducing power assay

The antioxidant activity (AA) of *C. cupreum* extracts was evaluated by potassium ferricyanide reducing power (PFRAP) assay.[19] A volume of 100 µL of different concentration was mixed with 2.5 mL phosphate buffer solution (0.2 M, pH 6.6). To this mixture, 2.5 mL potassium ferric cyanide (1% w/v) was added and the mixture was incubated in a water bath for 30 min at 50°C. After cooling, 2.5 mL of TCA (10% TAA v/v) was added to the mixture. From this mixture, 2.5 mL was added to 0.5 mL of ferric chloride solution (0.1% $FeCl_3$) and further 2.5 mL of distilled water was added to the mixture. Then, the absorbance of this mixture was measured at 700 nm using an ultravilolet-visible spectrophotometer. Rutin was used as standard. The control was prepared in the similar manner without the sample. The higher the absorbance of the reaction mixture, higher will be the reducing power of the fungal extract.

Determination of antioxidant activity by 2,2-diphenyl-1-picryl-hydrazyl activity assay

The antioxidant potential of *C. cupreum* was measured by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) free radical method.[20] The stock solution was prepared by mixing 5 mg of DPPH reagent in 10 mL of methanol. A volume of 1 mL of 0.1 mM DPPH reagent was mixed with 100 µL of the fungal extract and incubated for 20 min at 28°C. Methanol was added in the place of test sample in the control tube, and the absorbance was measured at 517 nm. The same procedure was followed for trolox standard, and the results were expressed as trolox equivalent (TE/g dry weight)

$$\% \text{ scavenging activity} = (A_c - A_t/A_c) \times 100$$

Where A_c is absorbance of control and A_t is the absorbance of the test sample.

Determination of anti-β-carotene bleaching (BCA) activity

The AA of different extracts of *C. cupreum* was measured by the β-carotene linoleate model system.[21] A β-carotene solution was prepared by mixing 2 mg of β-carotene in 10 mL of chloroform. From β carotene chloroform solution 2 ml was added to a flask containing linolic acid (40 mg), Tween 40 (400 mg) to form an emulsion. The chloroform was evaporated and 100 mL of distilled water was added to the flask with vigorous shaking. From this emulsion, 2.5 mL was transferred into different test tubes containing 0.1 mL of *C. cupreum* extracts with different concentrations. Each test tube was shaken well and then incubated in a water bath at 50°C, and the absorbance was recorded at 470 nm at zero time. After 20-min intervals, readings were taken until control sample changed its color. A blank without β-carotene was prepared for background subtraction. The butylhydroxytoluene (BHT) was used as standard. The AA was calculated using the following formula:

AA = (β-carotene content after 2 h/initial β-carotene content) × 100.

Determination of cupric ion reducing antioxidant capacity assay

The antioxidant capacity of *C. cuprem* extracts was estimated by CUPRAC assay.[22] In this assay, cupric oxide (Cu(II)) is reduced into cuprous oxide (Cu(I)) by the action of the electron-donating capacity of antioxidant compounds present in *C. cupreum* extracts. A solution of 1 mL of ammonium acetate buffer (1 mM, pH 7), 1 mL of cupric chloride (10 mM), and 1 mL of neocuproine (7.5 mM) was added into test tubes each containing 2 mL of distilled water. A volume of 100 µL of *C. cupreum* extracts with different concentrations was added to each test tube and incubated at room temperature for 30 min and the absorbance was measured spectrometrically against blank at 450 nm. Ascorbic acid was used as standard, and the results were expressed as ascorbic acid equivalent as mg/g dry weight. The AA percentage was calculated by the following formula:

$$\text{AA (\%)}: A_1 - A_0/A_1 \times 100$$

Where A_1 is the absorbance of control and A_0 is the absorbance of the test sample.

Determination of inhibition of lipid peroxidation by thiobarbituric acid-reactive substances

The effect of *C. cupreum* extracts in inhibiting lipid peroxide formed from egg yolk homogenate as lipid-rich media was estimated by TBA-reactive substances.[23] By adding a volume of 500 µL of egg yolk homogenate (10% in distilled water v/v), 100 µL of fungal extract with different concentrations,

and distilled water, the total volume was made up to 1 mL. To induce lipid peroxidation, 50 mL of ferrous sulfate ($FeSO_4$, 0.07 M) was added, and the mixture was incubated for 30 min. A volume of 1.5 mL of 20% acetic acid (pH 3.5), 1.5 mL of TBA (0.8%, w/v), sodium dodecyl sulfate (1.1% w/v), and 0.05 mL of (20% TCA) was added, and the the tubes containing the mixture were vortexed well and incubated at 95°C for 60 min. After incubation, 1-butanol (5 mL) was added to each tube and the tubes were centrifuged at 3000 rpm for 10 min. The absorbance of the supernatant was measured at 532 nm. The same procedure was followed for ascorbic acid standard and control (without sample). The percentage inhibition was calculated by the following formula:

$$\% \text{ inhibition of lipid peroxidation} = A_1 - A_0/A_1 \times 100$$

Where A_1 is the absorbance of control and A_0 is the absorbance of the test sample.

Inhibition of hydrogen peroxide-induced erythrocyte hemolysis

The AA of *C. cupreum* extracts was estimated by erythrocyte hemolysis inhibition assay.[24] In this experiment, venous blood samples were collected from healthy adult rats into glass centrifuge tube containing ethylenediaminetetraacetic acid as an anticoagulant. The tube was centrifuged for 10 min at 1500 rpm and the supernatant was discarded. The resulting pellet was washed three times carefully with phosphate-buffered saline (0.2 mM phosphate-buffered saline, sodium dihydrogen phosphate [NaH_2PO_4], sodium hydrogen phosphate [Na_2HPO_4], and sodium chloride, pH 7.4) to separate erythrocytes from the plasma and buffy coat and centrifuged at 1500 rpm for 5 min. The reaction mixture contained 100 µL of fungal sample of various concentrations and 200 µL of erythrocyte suspension, and the mixture was incubated for 30 min at 37°C. Hemolysis was induced by adding 100 µL of hydrogen peroxide (100 mM hydrogen peroxide in 0.1 mM phosphate-buffered saline) to induce oxidative degradation of membrane lipids, and the total volume was made up to 2 mL by adding phosphate-buffered saline followed by incubation at 37°C for 240 min. The erythocyte lysis in the presence of hydrogen peroxide without fungal extract was considered as 100% hemolytic activity. After incubation, the samples were centrifuged at 3000 rpm for 10 min, and the absorbance was measured at 540 nm spectrometrically. Ascorbic acid (0.1 mg/mL) was used as standard. The % hemolysis inhibition was calculated in the presence of extracts relative to control hemolysis as follows:

$$\% \text{ hemolysis inhibition} = ([A_0 - A_1/A_0] \times 100$$

Where A_0 is the absorbance of control sample (red blood cell [RBC] + H_2O_2) and A_1 is the absorbance of the test material.

Statistical analysis

The GraphPad Prism 6 software (Graphpad Software, Inc, United states). was used for data analysis with analysis of variance. The Pearson's correlation between AA and phytochemical classes was calculated to determine their relationship. $P < 0.05$ was considered statistically significant. The experimental results were expressed as mean ± standard deviation of three parallel measurements.

Results

Total flavonoid content

The TFC of *C. cupreum* was obtained from the calibration curve of standard rutin ($y = 0.38x - 1.828$; $r^2 = 0.986$) and expressed as milligram rutin equivalent per hundred gram dry weight of the extract [Figure 1a]. The TFC of different extracts of *C. cupreum* is depicted in Figure 1b. The TFC of chloroform extract of *C. cupreum* at 50 µg/mL was 5.52 ± 0.00 mg RE/g dry weight, ethyl acetate extract showed 9.98 ± 2.87 mg RE/g dry weight, n-butanol extract showed 10.23 ± 2.87 mg RE/g dry weight, and methanol extract showed 5.61 ± 0.19 mg RE/g dry weight. It was found that n-butanol extract possesses higher flavonoid content at different concentrations.

Ferric reducing antioxidant power assay

The ferric reducing AA of *C. cupreum* extracts was calculated from the calibration curve of rutin standard ($y = 0.247x - 0.772$; $r^2 = 0.996$) and expressed as milligram rutin equivalent per gram dry weight [Figure 2a]. The FRAP activity of

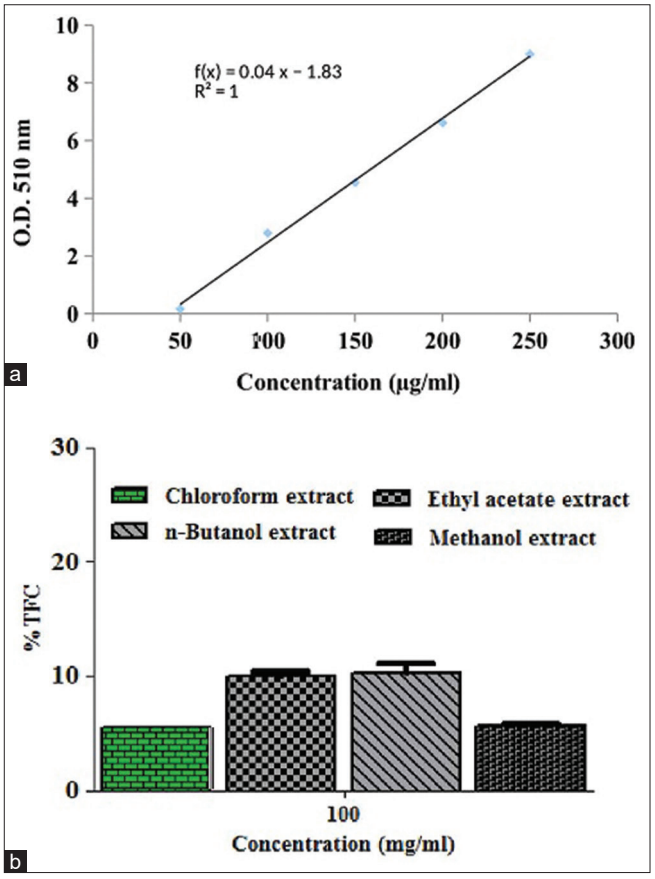

Figure 1: (a) Rutin standard calibration curve. (b) Determination of total flavonoid content by aluminum chloride method. Standard: Rutin. Experimental results are expressed as mean ± standard deviation ($n = 3$)

Table 1: The comparison of standard compounds and C. *cupreum* extracts in different antioxidant methods for IC_{50} value

Assay	Chloroform extract	*Ethyl acetate extract*	n-butanol extract	Methanol extract	Standard compounds
DPPH	840	100	150	1000	40 (Trolox)
β-carotene bleaching	367.66	285.11	-	-	28.08 (BHT)
CUPRAC	-	-	-	500	36.60 (Ascorbic acid)
Lipid peroxidation inhibition	47.60	48.0	45.13	49.01	47.16 (Ascorbic acid)

'-': No significant activity

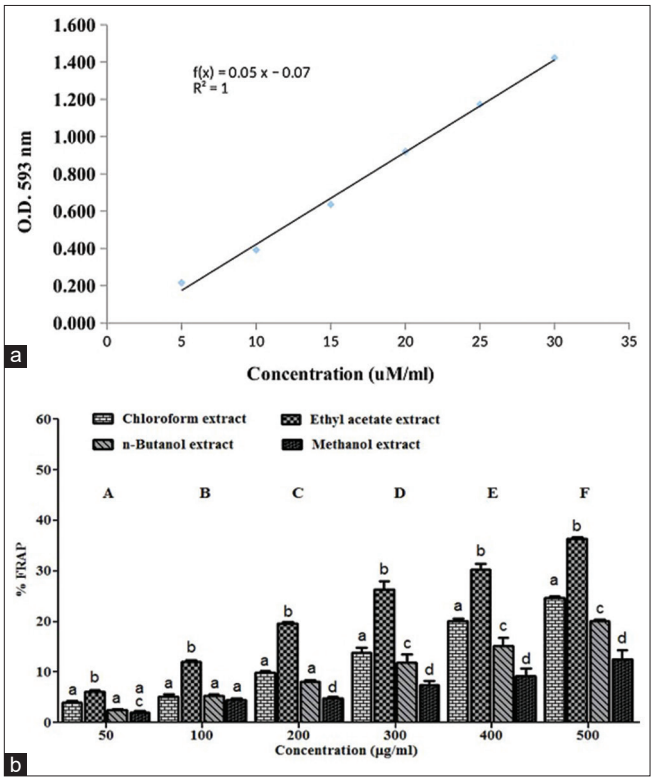

Figure 2: (a) Standard calibration curve of rutin for ferric reducing antioxidant power scavenging activity. (b) Effect of different extracts of *Chaetomium cupreum* on antioxidant activity by ferric reducing antioxidant power assay. Values are represented as mean ± standard deviation ($n = 3$). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

C. cupreum extracts was found highest in ethyl acetate extract (6.11 ± 0.32 mg RE/g dry weight), followed by chloroform extract (3.96 ± 0.31 mg RE/g dry weight), n-butanol extract (2.44 ± 0.99 mgRE/g dry weight), methanol extract (2.02 ± 0.28 mg RE/g dry weight), and rutin standard (52 ± 1.32 mg RE/g dry weight at 50 µg/ml concentration) [Figure 2b].

Potassium ferricyanide reducing power assay

The PFRAP assay of *C. cupreum* extracts and their comparison with rutin standard is presented in Figure 3. The potassium ferricyanide reducing AA was found higher in ethyl acetate extract (15.90%) followed by chloroform extract (9.50%),

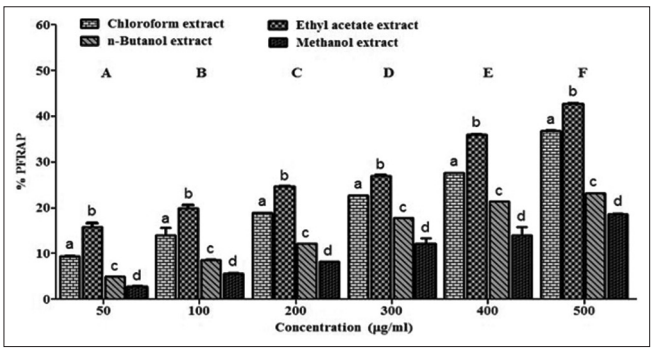

Figure 3: Effect of different extracts of *Chaetomium cupreum* on antioxidant activity by potassium ferricyanide reducing power assay. Values are represented as mean ± standard deviation ($n = 3$). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

n-butanol extract (4.93%), and methanol extract (2.92%), whereas rutin standard showed 78.06% at 50 µg/ml concentration.

2,2-diphenyl-1-picryl-hydrazyl radical scavenging activity

The DPPH of different extracts of *C. cupreum* and their comparison with trolox standard is given in Figure 4. The comparison of *C. cupreum* extracts and standard compounds in different antioxidant methods for IC_{50} value is presented in Table 1. The AA at 50 µg/mL was highest in the ethyl acetate extract of *C. cupreum* (36.13% ± 1.23%) followed by n-butanol extract (34.17 ± 1.30 mg), chloroform extract (15.04% ± 0.06%), methanol extract (4.71% ± 1.06%), and trolox standard (62.5% ± 1.5%). Whereas at 500 µg/mL concentration, the scavenging activity was highest in ethyl acetate extract (97.10% ± 1.34%) followed by n-butanol extract (93.30% ± 2.00%), chloroform extract (59.06% ± 2.40%), and methanol extract (48.19% ± 1.13%).

Anti-β-carotene assay

The rate of bleaching of β-carotene mixture was estimated by the difference between the initial reading of solution at the time 0 min (1st reading) and after 60 min (2nd reading) and again after 120 min (3rd reading). The absorbance increases because the antioxidant content found in *C. cupreum* extracts neutralizes the linoleate-generated free radical in the reaction

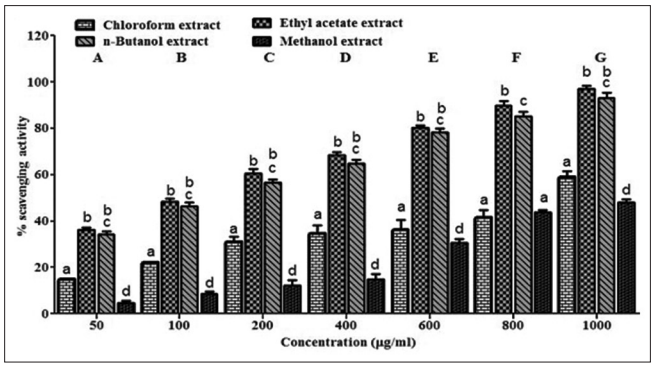

Figure 4: Effect of different extracts of *Chaetomium cupreum* on scavenging activity by 2, 2-diphenyl-1-picryl-hydrazyl radical assay. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

mixture. With regard to the AA, ethyl acetate extract of *C. cupreum* showed the highest activity (12.88 ± 0.03), followed by chloroform extract (9.82 ± 0.01), n-butanol extract (5.63 ± 0.01), methanol extract (3.76 ± 0.01), and the AA of BHT standard was 89.50 ± 1.50 at 50 µg/mL after 1 h of incubation [Figure 5a]. Similarly, after 2 h of incubation, the AA of chloroform extract of *C. cupreum* was 40.18 ± 0.11, ethyl acetate extract was 50.70 ± 0.13, n-butanol extract was 20.79 ± 0.13, and methanol extract was 9.53 ± 0.14 at 500 µg/mL concentration [Figure 5b and Table 1].

Cupric ion reducing antioxidant capacity assay

The CUPRAC of *C. cupreum* extracts and their comparison with ascorbic acid is presented in Figure 6 and Table 1. The highest antioxidant potential was estimated in methanol extract (18.62% ± 0.18%) followed by ethyl acetate extract (9.72% ± 0.01%), n-butanol extract (7.18% ± 1.83%), chloroform extract (2.46 ± 0.27), and standard ascorbic acid (68.30 ± 1.50 mg ACE/g dry weight at 50 µg/mL concentration. Whereas at 500 µg/mL, methanol extract showed the highest activity of CUPRAC (49.83% ± 0.27%) followed by ethyl acetate extract (45.48% ± 0.18%), n-butanol extract (42.93% ± 0.27%), and chloroform extract (41.48% ± 0.27%).

Lipid peroxidation

The inhibition effect of different extracts of *C. cupreum* on ferrous sulfate-induced lipid peroxidation in hen's egg yolk is presented in Figure 7 and their comparison for IC_{50} value for standard ascorbic acid is presented in Table 1. For the estimation of lipid peroxidation, the most commonly used method is thiobarbituric acid reactive substances (TBARS) method. In TBARS method, n-butanol extract showed higher lipid peroxidation inhibition percentage (55.39% ± 0.27%). This was followed by chloroform extract (50.51% ± 0.84%), ethyl acetate extract (46.27% ± 0.12%), and methanol extract with lowest lipid peroxidation inhibition percentage of 43.60% ± 0.40%, and standard ascorbic acid showed 40.51% ± 0.13% at 50 µg/mL concentration.

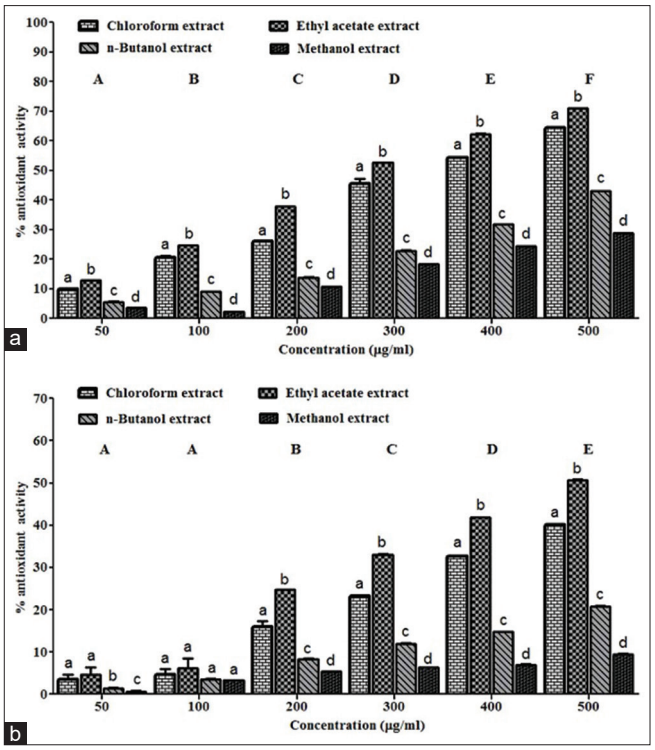

Figure 5: (a) Antioxidant activity of different extracts of *Chaetomium cupreum* by b-carotene bleaching method after 1 h. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$. (b) Antioxidant activity of different extracts of *Chaetomium cupreum* by b-carotene bleaching method after 2 h. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

Hydrogen peroxide-induced erythrocyte hemolysis inhibition assay

The ability of *C. cupreum* extract to inhibit hydrogen peroxide-induced erythrocyte hemolysis is presented in Figure 8. In antihemolysis activity, the highest hemolysis inhibition was observed in ethyl acetate extract (30.53%) followed by chloroform extract (26.42%) and n-butanol extract (9.16%) at 500 µg/mL. Whereas methanol extract did not exhibit antihemolysis activity. The standard ascorbic acid showed antihemolysis activity of 48.75% at 500 µg/mL.

Discussion

The results obtained showed higher TFC in n-butanol extract (10.23%) and ethyl acetate extract (9.98%) as compared to methanol extract (5.61%) and chloroform extract (5.52%) of *C. cupreum*. 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. There was a strong correlation for TFC between chloroform and methanol extracts

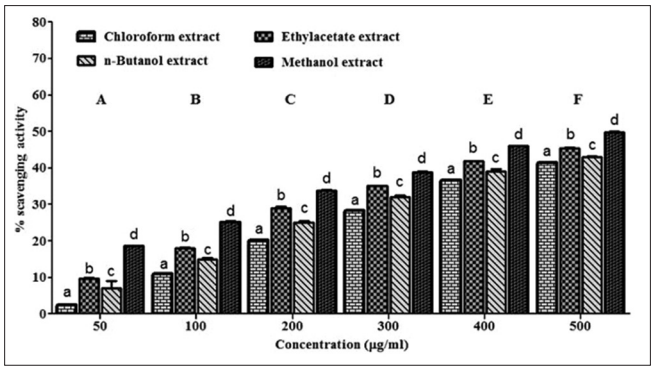

Figure 6: Determination of antioxidant capacity of different extracts of *Chaetomium cupreum* by cupric ion reducing antioxidant capacity assay. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

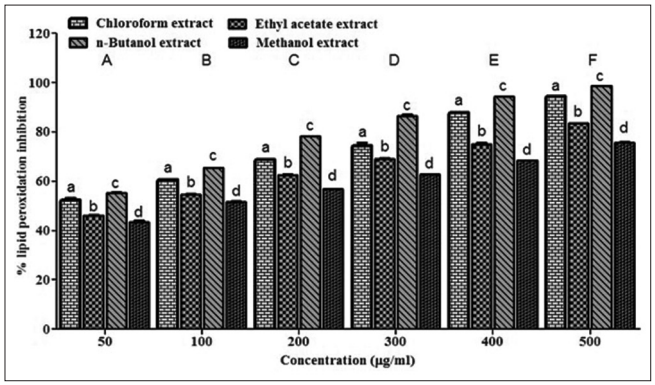

Figure 7: Determination of lipid peroxidation inhibition by *Chaetomium cupreum* extracts by thiobarbituric acid reactive substances method. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

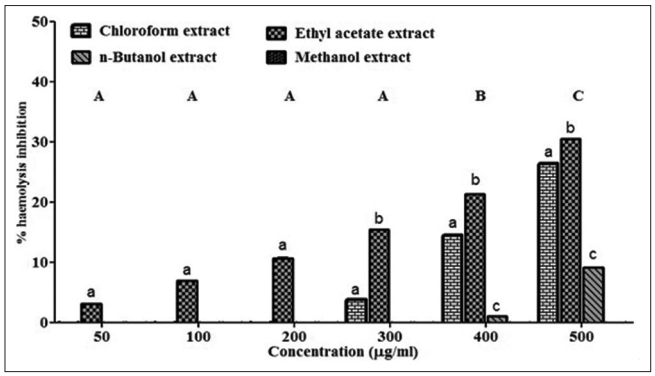

Figure 8: Determination of hydrogen peroxide-induced hemolysis inhibition by different extracts of *Chaetomium cupreum*. Values are represented as mean ± standard deviation (n = 3). Significance between the extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are statistically significantly different at $P < 0.05$

of *C. cupreum* (r^2 = 0.99, P = 0.0052). In addition, there was a strong correlation between TFC of ethyl acetate and n-butanol extract of *C. cupreum* (r^2 = 0.99, P = 0.0005). The TFC of *C. cupreum* extracts showed statistically significant differences ($P < 0.0001$) according to Bonferroni's *post hoc* tests. In previous studies, TFC of methanolic extracts of two endophytic fungi *Asperigillus* species (CPIL-1) and *Penicillium* species (CPIMR-2), in terms of quercetin equivalent, was 130.50 ± 6.11 and 94.91 ± 4.48 mg/g dry weight of fungal extract, respectively. These flavonoid compounds have a broad spectrum of chemical and biological activities including radical scavenging properties.[25] A previous study showed that exacerbation of van der Waals force inside the lipid-bilayer exerted by flavonoids is a source of membrane stabilization.[26]

The FRAP assay is more useful as it measures antioxidants directly than other assays which measure the inhibition of free radicals.[27] The FRAP assay measures the electron-donating capacity of antioxidants, which reduces ferric iron to ferrous iron (Fe^{3+} to Fe^{2+}).[27] In FRAP assay, the antioxidant compounds reduce ferric tripyridyltriazine (Fe (III)-TPTZ) to a blue-colored ferrous tripyridyltriazine (Fe (II)-TPTZ), which is measured spectrometrically at 593 nm. Previous studies[28] revealed that the FRAP of n-butanol fraction of *Fusarium sp*. JZ-Z6 was 308.26 ± 13.66 µM at 1.60 mg/mL and ethyl acetate fraction was 327.35 ± 67.48 µM, while the present study revealed that rutin standard (50 µg/mL) showed higher FRAP activity than ethyl acetate extract followed by chloroform extract, n-butanol extract, and methanol extract. The absorbance increased with the increase in FRAPS activity of *C. cupreun* extracts. In FRAP activity, there was a strong correlation between ethyl acetate and n-butanol extracts of *C. cupreum* (r^2 = 0.99, P = 0.0001).

In PFRAP assay, reaction mixture changes color from yellow, blue, or green depending on the antioxidant capacity of the extract. The antioxidant compounds upon reacting with potassium ferricyanide produce potassium ferrocyanide ($Fe^{3+} \rightarrow Fe^{2+}$). The potassium ferrocyanide further reacts with ferric chloride, which leads to the formation of ferric–ferrous complex, which can be measured at 700 nm spectrophotometrically. In reducing power measurement, ethyl acetate extract showed higher reducing power followed by n-butanol and chloroform, whereas methanol showed lower reducing power. However, the reducing power was less in all *C. cupreum* extracts as compared to that of rutin standard. The superoxide free radical acts as a precursor for various types of reactive species, which contributes in various diseases. The superoxide radical can be neutralized by superoxide dismutase.[29] A previous study showed that ethanol extract of *Armillaria mellea, Calocybe gambosa, Clitocybe odora,* and *Tricholima giganteum* posses PFRAP activity with IC_{50} value of 17.13, 11.46, 3.63, and 2.2 µg/mL, respectively.[30] In the present study, higher PFRAP was found in rutin followed by ethyl acetate, chloroform, n-butanol, and methanol extract at 50 µg/mL. In reducing power measurement, a

strong correlation was obtained between ethyl acetate and n-butanol extracts of *C. cupreum* (r^2 = 0.99, P = 0.0001). The antioxidant compounds in the reaction mixture reduce the purple chromogen radical DPPH to a hydrazine which is pale yellow and is measured spectrometrically at 517 nm. The more is the yellow color formation, the more is the AA of the extract. Previous studies have used DPPH scavenging assay for the evaluation of AA from secondary metabolites from the fungus *Cerrena unicolor* (ex-LMSI, ex-LMSII, and ex-LMIII) with IC_{50} – 20.39 µg/mL (ex-LMSI), 64.14 µg/mL (ex-LMSII), and 49.22 µg/mL (ex-LMSIII), whereas for standard trolox IC_{50} – 63.69 µg/mL and for ascorbic acid IC_{50} – 41.25 µg/mL.[31] In the present study, DPPH scavenging activity was highest in ethyl acetate extract (36.13%) followed by n-butanol (24.17%), chloroform extract (15.04%), and methanol extract (4.71%). A strong correlation was observed between ethyl acetate and n-butanol extracts of *C. cupreum* (r^2 = 0.98, P = 0.005).

In β-carotene bleaching (BCB) assay, oxidation of linoleic acid produces peroxyl-free radicals due to the abstraction of hydrogen atom from the diallylic methylene group of linoleic acid.[32] The free radicals will oxidize the highly unsaturated β-carotene, which will result in rapid discoloration in the solution due to the absence of an antioxidant. This will lead to decrease in absorbance without antioxidants, whereas in the presence of antioxidants, the color retain for longer duration and absorbance will increase with increase in concentration. The effect of oxidation by hydroperoxides on β-carotene will be minimized and neutralized in the presence of antioxidant compounds in *C. cupreum* extracts. The results showed that *C. cupreum* extracts can hinder the extent of β-carotene bleaching by neutralizing the linoleate-produced free radicals and other free radicals formed in the reaction.[33] The β-carotene is a naturally occurring carotenoid having orange color found in yellow-orange fruits and vegetables and dark green and leafy vegetables.[34] A study found that methanol extract of *Grifola frondosa* showed 65.22 ± 1.80, *Monascus purpureus* showed 83.94 ± 0.70, *Pleurotus*-32783 showed 83.13 ± 0.84, *Pleurotus scitrinopileatus* showed 53.60 ± 2.88, *Pleurotus eryngii* showed 53.72 ± 0.05, *Pleurotus salmoneo-str* showed 59.73 ± 0.70, and *Trametes versicolor* showed 63.94% ± 0.05% bleaching inhibition at 1 mg/mL concentration.[35] In β-carotene bleaching assay, the highest AA was observed by ethyl acetate extract (50.70%) followed by chloroform extract (40.18%), n-butanol extract (20.79%), and methanol extract (9.53%) at 500 µg/mL after 2 h of treatment. A statistically significant difference was observed between *C. cupreum* extracts (P < 0.05) according to Bonferroni's posttests.

The CUPRAC assay is used to measure antioxidant activity in food products, plants extracts, human serum, and to hydroxyl radical scavengers. This CUPRAC method is simple and can be completed in 30 min and widely used for measuring antioxidant capacity of flavonoids, phenolic acids, hydroxycinnamic acids, thiols, synthetic antioxidants, and Vitamins C and E.[36] Previous studies described[35] that the CUPRAC activity of methanol extract of *Grifola frondosa* showed 2.34 ± 0.05, *Monascus purpureus* showed 2.20 ± 0.03, *Pleurotus*-32783 showed 2.30 ± 0.02, *Pleurotus scitrinopileatus* showed 2.00 ± 0.05, *Pleurotus eryngii* showed 2.24 ± 0.02, *Pleurotus salmoneo-str* showed 2.25 ± 0.05, and *Trametes versicolor* showed 2.34 ± 0.09 EC_{50} value at 10 mg/mL concentration. In the present study, in CUPRAC assay, the results showed highest AA in methanol extract (49.83%) followed by ethyl acetate (45.48%), n-butanol extract (42.93%), and chloroform extract (41.48%) of *C. cupreum* at the concentration of 500 µg/mL concentration. A strong correlation (r^2 = 0.99, P < 0.05) was obtained for CUPRAC activity in *C. cupreum* extracts.

Malondialdehyde is a secondary product generated from the oxidation of polyunsaturated fatty acids, which has the capacity to interact with two molecules of TBA, and form a pinkish red chromogen which is measured at 532 nm,[37] For screening and monitoring lipid oxidation, the most commonly used method is TBARS.[38] In the previous study[39] the inhibition of lipid peroxidation by *Pleurotus ostreatus* acetone extract was 38%, dichloromethane 43% and hexane extract was 36% at 1.25 mg/mL concentration respectively by TBARS method. Whereas in the present study, with regard to the TBARS assay, the results showed that the highest lipid peroxidation inhibition was observed in n-butanol extract of *C. cupreum* (98.73%) followed by chloroform extract (94.57%), ethyl acetate extract (83.45%), and methanol extract (75.82%) at 500 µg/mL concentration. A strong correlation (r^2 = 0.99, P < 0.05) was obtained for TBARS assay in *C. cupreum* extracts.

In hydrogen peroxide-induced hemolysis, peroxyl radicals (ROO–) are generated which attack erythrocytes to induce the oxidation of lipids and proteins, disturbing the membrane structure and leading to hemolysis.[40] When *C. cupreum* extracts and RBCs were treated with hydrogen peroxide, reduction in hemolysis was observed. This may be due to the different secondary metabolites present in *C. cupreum* extracts, thus showing significant antihemolytic nature of the *C. cupreum* extracts.

The erythrocytes are susceptible to oxidative stress because of high polyunsaturated lipid acids such as linoleic and arachidonic acids and transition metals, particularly iron, which catalyze free radical production through Fenton reaction.[41] The iron-mediated oxidative stress of hemoglobin and membrane lipid can increase the severities of diseases such as thalassemia and sickle cell anemia and cause hemolysis.[42] The hemolysis can be neutralized by the therapeutic agents. In previous works,[43] it has been described that the inhibition of erythrocyte hemolysis of hot water extract of *Hericium erinaces* was 11.52 at 0.8 mg/mL to 38.94% at 4.0 mg/mL, whereas standard ascorbic acid was higher 46.27% at 0.2 mg/mL with an EC_{50} value of 0.17 mg/mL. Previous studies have described the inhibition of erythrocyte hemolysis by *Auricularia auricular* to be 6.67% at 2 mg/mL and 59.37% at 10 mg/mL with an EC_{50} value of 9.01 mg/mL concentration.[44] The antioxidant capacity and inhibition of hemolysis to erythrocytes induced by *C.*

cupreum crude extracts indicate the presence of different types of secondary metabolites. Whereas in the present study, the inhibition rate of H_2O_2-induced hemolysis was highest in ethyl acetate (30.53%), followed by chloroform (26.43%), n-butanol extract (9.16%), and ascorbic acid standard showed (45.75%) antihemolysis activity at 500 µg/mL concentration, whereas the methanol extract did not exhibit antihemolysis activity.

The flavonoid content was more in ethyl acetate and n-butanol extracts as compared to chloroform and methanol extracts. This indicates that middle polar solvents are more suitable for flavonoid compound extraction. Flavonoids are powerful antioxidants due to their proton-donating capacity.[45,46] The antioxidant properties of an extract or compound depend on its redox properties, which makes them as good reducing agents. The secondary metabolites have hydroxyl groups with electron- or atom-donating potential for free radicals. If more secondary metabolites are present, reduction will increase and subsequently antioxidant powder will also increase.[47] The higher the molecular weight of the solvent, the lower is the polarity, which allows similar molecular weight compounds to be easily extracted. The phytochemical compounds differ in the number and location of substitution of phenolic groups or hydroxyl groups, which donate electrons or atoms to free radicals and put a strong influence on the antioxidant properties of a compound. Thus, from the present investigation, the medicinal properties of *C. cupreum* can be identified based on the phytochemicals present in it. The results show that *C. cupreum* extracts poses significant antioxidant potential as determined by different methods. Overall, the antioxidant activities and presence of different kinds of secondary metabolites/pigments signify the potential of *C. cupreum* extract for its industry applications.

Conclusion

The results presented in this study showed significant antioxidant activities by the soil-isolated fungus *C. cupreum*-SS02 by different antioxidant methods as determined by spectrophotometric method. Thus, it can be concluded from the present study that different extracts of *C. cupreum* showed a significant antioxidant potential as determined by FRAP, PFRAP assay, DPPH, β-carotene bleaching (BCA), CUPRAC assay, metal chelating activity, TBARS assay, and hydrogen peroxide-induced erythrocyte hemolysis inhibition assay. The CUPRAC method is a simple and inexpensive antioxidant capacity method working at physiological pH and applicable for both hydrophilic and lipophilic antioxidant compounds. The scavenging activity is dependent on both reactivity and concentration of the antioxidant compound. These findings suggest further studies on the purification and characterization of principal compounds responsible for the antioxidant capacity of *C. cupreum*. Among the four solvent extracts of *C. cupreum* studied, ethyl acetate extract showed significant antioxidant potential. In addition, the extraction of extracellular compounds/pigments from *C. cupreum* is easier than that from other fungi and mushrooms, which indicates a new source of natural antioxidant compounds. Therefore, our results suggest further investigation on the antioxidant activities of *C. cupreum* extracts in order to develop new compounds useful for food and pharmaceutical industries.

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Conflicts of interest

There are no conflicts of interest.

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