Evaluation of Anticancer Activity of *Chaetomium cupreum* Extracts against Human Breast Adenocarcinoma Cell Lines

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Abstract

Background: Fungi are microorganisms which produce a wide variety of biologically active secondary metabolites that could be used for medicinal and pharmaceutical purposes. The *Chaetomium cupreum* is a natural source of different types of intracellular and extracellular pigments which exhibit various biological properties. **Objectives:** The main objectives of this study were to evaluate the anticancer property of *C. cupreum* extracts against human breast adenocarcinoma cell lines. **Materials and Methods:** The anticancer activity of different extracts of *C. cupreum* on human breast cancer cells was evaluated using MTT tetrazolium bromide assay. The measurement of mitochondrial membrane potential was evaluated using JC-1 fluorescent dye and reactive oxygen species (ROS) by 2,7-dichlorodihydrofluorescein diacetate dye. The partial purification and characterization of compound was done by chromatography and spectrometry methods. **Results:** It was found that ethyl acetate extract of *C. cupreum* showed significant cytotoxic activity against MCF-7 cells with IC_{50}-40 and against MDA-DB231 cells with IC_{50}-75 μg/ml concentration. In subfractions of ethyl acetate, the highest cytotoxic effect was shown by methanol subfraction (44.79%), followed by ethyl acetate subfraction (39.79%) and chloroform subfraction (37.07%) at the concentration of 25 μg/ml after 24 h. The anticancer activity of seco-chaetomugilin on MCF-7 cancer cells was 51.56% at the concentration of 25 μg/ml. In results, seco-chaetomugilin treatment increased depolarization of mitochondrial membrane potential to 16.45% at the concentration of 5 μg/ml and 32.25% at the concentration of 15 μg/ml as compared to 13.47% in untreated cells after 24 h. The results showed that the treatment of seco-chaetomugilin increased ROS generation to 19.6% at the concentration of 5 μg/ml and 26.2% at the concentration of 15 μg/ml as compared to 14% in untreated cells. **Conclusion:** These results showed that ethyl acetate extract and seco-chaetomugilin pigment induces mitochondria depolarization and ROS production, which leads to cell death. Thus, these results suggested that that pigment seco-chaetomugilin isolated from *C. cupreum* should further be studied for its anticancer activity on molecular level.

Keywords: Adenocarcinoma, azaphilones, *Chaetomium,* depolarization, seco-chaetomugilin

Introduction

Cancer represents one of the leading causes of deaths around the globe. The abnormal growth of tissues of milk ducts lining and the lobules in breast is known as breast cancer. The breast cancer which generates from ducts is called as ductal carcinoma and the one which generates from lobules is known as lobular carcinoma. The synthetic and natural compounds are developed for cancer treatment throughout the world with fewer side effects.[1-3] The 50% of women who have breast cancer in developing countries are 63 years old, whereas in developed countries are 50 years old.[4] In last few decades, it is found that the prevalence of breast cancer increased[5] with more incidence rate in Asian countries.[6] In India after cervical cancer, the second cause of deaths in India is the breast cancer with 53,592 cases being found in 2008.[7] The breast cancer is more at the age of forty in Indian women.[8] In India, in each year, 100,000 new cases of breast cancer are reported.[8,9] About 90% of people suffering with breast cancer require chemotherapy. The etiology of breast cancer includes multifactorial risk factors which include environmental and various biological factors such as early menarche, late

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menopause and delaying first pregnancy, oral contraception, hormonal imbalance, diet, and obesity.[10] About 1.35 million new cases of breast cancer are reported worldwide according to Harvard School of Public Health in 2009. Around 20%–30% increase in new cases and about 50% increase in breast cancer deaths in developing countries are reported by the International Agency for Research on Cancer (IARC) registry.[11] According to the ICMR-PBCR, the urban registries of Kolkata, Delhi, Mumbai, Trivandrum, and Ahmadabad show >30% cancers in females. In Barshi, it is found that the breast cancer is the second cancer after uterine cervix cancers in women.[12] The *Chaetomium* species belongs Chaetomiaceae family. It was in 1817 when Kunze first developed the genus and after that 350 Chaetomium species are reported so far.[13]

From the past few years, due to the toxicity and environmental issues associated with synthetic pigments, the natural pigment production from microbial source has increased due to consumers demand for natural color. Furthermore, previous studies have shown that pigments extracted from plant and animal source have certain drawbacks such as low solubility, instability, and seasonal variations.[14] The various ascomycetous fungi such as *Aspergillus, Fusarium, Penicillium, and Trichoderma* are natural source of pigments. Furthermore, fungal pigments have broad potential for designing new pharmaceutical compounds with various biological properties.[15]

The *Chaetomium* genus is natural of various types of secondary metabolites including pigments. Azaphilones have been identified in a variety of fungi. The various microorganisms such as Monascus*, Penicillium* and *Chaetomium* are significant producers of azaphilone pigments. Azaphilones are well known to exhibit various biological activities such as antifungal, antiviral, antioxidant, cytotoxic, nematicidal, and anti-inflammatory activities. Such characteristic biological potential of azaphilones are due to their production of vinylogous gamma-pyridone structures.[16] There are no previous reports on anticancer activity of *Chaetomium cupreum* extracts on human breast adenocarcinoma cell lines. In this study, an attempt was made to evaluate the cytotoxicity effect of *C. cupreum* extracts against human breast adenocarcinoma cell lines.

Materials and Methods

Procurement of fungal culture and extraction of secondary metabolites/pigments

The fungus *C. cupreum* culture was procured from the National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India, with accession number 3117. The extraction of pigments from *C. cupreum* fungal broth was carried out by liquid–liquid method as previously described by our group Nazir and Sharmila.[17]

Cell culture and maintenance

The human breast cancer cell lines such as MCF-7 and MDA-MB231 were procured from the National Cell Culture Science, Pune, India. Both the cells were cultured in cell culture flask in Dulbecco's Modified Eagle medium. The cells were maintained in CO_2 incubator at 37°C.

Cell viability-MTT assay

The cytotoxicity of *C. cupreum* extract on breast cancer cells was assessed using a MTT tetrazolium bromide assay.[18] The breast cancer cells were plated at 1×10^4 cells/well by adding 200 µl cell suspensions to each well of a 96-well microtiter plates (NUN96ft-Nunclon-96 Flat transparent) and incubated at 37°C in CO_2 incubator for 24 h to assure attachment and 80%–100% confluence. After 24 h, fresh media (200 µl) containing *C. cupreum* extract (25 µg/ml to 300 µg/ml) and reference standard doxorubicin (5 µg/ml) were added and again incubated for 24 h. After incubation, the media was removed and fresh media containing 20 µl MTT reagent (2 mg/ml PBS) for making up a total volume of 200 µl to each well and incubated at 37°C, with 5% CO_2, for 2–3 h. After incubation, MTT containing medium was removed gently and 200 µl of DMSO per well to dissolve the Formosan crystals was added. The control sample included all the reagents with cancer cells replacing the sample with DMSO. The absorbance was measured at 585 nm using a Model 680 Microplate Reader (Schimadzu UV-1800). The percentage of cell viability was calculated according to the following equation.[19] The percentage of cell viability = (OD of treated cells/OD of control cells) × 100.

Determination of cell morphology

Crystal violet assay

MCF-7 cells have a polygonal morphology with irregular dimensions and they have a habit of clumping into large aggregates. One simple method to detect maintained adherence of cells is the staining of attached cells with crystal violet dye, which binds to proteins and DNA. Cells that undergo cell death lose their adherence and are subsequently lost from the population of cells, reducing the amount of crystal violet staining in a culture. The MCF-7 cells were treated with methanol subfraction of *C. cupreum* for 24 h and then washed twice with PBS for 3 min. The treated cells were then stained with 0.5 ml crystal violet dye for 5 min. After washing with PBS for 3 times, the cells were observed under a fluorescent microscope by ×400 magnification.

DAPI (4, 6-diamidino-2-phenylindole) staining

The MCF-7 cells were treated with methanol subfraction for 24 h. The cells were washed with PBS, fixed with ice-cold 70% ethanol, and resuspended in DAPI and incubated for 15 min at 37°C wrapped in aluminum foil. The cells were then washed with phosphate buffer saline and examined under fluorescent microscope.

Acridine orange/ethidium bromide

The acridine orange (AO) binds with DNA and produces green fluorescence by staining live cells, whereas ethidium bromide (EtBr) produces orange fluorescence by staining dead

Table 1: The comparison of standard compound doxorubicin and *C. cupreum* extracts in anticancer activity for IC_{50} value

MDA-MB231 cancer cells	MCF-7 cancer cells	Sub-fractions of ethyl acetate extract (MCF-7 cancer cells)	Doxorubicin
Ethyl acetate IC_{50}-75 µg/ml	Ethyl acetate IC_{50}-40 µg/ml	Methanol sub-fraction (IC_{50}-37 µg/ml)	IC_{50}-7 µg/ml for MDA-MB231 cells and IC_{50}-5 µg/ml MCF-7 cancer cells
		Ethyl acetate sub-fraction (IC_{50}-43.5 µg/ml)	
		Chloroform sub-fraction (IC_{50}-50 µg/ml)	

cells. The MCF-7 cells were treated with methanol subfraction for 24 h. After washed with phosphate buffer saline for 3 min, 0.5 ml AO/EtBr was added for 5 min. Then, cells were washed again with PBS and observed under a fluorescent microscope by ×400 magnification.

Purification and characterization of pigment

Thin-layer chromatography

The analytical thin-layer chromatography (TLC) of ethyl acetate extract of *C. cupreum* were performed using silica gel plates (MERCK silica gel 60 PF_{254} aluminium sheets, 250 mm thick, 20 cm × 20 cm). The crude extracts were dissolved in 0.5 ml of methanol and spotted on the silica gel plates and spots were air dried. The silica gel TLC plates were run in the toluene: ethyl acetate: acetic acid: formic acid (7.5:2.5:1:1) solvent system for ethyl acetate extract of *C. cupreum*. The TLC silica gel plates were observed under visible light and ultraviolet (UV) light (254 nm and 366 nm).

Solvent subfractionation/solvent partitioning method

The subfractionation of ethyl acetate extract of *C. cupreum* was done into three solvent subfractions in a consecutive order. The ethyl acetate subfractions were run in the toluene: Ethyl acetate:acetic acid:formic acid (7.5:2.5:1:1) solvent system. The semi-purified TLC bands were scraped out and collected.

Analytical high-performance liquid chromatography

The purified TLC bands were further analyzed by analytical high-performance liquid chromatography (HPLC) (Waters, Ltd., Bangalore SunFire@C_{18} column, 5 µm, 46 mm × 250 mm). The chromatographic conditions are as follows. Flow rate: 1 ml/min, injection volume: 20 µl, run time 25 min, temp: 25°C and column temperature: 30°C. The solvent system consists of Solvent-A (acetonitrile [ACN]) and solvent-B (water-acetic acid [80:20]). The solvent system was filtered through a 0.45 µm nylon filter and sonicated in an ultrasonic bath for 15 min. The separation of the compounds is monitored at 254 nm using UV detector.

Ultraviolet-visible spectrophotometer analysis

The UV-Vis spectrometer technique was used for the analysis of the compounds in the scan range of 200–800 nm on spectrum mode.

Quadruple time of light liquid chromatography mass spectrometry

The quadruple time of light liquid chromatography mass spectrometry (Q TOF LCMS) was carried out using the following parameters; solvent A 5 mM NH4HCO2 in water, solvent B 5 mM NH4HCO2 in ACN, gradient (minutes %) 0 min–13%/1.80 min–60%/2.50 min–60, Injection volume – 10 µl, Flow rate 800 µl/min, UV Detector – 254 nm (4 nmBW on DAD), REF A (DAD) 400 nm (80 nm BW), Temp – 40°C, mass range (m/z) 100–1200, sheath gas temp: 250°C, sheath gas flow: 8 Lpm, Nebulizer pressure: 45 psi, Capillary voltage: 3500V, drying gas temp: 250°C, 3.5 µm, 100 Å, p/n 873700 902. The data acquisition and analysis were performed using MassLynx™ V4.1 software, SCN862 and ASC901 (Waters Corporation), respectively.

Cytotoxic effect of seco-chaetomugilin

Measurement of mitochondrial membrane potential using JC-1 dye

The changes during the loss of mitochondrial membrane potential were estimated using JC-1 (5,5'6,6-tetrachloro-1,1',3,3-tetraethylbenzimidazolylcarbocyaniodide) fluorescent dye.[20] The cells were seeded in 24 well plate at the density of 1×10^5 cells/ml. After 24 h of incubation, cells were treated with seco-chaetomugilin compound and incubated again for another 24 h. Then cells were collected by trypsinization and centrifuged at 2000 rpm for 5 min. Then around 1×10^6 cells were incubated with 500 µl of JC-1 dye for 15 min. The cells were washed twice with assay buffer before fluorescence-activated cell sorting (FACS) flow cytometry analysis. The estimation of depolarized cells were determined with excitation/emission at 488/525 nm in the FL1 and FL2 channels, respectively.

Measurement of reactive oxygen species production by 2,7-dichlorodihydrofluorescein diacetate labelling

In MCF-7 cancer cells the reactive oxygen species (ROS) measurement was determined using 2,7 dichlorodihydrofluorescein diacetate (DCFH DA) dye.[21] The cells were seeded in 24 well plate at the density of 1×10^5 cells/ml. Then 100 µl of DCF-DA (10 µM) were added in each well and incubated for 1 h. Then cells were washed with PBS three times and treated with 5 and 15 µl of seco-chaetomugilin and further incubated for 3 h. The fluorescence intensity produced was measured by FACS flow cytometry analysis with excitation/emission wavelengths of 485/528 nm, respectively.

Statistical analysis

For statistical analysis two-way analysis of variance, followed by Tukey's multiple comparison test with graph pad prism 6 software.

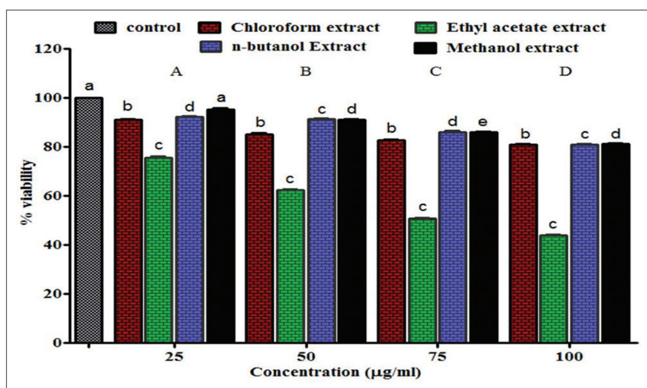

Figure 1: Cytotoxic effect of *Chaetomium cupreum* extracts on MDA-MB231 cancer cells after 24 h of treatment (ethyl acetate extract with IC50-75 µg/ml). Values are represented as mean ± SD ($n = 3$). Significance difference between the control and extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are significantly different at $P < 0.05$

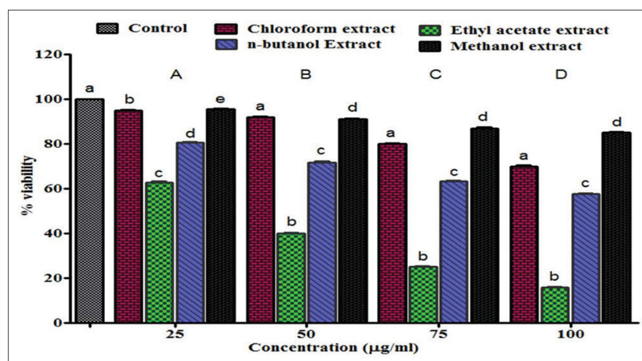

Figure 2: Cytotoxic effect of *Chaetomium cupreum* extracts on MCF-7 cancer cells after 24 h of treatment (ethyl acetate extract with IC50-40 µg/ml). Values are represented as mean ± SD ($n = 3$). Significance difference between the control and extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are significantly different at $P < 0.05$

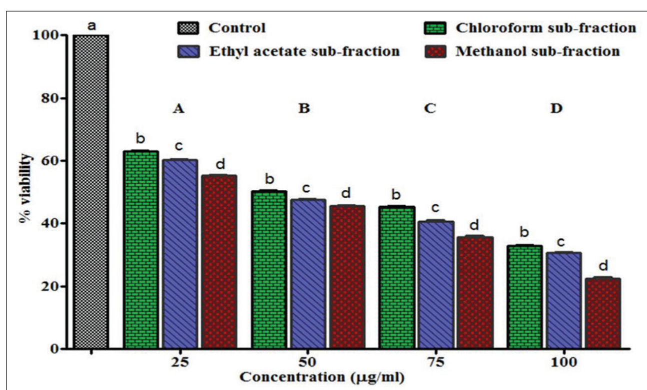

Figure 3: Cytotoxic effect of subfractions of ethyl acetate extract of *Chaetomium cupreum* on MCF-7 cancer cells after 24 h of treatment. Values are represented as mean ± SD ($n = 3$). Significance difference between the control and extracts is represented in lower case and between the concentrations in upper case. Those not sharing the same letter are significantly different at $P < 0.05$

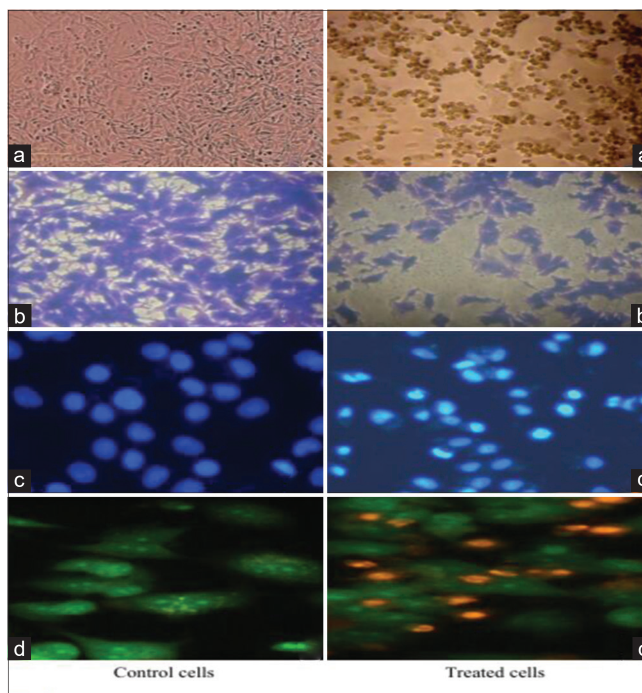

Figure 4: Morphological changes of MCF-7 cells treated with 25 µg/ml of methanol sub-fraction for 24 hrs viewed under an inverted light microscope (200 x magnification). a)A- Control and methanol sub-fraction treated MCF-7 cells, b) B-Crystal violet staining, c) C-DAPI (4, 6-diamidino-2-phenylindole) staining. d) D-Acridine orange/Ethidium bromide staining (AO/EtBr), Arrows indicate dead cells

Results

Anticancer activity by MTT assay

The anticancer activity of *C. cupreum* extracts on MDA-AB231 and MCF-7 cell lines are presented in Figures 1 and 2. In anticancer activity against two breast cancer cell lines, ethyl acetate extract showed highest cytotoxic activity on both the cancer cell lines. However, ethyl acetate extract of *C. cupreum* was more effective on MCF-7 cells with IC_{50}-40 µg/ml [Figure 2] as compared to MDA DB231 cells with IC_{50-}75 µg/ml [Figure 1]. The comparison of standard doxorubicin and *C. cupreum* extracts in anticancer activity for IC_{50} value is shown in Table 1.

Cytotoxic effect of subfractions of ethyl acetate extract

The ethyl acetate extract was further subfractionated into chloroform, ethyl acetate and methanol in a consecutive order. Then, these three subfractions were further studied for cytotoxic effect. The cytotoxic effect of methanol subfraction was 44.79%, followed by ethyl acetate subfraction 39.79% and chloroform subfraction showed 37.07% against MCF-7 cells at 25 µg/ml after 24 h of treatment [Figure 3]. Among three fractions, methanol subfraction showed the significant cytotoxic effect [Figure 3 and Table 1]. The morphological changes on MCF-7 cells treated with methanol subfraction for 24 h were further confirmed by different staining procedures such as crystal violet, DAPI and AO/EB staining [Figure 4].

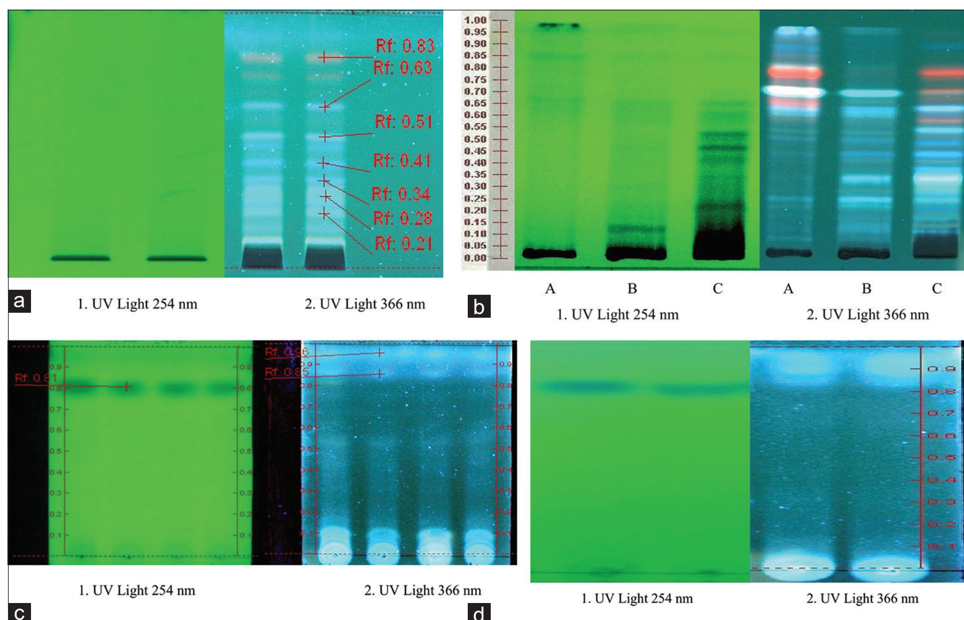

Figure 5: (a) Thin-layer chromatography of ethyl acetate extract of *Chaetomium cupreum.* (b) Thin-layer chromatography of subfractions of ethyl acetate extract of *Chaetomium cupreum*. A-chloroform fraction, B-Ethyl acetate fraction, C-Methanol fraction). (c) Preparative thin-layer chromatography image of semi-purified bands obtained from methanol subfraction. (d) Preparative thin-layer chromatography image of the most prominent band obtained from semi-purified band

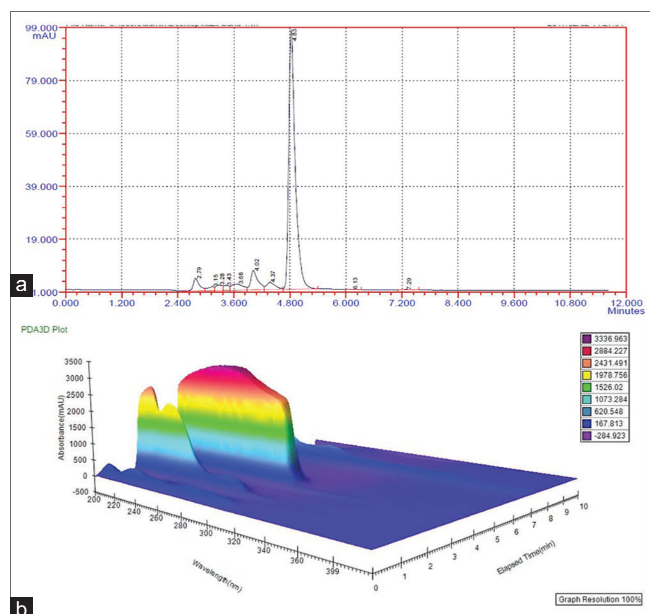

Figure 6: (a) Analytical high-performance liquid chromatography of purified band of thin-layer chromatography with one major peak having retention time 4.85. (b) Three-dimensional image of peak from high-performance liquid chromatography having retention time 4.85

Purification and characterization of seco-chaetomugilin

Thin-layer chromatography

The various solvent systems were used for the standardization of TLC in order to separate different compounds from *C. cupreum*. The mixture of compounds/pigments from ethyl acetate extract of *C. cupreum* was separated on the silica gel TLC plates with standardized first in solvent system of toluene/ethyl acetate/acetic acid/formic acid in the ratio of 7.5:2.5:1:1 [Figure 5a].

Subfractionation of ethyl acetate extract

As there was no clear separation or movement of compounds, the crude extracts were subjected to solvent subfractionation. Further, ethyl acetate extract of *C. cupreum* was solvent subfractionated in chloroform, ethyl acetate, and methanol subfractions in the consecutive order and run in the toluene/ethyl acetate/acetic acid/formic acid in the ratio of 7.5:2.5:1:1 [Figure 5b].

Preparative thin-layer chromatography

The semi purified bands of methanol subfraction were scraped and further purified on silica gel plates in solvent system of toluene/ethyl acetate/formic acid in the ratio of 7:3:1, and two UV visible bands were observed having Rf value of 0.81 cm and 0.96 cm [Figure 5c]. The most prominent UV band was scraped and preparative TLC was run on silica gel plate and only one UV visible band was observed with R_f value 0.85 cm [Figure 5d].

High-performance liquid chromatography

In the analytical HPLC, scraped and further purified band shows one major peak with retention time 4.83 nm was observed [Figure 6a and 6b].

Ultraviolet-spectrum analysis

The UV spectrum of purified compound showed λ_{max} at 274 nm [Figure 7].

Quadruple time of light liquid chromatography mass spectrometry analysis

The quadruple time of light liquid chromatography mass

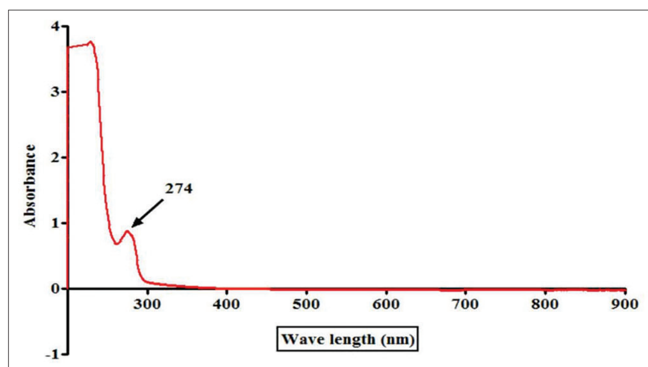

Figure 7: Ultraviolet (UV) spectrum of purified compound showing λmax at 274 nm

spectrometry was used for compound identification. The purified yellow-colored band was further identified as seco-chaetomugilin with molecular mass of 467.469 with molecular formula $C_{24}H_{32}$ $^{35}ClO_7$ based on Q-TOF LCMS analysis [Figure 8a and b].

Cytotoxic effect of seco-chaetomugilin on MCF-7 cells at different time points

The cytotoxic effect of seco-chaetomugilin is summarized in Figure 9a and b. The cytotoxic effect of purified compound seco-chaetomugilin on MCF-7 cells was 75.25%, 51.56%, 40.25%, 37.5%, and 25.25% at the concentration of 50, 25, 12.5, 6.25, and 3.12 µg/ml after 24 h of treatment, respectively [Figure 9a]. Similarly, after 38 h of seco-chaetomugilin treatment on MCF-7 cells, the maximum cytotoxic effect was 99%, 79%, 58.25%, 48.25%, and 41.5% at the concentration of 50, 25, 12.5, 6.25, and 3.12 µg/ml, respectively [Figure 9b]. Whereas, the cytotoxic effect of seco-chaetomugilin on breast epithelial (fr2) cell line was 21.14%, 18.2%, 14.6%, 10.67%, and 6.2% at the concentration of 50, 25, 12.5, 6.25, and 3.12 µg/ml, respectively [Figure 9b].

Depolarization of mitochondrial membrane potential

The loss of mitochondrial membrane potential is one of the earlier mitochondrial apoptotic biochemical pathways. The mitochondrial membrane potential diminishes with increase in mitochondrial membrane permeability. For the determination of JC 1 monomer and JC-1 dimer ratio, MCF -7 cells were treated with seco chaetomugilin pigment. The red (JC-1 dimer) to green (JC-1 monomer) shift is presented in Figure 10. The results showed that seco-chaetomugilin treatment increased depolarization of mitochondrial membrane potential to 16.45% at 5 µg/ml and 32.25% at 15 µg/ml as compared to 13.47% in untreated cells after 24 h [Figure 10]. The intensity of green fluorescence increased, whereas the intensity of red fluorescence decreased.

Analysis of reactive oxygen species generation

The ROS plays an essential role in apoptosis induction. To analyze changes associated with intracellular ROS level and involvement of ROS in apoptosis induced by

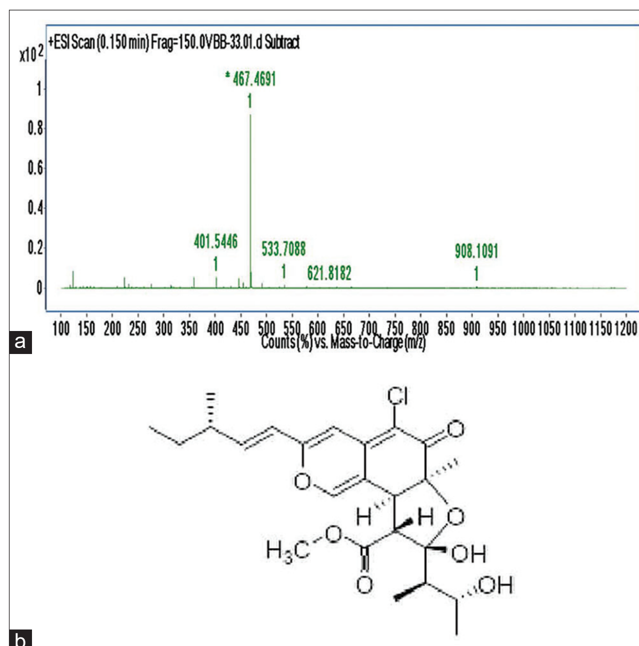

Figure 8: (a) The quadruple time of light liquid chromatography mass spectrometry analysis of the purified compound. The purified yellow colored compound was identified as "seco-chaetomugilin" with molecular mass of 467.469 [M+H]+ and molecular formula C24H32 35ClO7. (b) Structure of seco-chaetomugilin with molecular mass of 467.469 m/z

seco-chaetomugilin pigment, 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) fluorescent dye was used for the treatment of MCF-7 cells. The results showed that the treatment of seco-chaetomugilin increased ROS generation to 19.6% at the concentration of 5 µg/ml and 26.2% at the concentration of 15 µg/ml as compared to 14% in untreated cells [Figure 11]. These findings showed that ROS production increased significantly in comparison with control cells. These results indicated that seco-chaetomugilin is able to increase in ROS production in MCF-7 cells.

Discussion

A preliminary cytotoxicity study was performed to analyze the cytotoxicity of *C. cupreum* extracts against human breast adenocarcinoma cell line by MTT assay. In our previous studies, we demonstrated that *C. cupreum* extracts are a rich source of various secondary metabolites such as flavonoids, carbohydrates, saponins, tannins, glycosides, phytosterol, phenolic terpenoids, azaphilones, and coumarins. The flavonoid components were present in high ratio in all *C. cupreum* extracts.[22] In our antioxidant study on different extracts of *C. cupreum*, we demonstrated that different extracts of *C. cupreum* possess significant antioxidant properties. The results of our previous studies have revealed that *C. cupreum* extracts possess various biochemicals and thus have shown significant antioxidant properties in a dose-dependent manner as determined by different antioxidant methods.[17] In the view of these facts, this study was an attempt to evaluate the *in vitro* anticancer activity of ethyl acetate extract of *C. cupreum* extract against

breast adenocarcinoma cells. We also isolated and identified seco-chaetomugilin from *C. cupreum* for the first time.

In the present study, ethyl acetate extract of *C. cupreum* was more effective on MCF-7 cells with IC_{50}-40 µg/ml as compared to MDA-DB231 cells with IC_{50}-75 µg/ml concentration. Previous studies reported that the anticancer activity of ethyl acetate extract of *C. globosum* against MCF-7 and HEPG-2 cell line showed IC_{50} value of 147.87 and 150.70 µg/ml, respectively.[23] Previous studies also showed anticancer activity of methanol extract of *C. cupreum*-RY202 against KB cell lines with IC_{50} value of 3.24 µg/ml.[24] In other previous studies, two new azaphilones isochromophilonol and ochrephilonol were isolated from *C. cupreum* RY202 strain showed moderate cytotoxicity against KB and NCI-H187 cell lines with IC_{50} value of 9.63–32.42 µg/ml.[24] Another previous study[25] found a new compound seco chaetomugilin from *C. globosum* isolated from fish *Mugil cephalus* showed moderate cytotoxic effect against murine P388 leukemia cell with IC_{50} of 38.6 µM and against human KB epidermoid carcinoma cell lines with IC_{50} of 42.2 µM.

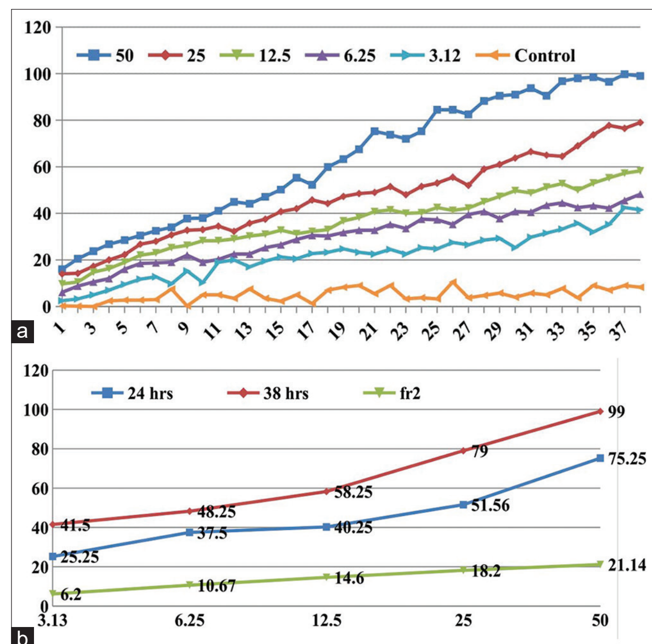

Figure 9: (a) Cytotoxic effect of seco-chaetomugilin on MCF-7 cancer cells at different time points. Experimental results are expressed as mean ± SD (n = 3). (b) Cytotoxic effect of Seco-chaetomugilin on MCF-7 cells and normal fr2 after 24 and 38 h of treatment (IC50 at 25 µg/ml). Experimental results are expressed as mean ± SD (n = 3)

In chromatographic separation of ethyl acetate extract of *C. cupreum,* seco-chaetomugilin pigment was identified. The different solvent systems were standardized with different concentrations for compound purification. The compound was purified by TLC in the solvent system of toluene/ethyl acetate/acetic acid in the ratio of 7:3:1 and one band was observed at 254 nm having Rf value of 0.81. The purified compound was yellow-colored pigment characterized as seco-chaetomugilin with molecular mass of 467.469 m/z based on Q-TOF LCMS analysis. The retention time of the compound was found to be 4.83 nm by HPLC, and UV absorbance was recorded at 274 nm. Previously, the pigment seco-chaetomugilin was reported from other species of *Chaetomium*, but in the present study, pigment seco-chaetomugilin was isolated from *C. cupreum*. In the present study, the isolated pigment seco-chaetomugilin showed significant cytotoxic effect on MCF-7 cells with IC_{50}-25 µg/ml after 24 h and IC_{50-}10 µg/ml concentration after 38 h. A strong correlation was observed between *C. cupreum* extracts and control cells (r^2 = 0.94, P = 0.0001).

For treating different cancers including breast cancer, apoptosis is one of the drug targets.[26] Apoptosis is one of the main mechanisms on which most of the drugs induce their effects.[27]

The changes associated with mitochondrial membrane potential in MCF-7 cells treated with seco-chaetomugilin

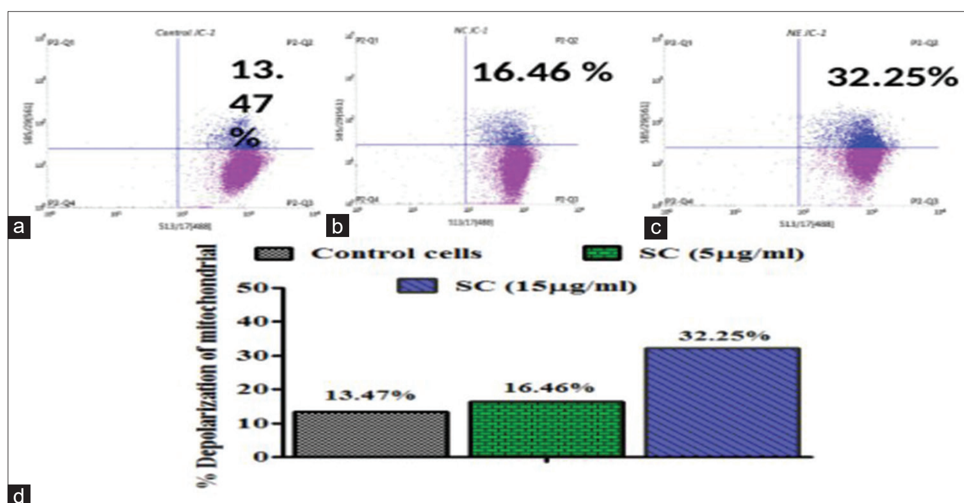

Figure 10: Depolarization of mitochondrial membrane potential of MCF-7 cells after treatment with seco-chaetomugilin (SC) for 24 h. (a) Control, (b) SC-treated cells at 5 µg/ml, (c) SC treated at 15 µg/ml. (d) = % at different concentrations

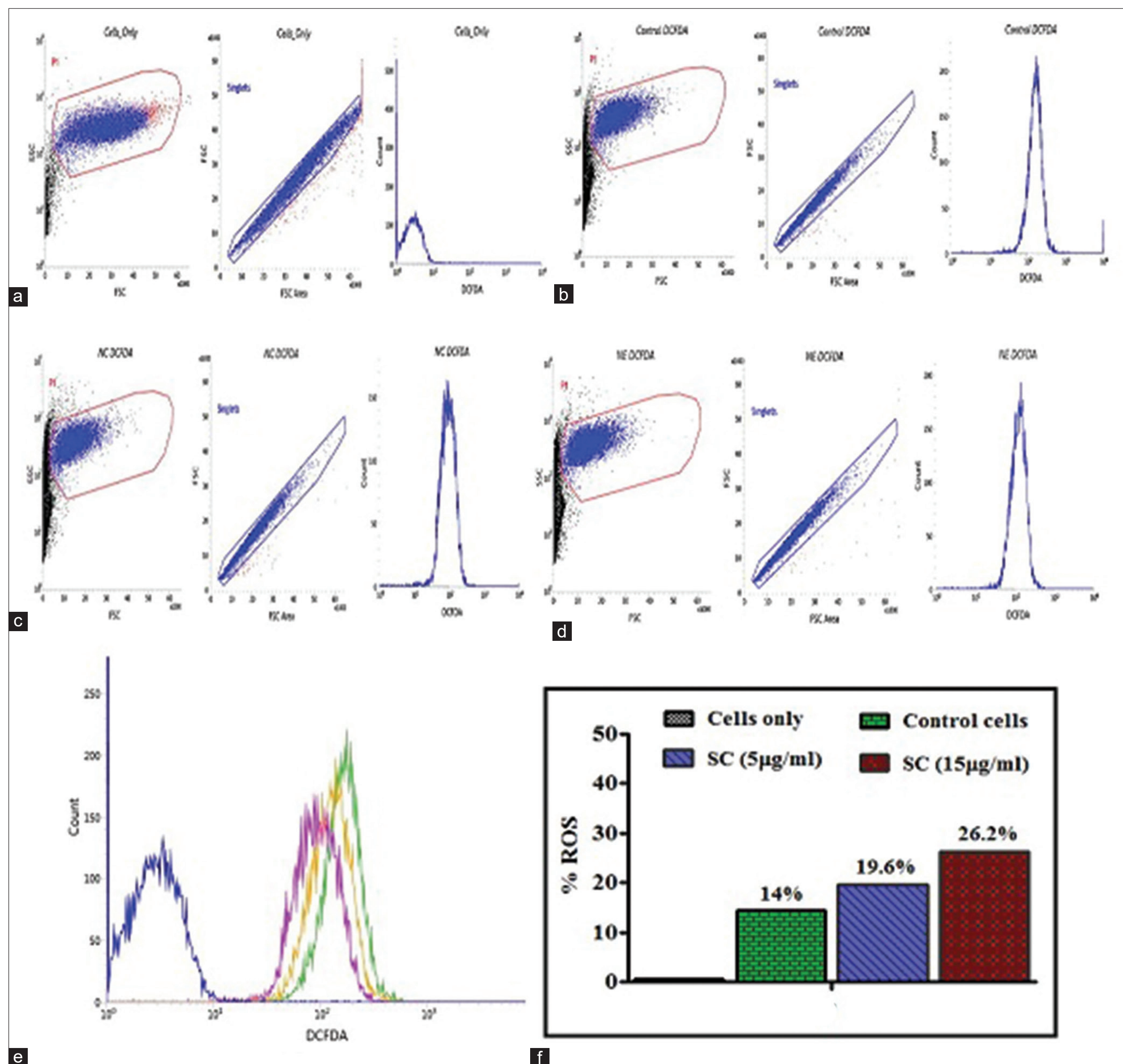

Figure 11: Analysis of reactive oxygen species production in MCF-7 cells treated with seco-chaetomugilin after 3 h by flow cytometry. (a) Cells only, (b) control, (c) SC-treated cells at 5 µg/ml, (d) SC treated at 15 µg/ml. (e and f)- % of ROS

pigment were measured using JC-1 as fluorescent dye. In nonapoptic cells, JC-1 exists as dimer form and accumulates as aggregates in mitochondria and this emits red fluorescence. Whereas, in case of apoptotic cells, JC-1 exists in monomeric form in cytosol and generates green fluorescence. This shift of green to red fluorescence is proportional to the strength of mitochondrial membrane potential. Some anticancer drugs such as doxorubicin induce mitochondrial depolarization, ROS production, inhibition of mitochondrial respiration, and ATP depletion; these changes are associated with cell cycle arrest and cell death.[28] Similarly, the results of the present study showed that there was an increase in depolarization of mitochondrial membrane potential after treatment with seco-chaetomugilin. These results indicates the involvement of mitochondrila apoptotic mitochondrial pathway in breast cancer cells induced by seco chaetomugilin. A strong correlation was observed between seco-chaetomugilin pigment and control cells (r^2 = 0.96, P = 0.0001).

The generation of oxidative stress is one of the key events inducing apoptosis.[29] The 2,7-dichlorodihydrofluorescein diacetate (DCFH-DA) is used for directly measuring the redox state of cell. This fluorescent dye enters inside cells by simple diffusion and gets hydrolyzed into nonfuorescent dichlorodihydrofluorescein (DCFH) by intracellular esterases, which is rapidly oxidized by ROS to a highly fluorescent

dichlorofluorescein (DCF), which emits green fluorescence. Thus, the green fluorescence intensity of DCF is proportional to the mount of ROS generated in the cells. The dead or dying cells produce ROS. The reactive species such as nitric oxide, carbonate, hydroxyl radical, peroxyl, alkoxyl, and peroxynitrite can oxidize DCFH into DCF, which produces green fluorescence.[30] Whereas, DCFH is not sensitive to hydrogen peroxide, lipid peroxides, and singlet oxygen.[31] Many anticancer drugs such as cisplatin, anthracyclines, and bleomycin are used for cancer treatment, which are working by increasing intracellular ROS production.[32] The results of the present study also showed that ROS production increased significantly in comparison with control cells. The present study further indicates that seco-chaetomugilin exerts its cytotoxicity effect through ROS-dependent manner in MCF-7 cells. A strong correlation was observed between seco-chaetomugilin pigment and control cells ($r^2 = 0.99$, $P = 0.0001$) of *C. cupreum*. The collapse of mitochondrial membrane potential and ROS production from mitochondria indicates mitochondrial dysfunction induced by seco-chaetomugilin, which leads to cell death.

Conclusion

The results of the study evaluated the anticancer activity of different extracts of C. *cupreum* on two human breast cancer cell lines (MCF-7 and MDA MB231) by MTT assay. The results showed that ethyl acetate extract of *C. cupreum* exhibited significant anticancer activity against MCF-7 cells. The cytotoxic fraction ethyl acetate extract was further subfractionated into chloroform, ethyl acetate, and methanol. The methanol subfraction showed significant cytotoxic activity against MCF-7 cell line. The present study purified and characterized 'seco-chaetomugilin' pigment from *C. cupreum* with molecular mass of 467.469 m/z based on Q-TOF LCMS analysis. The seco-chaetomugilin showed significant cytotoxic activity against MCF-7 cells. Further, the present study indicates that seco-chaetomugilin exerts its cytotoxicity effect through collapse of mitochondrial membrane potential and ROS production from mitochondria indicates mitochondrial dysfunction induced by seco-chaetomugilin, which leads to cell death. Therefore, our results suggest for carrying out further investigations on the anticancer activity of *C. cupreum* extracts in order to develop new therapeutic agents useful for food, pharmaceutical, and medical applications.

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Conflicts of interest

There are no conflicts of interest.

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