Research and Reviews: Journal of Pharmacognosy and Phytochemistry

e-ISSN:2321-6182
p-ISSN:2347-2332

In-Vitro Inhibitory Effect Of Different Extracts of *Chaetomium Cupreum* on Alpha Glucosidase, Alpha Amylase and Hepg2 Cancer Cells

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Review Article

Received date: 17/11/2017
Accepted date: 20/11/2017
Published date: 05/01/2018

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Keywords: α-glucosidase, α-amylase, MTT, Chaetomium cupreum, postprandial, hyperglycemia, diabetes, cancer activity

ABSTRACT

Objective: The present study investigates the effect of soil isolated fungus *C. cupreum* on alpha glucosidase, alpha amylase and HepG2 cancer cells.

Methods: The antidiabetic effect of *C. cupreum* extracts was determined by α-glucosidase and α-amylase inhibition assays and anticancer activity was done by MTT assay.

Results: The inhibitory effect on α-glucosidase enzyme by *C. cupreum* extracts was observed in ethyl acetate extract with the value of 57.88 ± 0.05, followed by n-butanol extract 21.27 ± 0.04, methanol extract 15.19 ± 0.05 and chloroform extract 12.34 ± 0.01 at 100 µg/ml. Whereas, the inhibitory effect on α-amylase enzyme by n-butanol extract with a value of 53.70 ± 0.10, followed by ethyl acetate extract 31.81 ± 0.05, methanol extract 20.51 ± 0.05 and chloroform extract 16.00 ± 0.05 at 100 µg/ml concentration. In anticancer activity, n-butanol extract of *C. cupreum* showed significant cytotoxic effect against HepG2 cancer cells with IC50 value at 300 µg/ml.

Conclusion: Our study concludes that *C. cupreum* extracts possess potential phytochemicals that will be useful in antidiabetic and anticancer treatment after their purification and characterization

INTRODUCTION

Diabetes is a complex metabolic disorder characterized by hyperglycemia caused by lack of insulin hormone affecting numerous people worldwide. In 2011 it was estimated that 366 million people are suffering with diabetes and by 2030 it is predicted to be 552 million reports by International Diabetes Federation 2030 [1]. In India 40.9 million people had diabetes, which is predicted to grow to 60.9 million by 2025 [2]. In 2014, 387 million people were suffering with diabetes and are predicted to be 592 million by 2035 [3-5]. It was estimated that in the year 2013 in Africa 19.8 million people had diabetes and is predicted to be 41.4 million by 2035 [6]. In 2014 in Eritrea, the diabetic rate in adults was found to 4.89% [7]. In the United States, 8% of the adult population is suffering with diabetes and cause two-fold increase in age dependent mortality [8]. A diabetic study in Saudi Arabia reported that 4004 out of 16917 (23.7%) were suffering with diabetes between the ages groups of 30-70 years and was found more in urban areas of 25.5% compared to rural Saudis of 19.5% [9]. One possible approach for the prevention of diabetes is by controlling postprandial hyperglycaemia by the inhibition of *α-glucosidase* and *α-amylase* enzyme present in the gastrointestinal tract [10]. The inhibition of these two enzymes reduces carbohydrate hydrolysis, glucose absorption and consequently reduces the postprandial plasma glucose rise [11].

The function of enzymes *α-amylase* is the digestion of starch whereas *α-glucosidase* helps in absorption of glucose and thus both enzymes are involved in the control of postprandial hyperglycaemia [12-15]. *α-amylase* secreted by pancreas and salivary glands catalyses the hydrolysis of α-D-(1-4)-glucosidic linkages of starch to produce maltose and glucose, whereas another intestinal enzyme *α-glucosidase* (maltase, isomaltase and sucrose) releases glucose from maltose and sucrose [16,17]. Thus, the

e-ISSN:2321-6182
p-ISSN:2347-2332

inhibition α-amylase and *α-glucosidase* is one of the method to control glucose level [18,19]. There are many synthetic inhibitors such as acarbose, voglibose and miglitol are used for type II diabetes [20]. However, these inhibitors have several side effects, such as liver disorders, flatulence, diarrhea and other intestinal disturbances.

According to the global cancer statistic, hepatocellular carcinoma (HCC) is the third most common cause of cancer-related death worldwide [21] and it is estimate that out of 782,000 people diagnosed for liver cancer, among them 746,000 people died [22]. The liver cancer incidence in Western countries is 4 to 15 per 100,000, whereas Asia and Africa it is 120 per 100,000 [23]. Liver carcinogenesis is caused by various factors such as Hepatitis-B virus, Hepatitis-C infection, nonalcoholic fatty liver disease (NAFLD), aflatoxin- B1, diabetes, alcohol and iron accumulation [24].

Filamentous fungi have been used for the production of different natural products, such as enzymes, antibiotics, colorants, additives and many others which are used in cosmetic, food and pharmaceutical applications [25]. The natural products derived from plant and animal source exhibit some disadvantages such as low solubility and instability [26]. On the other hand, due to the environmental and toxicity issues of synthetic pigments/compounds industries are looking for microorganisms for the alternative source of natural compounds [27-31]. The fungal members of *Fusarium, Monascus, Aspergillus, Penicillium* and *Chaetomium* species are reported to produce high pigment production both intracellular and extracellular pigments with different colors ranging from red, yellow and orange [29-35]. Most of these fungal strains are non-pathogenic and non-mycotoxigenic to humans. Fungi produce different types of enzymes and secondary metabolites with various activities to survive and compete with the environment.

Due to the increasing drug toxicity and drug resistance it is important to search an alternative for new drugs from natural sources, such as fungi. Natural compounds are the one of the research alternative with potent inhibitory properties and minimal side effects. *Chaetomium* species produce a class of secondary metabolites called as azaphilones. Azaphilones are fungal pigments with pyrone-quinone structures and a highly oxygenated bicyclical core with a chiral quaternary centre [36,37]. Azaphilones possesses antimicrobial, anticancer, antiviral, antioxidant, antifungal and anti-inflammatory activities by the production of vinylogous χ-pyridones [38,39]. The aim of the study was to investigate the different extracts of *C. cupreum* for their antidiabetic and anticancer activities.

MATERIALS AND METHODS

Isolation and Identification of fungus

Chaetomium 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 isolated fungus was identified as *C. cupreum* -SS02 based on morphological and microscopic characteristics [40]. 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) region using universal primers; ITS-1 (TCCGTAGGTGAACCTGCGG) was the forward primer and ITS-4 TCCTCCGCTTATTGATATGC) was the reverse primer was performed [41]. Search for homologous sequences in Gen Bank was performed using BLAST (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/ blast) [42]. BLAST search performed for the sequence of ITS analysis, showed 99% homology with other strains of *C. cupreum* available in Gen bank. The sequence was deposited in NCBI Genbank with accession Number KF668034. The Culture was deposited in the National Fungal Culture Collection of India (NFCCI), with accession Number NFCCI 3117. The isolated fungus was maintained on potato dextrose agar (PDA) plates and potato dextrose agar slants at 4°C.

Inoculum preparation and fermentation

For inoculum preparation, the fungus was grown at 25°C on a PDA plate for 7 days, then 5 mm mycelial discs were bored out from the periphery of the colony and transferred to 250 ml Erlenmeyer flasks containing 100 ml of potato dextrose broth (PDB) and incubated at 26 ± 2°C on a rotary shaker at 120 rpm for 20 days to achieve the highest pigment production.

Extraction of extracellular pigments

The extraction of pigments was carried out according to the method described previously [43]. After 20 days of incubation biomass was removed by filtration through Whatman No. 1 filter paper and the broth containing the extracellular pigment/ metabolites was obtained. The cultural broth obtained was used for extraction of pigments/compounds by liquid-liquid method in 500 ml of separating funnel using four different organic 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 minutes 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 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.

e-ISSN:2321-6182
p-ISSN:2347-2332

α-Glucosidase inhibition assay

The effect of *C. cupreum* extract on the inhibition of α-glucosidase activity was determined according to the method as described by Kim [44]. Fungal extracts were prepared as a stock concentration by dissolving 5 mg/ml of DMSO. The reaction mixture consists of 250 µl of 3.0 mM 4-nitrophenyl α-D-glucopyranoside (PNPG) as a substrate dissolved in 20 mM phosphate buffer (pH 6.9), 100 µl (100 µg/ml in DMSO) from each *C. cupreum* extract (chloroform, ethyl acetate, n-butanol and methanol extract) and 100 µl of α-glucosidase solution (1.0U/ml) in 0.01 M phosphate buffer (pH 7) and the reaction mixture was incubated at 25 °C for 20 minutes. After incubation, the reaction was stopped by adding 2 ml of 0.1M sodium carbonate (Na_2CO_3). The α-glucosidase activity was determined by measuring the yellow colored paranitrophenol release from paranitrophenol glucopyranoside (PNPG) at 420 nm. The control sample included all the reagents with enzyme, replacing the sample with DMSO. The inhibitory activity of α-glucosidase is calculated by the following formula:

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100 \quad \ldots\ldots 1$$

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

α-Amylase inhibition assay

The effect of *C. cupreum* extracts on the inhibition of *α-amylase* activity was determined according to the method as described previously [45]. The reaction mixture consists of 250 µl (100 µg/ml DMSO) of *C. cupreum* extract (chloroform, ethyl acetate, n-butanol and methanol extract), 250 µl of 0.02 M sodium phosphate buffer (pH 6.9) and 100 µl of α-*amylase* solution (0.5mg/ml). This solution was incubated at 25^{0}C for 10 minutes. After incubation 250 µl of 1% starch solution in 0.02M sodium phosphate buffer (pH 6.9) was added and was further incubated at 25 ˚C for 10 minutes. The reaction was terminated by adding 500 µl of 3,5-dinitrosalicylic acid (DNS) reagent. The reaction tubes were then incubated in boiling water for 5minutes and cooled to room temperature. The α-*amylase* activity was determined by measuring the absorbance at 540 nm using spectrophotometer. Control sample included all the reagents with enzyme, replacing the samples with DMSO. The α-*amylase* inhibition by *C. cupreum* extracts was calculated by the given formula:

$$\% \text{ Inhibition} = ((A_0 - A_1)/A_0) * 100 \quad \ldots\ldots 2$$

where, A_0 is the absorbance of the control and A_1 is the absorbance of the test extract.

Anticancer activity

Cell culture and maintenance

The cancer cell lines HepG2 (Hepatocellular carcinoma) was obtained from National cell culture science (NCCS), Pune, India. The cells were cultured in a cell culture flask using Dulbecco's Modified Eagles Medium (DMEM) Nutrient Mixture F-12 HAM supplemented with 10% fetal bovine serum (FBS), 15mM HEPES, $NaHCO_3$, pyridoxine and L-glutamine. The cells were maintained in a 5% CO_2 incubator at 37ºC.

Cell viability assay

The cytotoxicity of *C. cupreum* extracts on HepG2 liver cancer cells was assessed using a MTT (3-(4,5-dimetylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) assay as described previously [46]. HepG2 liver cancer cells were plated (1×10^4 cells/well) by adding 200 µl cell suspension to each well of a 96-well microtiter plates (NUN96ft-Nunclon-96 Flat transparent). The plate was incubated at 37 °C in humidified atmosphere with 5% CO_2 for 24 hrs to assure attachment and 80% to 100% confluence. After 24 hrs, media was aspirated off and replaced with fresh media (200 µl) containing *C. cupreum* extract of different concentrations (25 µg/ml, 50 µg/ml, 100 µg/ml, 150 µg/ml, 200 µg/ml, 250 µg/ml and 300 µg/ml). The plate was incubated at 37 °C with 5% CO_2 for 24 hours. After incubation, the media was aspirated off and replaced with fresh media containing 20 µl MTT reagent (2 mg/ml PBS) for a total volume of 200 µl was added to each well and incubated at 37 °C, with 5% CO_2, for 2 to 3 hours. After incubation, MTT containing medium was removed gently and 200 µl of DMSO per well to dissolve the formazan crystals was added. The control sample included all the reagents with HepG2 cells replacing the sample with DMSO. The absorbance was measured at 585 nm using a Model 680 Microplate Reader (Shimadza UV-1800). Percentage of cell viability was calculated according to the following equation [47].

$$\text{Percentage of cell viability} = (\text{OD of treated cells}/\text{OD of control cells}) * 100 \quad \ldots\ldots 3$$

The concentration of drug that inhibits 50% of cells (IC_{50} values) for these samples were calculated.

Statistical analysis

All the measurements were taken in triplicate and expressed as mean value ± standard deviation. The data were analyzed for statistical significance using one-way analysis of variance (ANOVA) followed by Bonferroni Multiple Comparisons Test. with Graph Pad Prism 6 software (Graph Pad Software, Inc., USA). The probability values of $p<0.05$ were considered as statistically significant.

e-ISSN:2321-6182
p-ISSN:2347-2332

RESULTS

Identification of *C. cupreum*

The fungus was identified on the basis of morphological, microscopic and molecular phylogenetics involving an ITS-5.8S region of the rDNA and named as *C. cupreum* SS02. In morphology *C. cupreum* exhibited typical white cottony mycelial colonies on the PDA.

Effect of *C. cupreum* extract on α-Glucosidase Inhibition

The *C. cupreum* extracts were studied for their *α-glucosidase* inhibition property. The percentage inhibition displayed by each *C. cupreum* extracts are presented in **Figure 1**. Among the four different extracts of *C. cupreum* studied, ethyl acetate extract showed inhibition percentage with a value of 57.88 ± 0.05, followed by n-butanol extract 21.27 ± 0.04, methanol extract 15.19 ± 0.05 and chloroform extract 12.34 ± 0.01 at 100 µg/ml. The *α-glucosidase* inhibitory activity was displayed by ethyl acetate extract with IC_{50} value at 86.38 µg/ml concentration. The highest inhibition percentage of *α-glucosidase* enzyme was shown by ethyl acetate extract, whereas methanol extract shows lowest *α-glucosidase* inhibition percentage. The *C. cupreum* extracts shows a significant difference (p<0.0001) followed by Bonferroni's multiple comparison test.

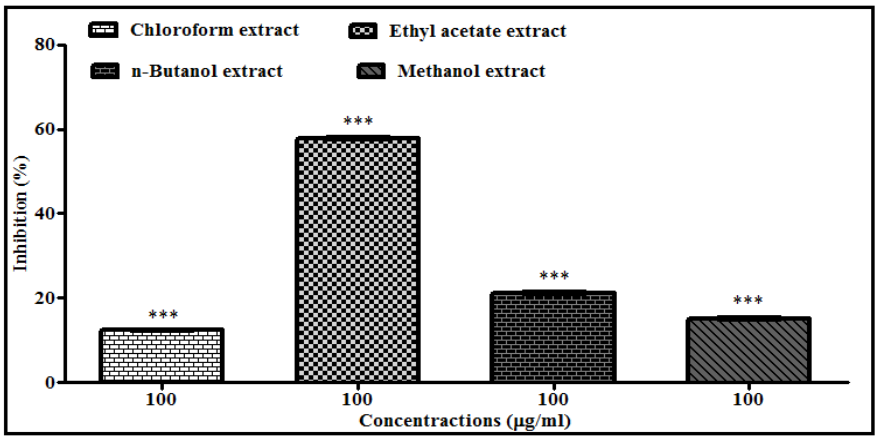

Figure 1. The Inhibitory effect of *C. cupreum* extracts on *α-glucosidase* activity. The ethyl acetate extract showed IC_{50} value at 86.38 mg/ml concentration. The ***p indicates a significant difference between the columns analyzed by using the Bonferroni's Multiple Comparison Test (p<0.0001).

Effect of *C. cupreum* extract on α-amylase inhibition

The percentage inhibition of *α-amylase* enzyme displayed by each *C. cupreum* extracts are presented in **Figure 2**. Among the different extracts of *C. cupreum* studied, the inhibition percentage on *α-amylase* enzyme was shown by n-butanol extract with the value of 53.70 ± 0.10, followed by ethyl acetate extract 31.81 ± 0.05, methanol extract 20.51 ± 0.05 and chloroform extract 16.00 ± 0.05 at 100 µg/ml. The highest *α-amylase* inhibitory activity was shown by n-butanol extract of *C. cupreum* with IC_{50} value at 93.10 µg/ml concentration. The highest inhibition percentage of *α-amylase* was shown by n-butanol extract whereas chloroform extract shows the lowest *α-amylase* inhibition percentage. In the results, it was observed that *C. cupreum* extracts shows a significant difference (p<0.0001) followed by Bonferroni's multiple comparison test.

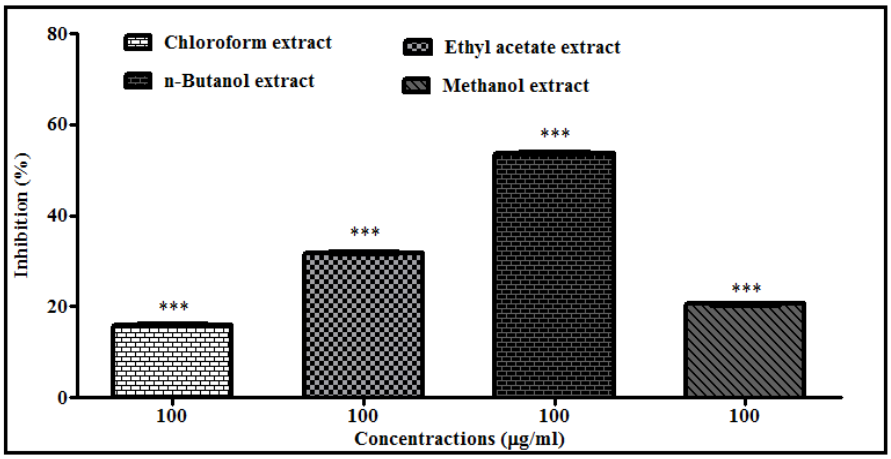

Figure 2. Inhibitory activity of *C. cupreum* extracts on *α-amylase* activity. The n-butanol extract of *C. cupreum* showed IC_{50} value at 93.10 mg/ml. The ***p indicates a significant difference between the columns analyzed by using the Bonferroni's Multiple Comparison Test (p<0.0001).

e-ISSN:2321-6182
p-ISSN:2347-2332

Effect of *C. cupreum* extract on HepG2 cells

The *in-vitro* effect of *C. cupreum* extracts on HepG2 is presented in **Figure 3**. A significant decrease was observed in percentage cell viability against HepG2 cancer cells at different concentrations after 24 hours of treatment. The maximum decrease in percentage cell viability by *C. cupreum* extracts was observed in n-butanol extract with the value of 51.68 ± 0.01, followed by ethyl acetate extract 43.45 ± 0.01, methanol extract 30.94 ± 0.01 and chloroform extract 24.04 ± 0.07 at 300 µg/ml concentration after 24 hours. The IC_{50} values of n-butanol extract of *C. cupreum* was found at 300 µg/ml after 24 hours of treatment. The results from our study revealed that the compounds from n-butanol extract of *C. cupreum* could be a possible target against hepatocellular carcinoma treatment. There was a significant difference (p<0.0001) followed by Bonferroni posttests test.

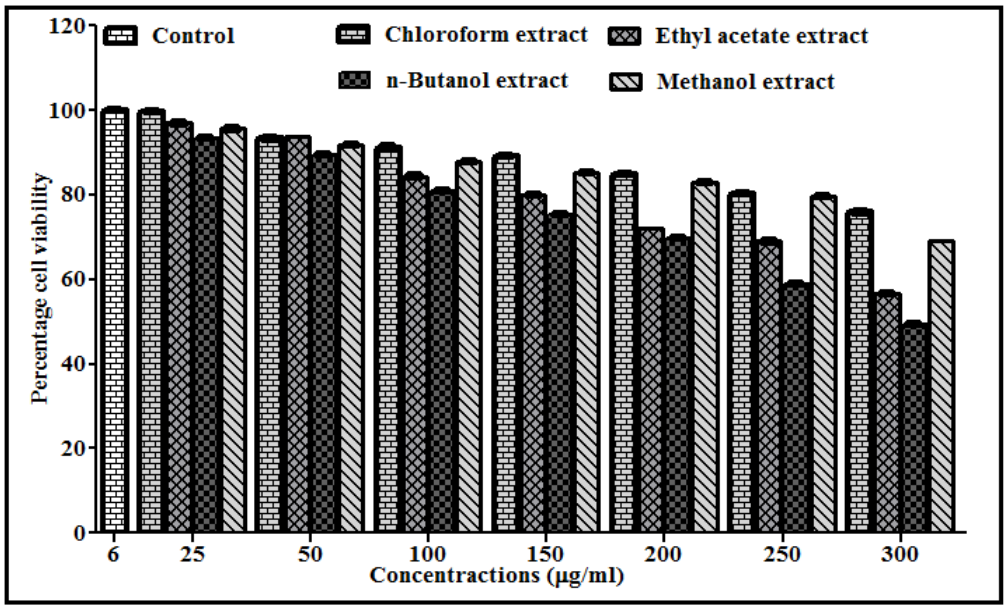

Figure 3. Cytotoxic effect of *C. cupreum* extracts on HepG2 liver cancer cells after 24 hrs treatment by MTT assay. (IC50 value at 300 µg/ml in n-butanol extract). All *C. cupreum* extracts indicates significant differences (p<0.0001) according to Bonferroni posttests.

DISCUSSION

In diabetes the therapeutic approach involves the inhibition of α-*amylase* and α-*glucosidase* this will result in delaying the carbohydrate digestion and reduction in glucose absorption thereby lowering postprandial glucose level hyperglycemia [48]. Therefore, the inhibition of carbohydrate digesting enzymes could be one of the most effective approaches to control diabetes [49]. At present, the inhibitors of *α-glucosidase* and α-*amylase* used are acarbose, miglitol and voglibose [50]. In carbohydrate digestion they inhibit the conversion of maltose to glucose and thus decrease the rate of glucose into the blood circulation [51]. Our study showed that all the *C. cupreum* extracts have exhibited inhibition of *α-glucosidase* and *α-amylase* activity. However, the maximum *α-glucosidase* inhibition was shown by ethyl acetate extract of *C. cupreum* whereas, the maximum *α-amylase* inhibition was shown by n-butanol extract of *C. cupreum*. In the literature there was no information available about the *α-glucosidase* and *α-amylase* inhibitory activity of the fungus *C. cupreum*. Hence, our study evaluated the *in vitro α-glucosidase* and *α-amylase* inhibitory activity of soil isolated fungus *C. cupreum*. The inhibition of *α-glucosidase* and *α-amylase* by *C. cupreum* extracts would result in the slow hydrolysis of carbohydrates thereby reduces the rate of glucose uptake from small intestine and thus control of hyperglycaemia condition could be achieved through the inhibition of carbohydrate digesting enzymes [52, 53].

In our report, we have demonstrated for the first time that *C. cupreum* extract acts as an anticancer agent by induction of dose dependent apoptosis in HepG2. The results of this study suggest further in depth in vitro and in vivo studies. The positive outcomes of future studies could be useful for developing *C. cupreum* compounds as a novel compound for hepatocellular carcinoma treatment. Thus, these biological activities of *C. cupreum* extracts may be due to the phytochemicals present in it. The positive outcomes of future studies could be useful for developing *C. cupreum* compounds as a novel compound for antidiabetic and hepatocellular carcinoma treatment.

CONCLUSION

These findings suggest that the *C. cupreum* extracts are effective inhibitors for α-glucosidase and α-amylase enzymes, which may helpful to reduce the postprandial glucose levels. Also, *C. cupreum* has displayed significant anticancer activity against HepG2 cancer cells. The results of this study suggest further in depth *in-vitro* and *in-vivo* studies on *C. cupreum* extracts and purification and characterization of their compounds.

e-ISSN:2321-6182
p-ISSN:2347-2332

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGMENT

The authors are grateful to the UGC (University Grants Commission), New Delhi, Govt. of India, for UGC-MRP Grants for financial support and also to the Head, Department of Microbiology and Biotechnology (MB & BT), Bangalore University, Bengaluru (BUB), Karnataka, India, for the use of laboratory facilities.

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