Biological evaluation of ethyl acetate extract of *Chaetomium cupreum* against Ehrlich ascites carcinoma cells in Swiss albino mice

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Background
Chaetomium genus is a natural source of different types of secondary metabolites or pigments. These secondary metabolites display a broad spectrum of biological properties including antimicrobial, antioxidant, anti-inflammatory, and anticancer activity.
Objective
The objective of the study was to evaluate the anticancer activity of ethyl acetate extract of *Chaetomium cupreum* against Ehrlich ascites carcinoma (EAC) cells in Swiss albino mice.
Materials and methods
Methods involved are evaluation of acute toxicity study, tumor induction using EAC cells, estimation of various hematobiochemical parameters, and evaluation of antioxidant enzymes and markers of oxidative stress.
Results
The ethyl acetate extract of *C. cupreum*-treated EAC-bearing mice at the concentration of 200 mg/kg body weight (bwt) reduced ascitic fluid volume (1.65 ±0.70 ml) and ascitic fluid weight (1.32±0.69 g) as compared with ascitic fluid volume (4.79±0.52 ml) and ascitic fluid weight (3.93±0.57 g) in EAC control group. Similarly, the cell apoptosis was higher in EAC-bearing mice treated with standard 5-fluorouracil at 50 mg/kg bwt (96.04%) as compared with treatment with ethyl acetate extract at 50 mg/kg bwt (21.92%) followed by 100 mg/kg bwt (36.63%) and increased further at 200 mg/kg bwt (47.48%) in treated groups. In hematological estimation, the EAC-bearing mice treated with ethyl acetate extract at 200 mg/kg bwt showed increased red blood cell count (3.78 ±0.07×10^6/μl) and hemoglobin content (6.02±01 g/dl) and decreased white blood cells count (5.45±0.01×10^3/μl). In biochemical estimation, ethyl acetate extract treatment in EAC-bearing mice at 200 mg/kg bwt decreased aspartate aminotransferase activity (64.10±0.07 U/l), alanine aminotransferase (55.71 ±0.65 U/l), alkaline phosphatase (107.04±0.02 U/l), cholesterol (124.38±0.04 mg/dl), and triglycerides (155.38±0.04 mg/dl). Similarly, in enzymatic antioxidants and oxidative stress, the ethyl acetate extract-treated EAC-bearing mice at 200 mg/kg bwt increased superoxide dismutase (27.10±0.03 U/mg protein), catalase (20.20 ±0.02 U/mg protein), and reduced glutathione (24.04±0.03 U/mg protein), whereas decreased glutathione peroxidase (38.04±0.07 U/g hemoglobin) and malondialdehyde content (170.50±0.06 nmol/mg protein) significantly.
Conclusion
The results of the present finding showed that ethyl acetate extract of *C. cupreum* possesses significant anticancer potential.

Keywords:
antioxidant enzymes, Ehrlich tumor, hematobiochemical markers, malondialdehyde, oxidants

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Introduction

In recent years, a significant development has been made in cancer treatment globally. However, still the development of effective cancer prevention remains the greatest challenges in the area of cancer chemotherapy. Cancer causes most of the morbidity and mortality in the world. The incidence of cancer percentage is increasing globally. The main objective of cancer therapy is to induce death of cancer cells without causing damage or little damage to normal cells [1]. Most of the anticancer drugs are carcinogenic, mutagenic, and expensive. One of the most effective methods of treatment of cancer is chemotherapy. Unfortunately, the presently available chemotherapeutic synthetic anticancer molecules used in the treatment of cancer

have developed drug resistance and cause various adverse effects in patients [2]. Owing to the limitations of synthetic anticancer drugs, there is an urgent need of molecules derived from natural sources such as microorganisms, plants, marine organisms, and fungi for the development of anticancer drugs for the treatment of human cancer [3]. More recently, it was found that tumor cells develop resistance to a single compound easily than multiple compounds. Therefore, treatment of cancer involving combination of drugs is preferred over a single drug treatment, and combination of specific phytochemicals may be more effective than a single compound in preventing against cancer [4].

The phytochemicals from natural source possess various biological activities such as antimicrobial, antioxidant, and anticancer activity [5,6]. Approximately 60% of presently used anticancer drugs are derived from natural source including plants, marine organisms, and microorganisms [7,8]. The recent studies have shown that the naturally occurring phytochemicals are more effective with fewer adverse effects than synthetic drugs [9]. The filamentous fungi are known to produce various phytochemicals such as cyclosporine, lovastatin, and penicillin and also a rich resource of novel compounds of therapeutic importance [10].

Chaetomium species belongs to chaetomiaceae family, which are the largest genus of saprophytic ascomycetes and a natural source of different types of metabolites. Since Kunze was the first to establish this genus in 1817 and till now more than 350 *Chaetomium* species are known [11]. The *Chaetomium cupreum* was described by Lawrence Marion Ames in 1949 as part of a military effort to identify the organisms responsible for the biodeterioration [12]. In previous studies, methanol extract of *C. cupreum*-RY202 exhibited in-vitro anticancer activity against KB cell line having IC_{50}-3.25 µg/ml concentration. Further study revealed that isochromophilonol, ochrephilonol, and azaphilones were isolated from *C. cupreum*-RY202, and both the compounds exhibit moderate anticancer against KB and NCI-H187 cell line having IC_{50} of 9.63–32.42 µg/ml [13]. The *C. cupreum* possess various secondary metabolites with various biological properties [14]. For the present study, Ehrlich ascites carcinoma (EAC) cells were used as the cell line. The Ehrlich tumor is a spontaneous murine mammary adenocarcinoma [15]. The Ehrlich tumor is a rapidly growing carcinoma in all strains of mice. In ascites form in mice, it is used to study the effect of anticancer molecules [16]. To the best of our knowledge, there are no previous reports on the anticancer activity of *C. cupreum* extracts. The purpose of the study was to investigate the anticancer properties of ethyl acetate extract of *C. cupreum* against EAC cells in Swiss albino mice.

Materials and methods

Procurement of fungal culture

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

Inoculum preparation and extraction

The *C. cupreum* was inoculated on potato dextrose agar plates and slants and maintained at 4°C for future use. For inoculum preparation, the culture was inoculated into potato dextrose broth and incubated at room temperature for 20 days on a rotary shaker at 120 rpm. The extraction involves liquid-liquid method [17]. After incubation, broth was filtered to remove mycelium. Then solvent and broth were taken in equal volume and shaken well for 15 min in a separating funnel. Then solvent layer was collected, and a crude dried extract powder was obtained.

Animal in-house testing

Male adult Swiss albino mice weighing between 25 and 30 g (n=60) were housed in well-ventilated large spacious polypropylene cages under standard conditions of humidity (50–60%), temperature (30 ±5°C), and light (12 h light/dark). Animals were provided with dry pellet rat feed along with tap water *ad libitum*. Animal experiments were done at the animal facility of KM College of Pharmacy, Madurai, Tamil Nadu, India. The present study involving animals was approved by Institutional Animal Ethical Committee (IAEC) and was conducted according to the guidelines of the committee for the purpose of control and supervision of experiments on animals, with Regd. No.: 661/02/C/CPCSEA/29/09/2017.

Toxicity study of ethyl acetate extract

The toxicity test in male Swiss albino mice was performed in accordance with the Organization for Economic Co-operation and Development (OECD) guidelines 425 [18]. The Swiss albino mice (n=6) were deprived of food for 18 h and then administered intraperitoneally 0.25 ml of various doses [250, 500, and 1000 mg/kg body weight (bwt)] of ethyl acetate extract of *C. cupreum* once only. The toxicity symptoms were recorded for 6, 12, 24 h, and 48 h, after which time, the number of survival animals was recorded. The

general behaviors that were observed during acute toxicity study are sedative, hypnosis, convulsion, motor activity, lacrimal secretion, ptosis, change in skin color, and stool consistency.

Experimental design for intraperitoneal dosing of ethyl acetate extract

Group I: animals were treated with normal saline.
Group II: animals were treated with 250 mg/kg bwt of ethyl acetate extract.
Group III: animals were treated with 500 mg/kg bwt of ethyl acetate extract.
Group IV: animals were treated with 1000 mg/kg bwt of ethyl acetate extract.

Induction of tumor using Ehrlich ascites carcinoma cells

The EAC cells were procured from Amala Cancer Research Center, Thrissur, Kerala, India, and were maintained and subcultured in Swiss albino mice after every 10th day through intraperitoneally. The cancer formation was done by Jagetia *et al*. [19] method. After 8 days, ascitic fluid from peritoneal cavity was withdrawn from EAC-bearing mice. The cancer cells were diluted with normal saline and adjusted in a suspension of 1×10^6 cells/ml. All animals received 100 µl EAC cells except group I. After 2 h, cancer-developed animals were divided into six groups, with six animals in each group (n=6).

Experimental design

Group I (normal control): animals as normal control received with normal saline (100 µl intraperitoneally) from day 1 to 9.
Group II (EAC control): animals received EAC cells (100 µl intraperitoneally) from day 1 to 9.
Group III (standard control): animals received 5-fluorouracil at 50 mg/kg bwt (100 µl intraperitoneally) from day 1 to 9.
Group IV (EAC+extract): animals received ethyl acetate extract at 50 mg/kg bwt (100 µl intraperitoneally) from day 1 to 9.
Group V (EAC+extract): animals received ethyl acetate extract at 100 mg/kg bwt (100 µl intraperitoneally) from day 1 to 9.
Group VI (EAC+extract): animals received ethyl acetate extract at 200 mg/kg bwt (100 µl intraperitoneally) from day 1 to 9.

Collection of ascitic fluid and blood from animals

After 24 h of last dose and 18 h of fasting (12 days of ethyl acetate extract treatment), the blood was collected in an ethylenediaminetetraacetic acid vial from retro-orbital plexus by sterile capillary for estimation of blood markers. The animals were anesthetized with diethyl ether, and ascitic fluid was collected.

Estimation of ascitic fluid volume, ascitic fluid weight, and cell viability

The ascitic fluid was measured in graduated centrifuge tube and expressed in milliliters, whereas ascitic fluid weight was measured by weighing ascitic fluid and expressed in grams. The cell viability was measured by diluting ascitic fluid with phosphate buffer saline, and 10 µl was mixed with 90 µl of Trypan blue dye and then 10 µl was placed on hemocytometer. Then viable and dead cells were counted, and percentage viability was calculated as follows:

$$\%\text{viability} = \frac{\text{Total viable cells}}{\text{Total cells}} \times 100.$$

Estimation of hematological parameters in control and experimental animals

Various hematological parameters such as red blood and white blood cell count and hemoglobin (Hb) were estimated.

Estimation of red blood cell count

Estimation red blood cell was carried out by using Neubauer hemocytometer counting chambers by Davidson's method [20]. A volume of 0.5 ml of ethylenediaminetetraacetic acid-anticoagulated blood in red blood pipette was diluted to 1 : 200 with Hayem's diluting fluid (mercuric chloride 0.25 g, sodium chloride 0.50 g, sodium sulfate 2.50 g, and distilled water 100 ml, pH 5.9). After incubation at room temperature for 3 min, the sample was placed on counting chamber and the number of red blood cell per cubic millimeter (cumm) was calculated.

$$\text{Number of red blood cells/cumm of blood} = \frac{\text{Number of cells} \times \text{dilution factor} \times \text{depth factor}}{\text{Area counted}}.$$

where the dilution factor is 200, area counted is 4 mm^2 and depth is 0.1 mm.

Estimation of white blood count

Estimation of white blood cells was carried by using Neubauer hemocytometer counting chamber method [20]. A volume of 0.5 ml of ethylenediaminetetraacetic acid-anticoagulated blood was diluted to 1:20 with Jurks's diluting fluid (glacial acetic acid 2.0 ml, gentian violet 1%, distilled water 97 ml, pH 2.2). The sample was placed on counting chamber, and

the number of white blood cells per cumm was calculated as follows:

$$\text{Number of white blood cells/cumm of blood} = \frac{\text{Number of WBC} \times \text{dilution factor} \times \text{depth of fluid}}{\text{Area counted}}.$$

where the dilution factor is 20, area counted is 4 mm^2, and depth is 0.1 mm.

Estimation of hemoglobin

The Hb concentration was measured by the acid-hematin method [21]. The graduated Hb tube was filled with 0.1 N hydrochloric acid by a dropper. Then blood was filled in the Hb pipette up to mark 20 mm^3 and transferred to graduated Hb tube containing 0.1 N hydrochloric acid and further diluted by 0.1 N hydrochloric acid drop by drop with constant stirring by glass rod till the color matches with that of standard brown glass rod. The results were recorded on the Hb tube showing concentration of Hb in grams per deciliter.

Estimation of biochemical parameters in control and experimental animals

Aspartate aminotransferase

The aspartate aminotransferase was estimated by Reitman method [22]. A volume of 4.0 ml of solution 1 (each of 1.0 ml of tris buffer 100 mmol/l), L-aspartate (300 mmol), lactate dehydrogenase (1.5 U/ml), and malate dehydrogenase (0.53 U/ml)) was added to 1.0 ml of solution 2 (each of 0.05 ml of α-ketoglutarate; 75 mmol/l), and nicotinamide adenine dinucleotide (0.23 mmol/l)). Then 1 ml of this reaction mixture was added to 100 µl of test sample, and sample tubes were incubated for 1 min at 37°C, and absorbance was measured at 340 nm. The enzyme activity was expressed as micromole of pyruvate formed/mg protein/h.

Alanine aminotransferase

The alanine aminotransferase was estimated by Reitman method [22]. A volume of 3.0 ml of solution 1 (each of 1.0 ml of tris buffer 100 mmol), L-alanine (500 mmol/l), and lactate dehydrogenase (1.5 U/ml) was added to 1.0 ml solution 2 (each of 1.0 ml of α-ketoglutarate; 15 mmol/l), and nicotinamide adenine dinucleotide (0.18 mmol/l). Then 1 ml of this reaction mixture was added to 100 µl of test sample, and reaction tubes were incubated for 1 min at 37°C and absorbance was read at 340 nm. The enzyme activity was expressed as micromole of pyruvate formed/mg protein/h.

Assay of alkaline phosphatase

The alkaline phosphatase was estimated by King's method [23]. A volume of 100 µl of test sample was mixed well with 0.5 ml enzyme solution, 1.5 ml buffer, and 1.0 ml substrate. After incubation for 15 min at 37°C, 1.0 ml of Folin's phenol reagent was added and centrifuged for 10 min at 3000 rpm. Then supernatant (1.0 ml) was collected and 1.0 ml substrate, 1.0 ml sodium carbonate (15%), and 0.1 ml magnesium chloride were added followed by 37°C incubation for 10 min, and absorbance was measured at 640 nm. The enzyme activity in serum was expressed as micromoles of phenol liberated/min/mg of protein.

Estimation of total cholesterol

The total cholesterol was estimated by Allain method [24]. A volume of 10 µl of serum sample was added to 1.0 ml of reagent 1 (tris buffer 100 mM, pH 7.7, sodium cholate 10 mM, phenol 6 mM, cholesterol hydrolase 33 U/l, cholesterol oxidase 150 U/l, and horseradish peroxidase 200 U/l). The solution was incubated at 37°C for 10 min, and absorbance was measured at 500 nm. The concentration of cholesterol was measured from standard cholesterol calibration curve. The standard cholesterol consists of 100 mg of cholesterol dissolved in 100-ml ice cold acetic acid, and 10 ml was further diluted with 100-ml acetic acid. The cholesterol content was expressed as mg/dl serum or mg/100 g wet tissue.

Estimation of triglycerides

The triglyceride content was estimated by Werner method [25]. A volume of 10 µl of test sample was incubated at 37°C for 5 min and added to 1.0 ml of reagent 1 (tris-HCl buffer 100 mM, pH 7.8, glycerophosphate oxidase 2000 U/l, lipase 1000 U/l, glycerol kinase 800 U/l, peroxidase 150 U/l, sodium azide 0.1%, magnesium chloride 17 mM, 4-aminoantipyrene 0.4 mM, ATP 0.55 mM, and 3-hydroxyl-2,4,6-tribromobenzoic acid 1.6 mM), and absorbance was measured at 540 nm. The triglyceride content was expressed as mg/dl.

Estimation of enzymatic antioxidants and markers of oxidative stress in control and experimental animals

Estimation of superoxide dismutase

The superoxide dismutase was estimated by Misra's method [26]. A volume of 1.0-ml plasma in potassium phosphate buffer was mixed with 1.0 ml of sodium carbonate (50 mM), 0.2 ml of hydroxylamine hydrochloride (0.1 mM), and 0.4 ml of nitroblue tetrazolium (75 mM). The reaction mixture was mixed well, and development of blue color was monitored at 560 nm spectrometrically. The

superoxide dismutase activity was expressed as U/mg protein.

Estimation of catalase

The catalase was estimated by Aebi method [27]. A volume of 0.1 ml of plasma was mixed well with 1.0 ml of hydrogen peroxide (0.5 mM) and 1.9 ml pf phosphate buffer (10 mM). The absorbance was read at 240 nm, keeping 1-min interval for 3 min. The enzyme activity was expressed as micromoles hydrogen peroxide consumed/min/mg protein.

Estimation of reduced glutathione

The reduced glutathione was estimated by Ellman method [28]. A 1.0-ml test sample was added to 1.0 ml of 20% trichloroacetic acid, which contains 1 mM ethylenediaminetetraacetic acid for precipitating proteins. The solution was incubated for 5 min at room temperature and then centrifuged for 10 min at 2000 rpm. In 200 µl of supernatant, 1.8 ml of Ellman's reagent (0.1 mM, 5, 50-dithiobis-2-nitrobenzoic acid) was added, and absorbance was measured at 412 nm. The reduced glutathione content was calculated from standard graph from known reduced glutathione. The reduced glutathione was expressed as nanomoles/mg protein or U/mg protein.

Estimation of glutathione peroxidase

The glutathione peroxidase activity was estimated by Hafemann *et al*. [29]. Volumes of 0.1 ml of 5 mM reduced glutathione, 0.02 ml of plasma, 0.1 ml of 1.25 mM H_2O_2, 0.1 ml of 25 mM sodium azide, and 1.0 ml of 0.05 mM phosphate buffer were incubated for 10 min for 37°C. The reaction was terminated by adding 2 ml of 1.65% of hydrogen phosphite and centrifuged at 1500 rpm for 10 min. Then 2.0 ml of supernatant was added to 2 ml of 0.4 M disodium hydrogen phosphate and 1 ml of 1 mM Ellman's regent (DTNB) and incubated at 37°C for 10 min, and absorbance was measured at 412 nm. The results are expressed as U/g Hb.

Estimation of malondialdehyde

The malondialdehyde (MDA) content is estimated by Draper method [30]. A volume of 0.5 ml of test sample was added to 1 ml of trichloroacetic acid. This mixture was centrifuged at 4000 rpm for 10 min. Then 0.5 ml of supernatant was mixed with 1.0 ml of 0.67% thiobarbituric acid reagent followed by incubation for 15 min at 95°C, and absorbance was read at 532 nm. The MDA was expressed as nanomoles of MDA/mg protein.

Statistical analysis

The two-way analysis of variance followed by Tukey's multiple comparison tests with graph pad prism 6 software (Graphpad Software, Inc., United states) was used.

Results

Toxicity study of ethyl acetate extract

The intraperitoneal toxicity test was investigated to assess the therapeutic index, which is the ratio of lethal dose and pharmacologically effective dose for the survival and treatment of mice. The behavior of the animals was closely monitored for 6, 12, 24 h, till 48 h. It was found that ethyl acetate extract of *C. cupreum* did not induce behavioral changes such as hypnosis, convulsion lacrimal secretion, ptosis, change in skin color, stool consistency, diarrhea, locomotor ataxia, weight loss, and mortality in mice during the 48 h of observation. However, a mild sedation was observed (Table 1).

Ascitic fluid volume, ascitic fluid weight, cell viability (%), and cell death (%) in control and experimental animals

The anticancer activity of ethyl acetate extract of *C. cupreum* against EAC cancer-bearing mice was evaluated by measuring the ascitic fluid volume, ascitic fluid weight, and cell viability. From the results, the ascitic fluid volume (4.79±0.52 ml) and ascitic fluid weight (3.93±0.57 g) increased significantly in EAC control group (Table 2). The treatment of EAC-bearing mice with ethyl acetate extract of *C. cupreum* reduced ascitic fluid volume to 2.78±0.44 ml and ascitic fluid weight to 2.81±0.27 g at 50 mg/kg bwt, whereas standard 5-fluorouracil decreased ascitic fluid volume to 0.57±0.22 ml and ascitic fluid weight to 0.44±0.22 g at 50 mg/kg bwt. The cell count (% viable) and cell death (% nonviable) were evaluated by trypan blue assay. The cell viability count was higher in EAC control group (98.02%) than

Table 1 Acute toxicity test of ethyl acetate extract of *Chaetomium cupreum*

Serial numbers	General behavior	Ethyl acetate extract
1	Sedative	+
2	Hypnosis	–
3	Convulsion	–
4	Ptosis	–
5	Motor activity	–
6	Change in skin color	–
7	Lacrimal secretion	–
8	Diarrhea	–
9	Weight loss	–
10	Mortality	–

+, positive;–, negative.

Table 2 Effect of ethyl acetate extract of *Chaetomium cupreum* on ascitic fluid volume, ascitic fluid weight, viability (%), and inhibition (%) between Ehrlich ascites carcinoma control and experimental groups.

	12 days treatment			
Treatment	Volume of ascitic fluid (ml)	Weight of ascitic fluid (g)	% cell viability	% cell death
EAC control	4.79±0.52^{a}	3.93±0.57^{a}	98.02 ±0.20^{a}	2±0.001^{a}
EAC+5-fluorouracil (50 mg/kg bwt)	0.57±0.22$^{b\#}$	0.44±0.22$^{b\#}$	3.96± 0.13$^{b\#}$	96.04±0.53$^{b\#}$
EAC+ethyl acetate extract (50 mg/kg bwt)	2.78±0.44$^{c\#A}$	2.81±0.27$^{c\#A}$	78.08±0.37$^{c\#A}$	21.92±0.17$^{c\#A}$
EAC+ethyl acetate extract (100 mg/kg bwt)	1.87±0.37 ^{dB}	1.02±0.34$^{d\#B}$	63.37±0.54$^{d\#B}$	36.63±0.27$^{d\#B}$
EAC+ethyl acetate extract (200 mg/kg bwt)	1.65±0.70^{eCB}	1.32±0.69$^{e\#CB}$	52.52±0.21$^{e\#C}$	47.48±0.01$^{e\#C}$

Values are represented as mean±SD (n=3). bwt, body weight; EAC, Ehrlich ascites carcinoma. Significance difference between the untreated control and experimental groups are represented in lower case and between the standard fluorouracil and experimental groups in hashes. Significance difference between the concentrations is represented in upper case. Those not sharing the same letter are significantly different at P value less than 0.05.

Table 3 Effect of ethyl acetate extract of *Chaetomium cupreum* on hematological parameters between Ehrlich ascites carcinoma control and experimental groups

Treatment	RBC count (cells ×10^6/µl)	Total WBC (cells ×10^3/µl)	Hb (g/dl)
Untreated	5.95±0.08^{a}	4.85±0.47^{a}	10.75±0.10^{a}
EAC control	2.50±1.2^{b}	8±1.1^{b}	5.10±1.0^{b}
EAC+5-fluorouracil (50 mg/kg bwt)	4.43±0.07$^{c\#}$	5.42±1.08$^{a\#}$	9.11±0.03$^{c\#}$
EAC + ethyl acetate extract (50 mg/kg bwt)	2.10±0.06$^{d\#A}$	6.91±0.78$^{c\#A}$	3.45±0.07$^{d\#A}$
EAC+ethyl acetate extract (100 mg/kg bwt)	3.08±0.07$^{e\#A}$	6.71±0.65$^{d\#A}$	5.04±0.04$^{e\#B}$
EAC+ethyl acetate extract (200 mg/kg bwt)	3.78±0.07^{fBA}	5.45±0.01^{aB}	6.02 ±0.1$^{f\#C}$

Values are represented as mean±SD (n=3). bwt, body weight; EAC, Ehrlich ascites carcinoma; Hb, hemoglobin; RBC, red blood cells count; WBC, white blood count. Significance difference between the untreated control and experimental groups is represented in lower case and between the standard fluorouracil and experimental groups in hashes. Significance difference between the concentrations is represented in upper case. Those not sharing the same letter are significantly different at P value less than 0.05.

the standard 5-fluorouracil-treated group (3.96%). However, ethyl acetate extract treatment reduced cell viability count to 78.08% at 50 mg/kg bwt, followed by 63.37% at 100 mg/kg bwt, and 52.52% at 200 mg/kg bwt in the treated groups. Similarly, the cell death in ethyl acetate extract treatment was 21.92% at 50 mg/kg bwt, followed by 36.63% at 100 mg/kg bwt and 47.48% at 200 mg/kg bwt in treated groups. However, cell death was 96.04% in standard 5-fluorouracil at 50 mg/kg bwt (Table 2).

Hematological parameters in control and experimental animals

All hematological parameters were evaluated in treated and control animals and are presented in Table 3. In EAC control group, red blood cells count (2.50 ±1.2×10^6/µl) and Hb content (5.10±1.0 g/dl) decreased, whereas white blood count increased (8 ±1.1×10^3/µl) compared with normal group. Similarly, ethyl acetate treatment at 50 mg/kg bwt increased red blood cells count to 2.10±0.06×10^6/µl and Hb content to 3.45±0. 07 g/dl, whereas decreased white blood count to 6.91±0.78×10^3/µl in EAC-bearing mice. However, the 5-fluorouracil treatment at 50 mg/kg bwt increased red blood cells count to 4.43±0.07×10^6/µl and Hb content to 9.11±0.03 g/dl, whereas reduced the white blood count to 5.42±1.08×10^3/µl in EAC-bearing mice significantly (Table 3).

Biochemical parameters in control and experimental animals

In the results, treatment with ethyl acetate extract of *C. cupreum* at 50 mg/kg bwt decreased aspartate aminotransferase activity (81.50±0.06 U/l), alanine aminotransferase (66.91±0.78 U/l), alkaline phosphatase (123.45±0.07 U/l), cholesterol (141.01 ±0.04 mg/dl), and triglycerides (176.01±0.04 mg/dl) significantly. However, standard 5-fluorouracil at 50 mg/kg bwt decreased aspartate aminotransferase (50.43±0.07 U/l), alkaline phosphatase (40.23 ±1.08 U/l), alanine aminotransferase (70.11±0.03 U/ l), cholesterol (112.09±0.07 mg/dl), and triglycerides (145.09±0.07 mg/dl) significantly (P=0.05) (Table 4).

Enzymatic parameters in control and experimental animals

The results showed that all enzymatic antioxidant and oxidative stress markers increased in EAC-bearing mice compared with normal group (Table 5). The ethyl acetate extract of *C. cupreum* treatment at 50 mg/kg bwt increased superoxide dismutase (15.50 ±0.06 U/mg protein), catalase (9.91±0.78 µmol H_2O_2 consumed/min/mg protein), and reduced glutathione (11.45±0.07 U/mg protein), whereas decreased glutathione peroxidase (54.45±0.10 U/g Hb) and MDA (230.01±0.04 nmol/mg protein) significantly (P=0.05) in EAC-bearing mice. However, standard 5-fluorouracil treatment at 50 mg/kg bwt increased

Table 4 Effect of ethyl acetate of *Chaetomium cupreum* on serum biochemical parameters between Ehrlich ascites carcinoma control and experimental groups

Treatment	AST (U/l)	ALT (U/l)	ALP (U/l)	Cholesterol (mg/dl)	Triglycerides (mg/dl)
Untreated	53.95±0.08^{a}	41.85±0.47^{a}	92.75±0.10^{a}	105±0.02^{a}	125±0.02^{a}
EAC control	93.50±1.2^{b}	70±0.2^{b}	130.10±1.0^{b}	150. 86±0.01^{b}	190.86±0.01^{b}
EAC+5-fluorouracil (50 mg/kg bwt)	50.43±0.07$^{c\#}$	40.23±1.08$^{c\#}$	70.11±0.03$^{c\#}$	112.09±0.07$^{c\#}$	132.09±0.07$^{c\#}$
EAC+ethyl acetate extract (50 mg/kg bwt)	81.50±0.06$^{d\#A}$	66.91±0.78$^{d\#A}$	123.45±0.07$^{d\#A}$	141.01±0.04$^{d\#A}$	176.01±0.04$^{d\#A}$
EAC+ethyl acetate extract (100 mg/kg bwt)	75.80±0.07$^{e\#B}$	61.71±0.65$^{e\#B}$	115.04±0.04$^{e\#B}$	135.38±0.04$^{e\#B}$	165.38±0.04$^{e\#B}$
EAC+ethyl acetate extract (200 mg/kg bwt)	64.10±0.07$^{f\#C}$	55.71±0.65$^{f\#C}$	107.04±0.02$^{f\#C}$	124.38±0.04$^{f\#C}$	155.38±0.04$^{f\#C}$

Values are represented as mean±SD (n=3). ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, Aspartate aminotransferase; bwt, body weight; EAC, Ehrlich ascites carcinoma. Significance difference between the untreated control and experimental groups is represented in lower case and between the standard fluorouracil and experimental groups in hashes. Significance difference between the concentrations is represented in upper case. Those not sharing the same letter are significantly different at P value less than 0.05.

Table 5 Effect of ethyl acetate extract of *Chaetomium cupreum* on the enzymatic antioxidants and markers of oxidative stress between Ehrlich ascites carcinoma control and experimental groups

Treatment	Superoxide dismutase (units/mg protein)	Catalase (μmol H_2O_2 consumed/min/mg protein)	Reduced glutathione (U/mg protein)	Glutathione peroxidase (U/g Hb)	Malondialdehyde (nmol/mg protein)
Untreated	35.95±0.08 ^{a}	28.85±0.47^{a}	34.75±0.10^{a}	22.75±0.10^{a}	130.78±0.02^{a}
EAC control	10.50±1.2^{b}	8±1.1^{b}	15.10±1.0^{b}	60.10±1.0^{b}	258. 86±0.01^{b}
EAC+5-fluorouracil (50 mg/kg bwt)	30.43±0.07$^{c\#}$	25.42±1.08$^{c\#}$	27.11±0.03$^{c\#}$	35.11±0.02$^{c\#}$	160.09±0.07$^{c\#}$
EAC+extract (50 mg/kg bwt)	15.50±0.06$^{d\#A}$	9.91±0.78$^{d\#A}$	11.45±0.07$^{d\#A}$	54.45±0.10$^{d\#A}$	230.01±0.04$^{d\#A}$
EAC+extract (100 mg/kg bwt)	21.780±0.07$^{e\#B}$	15.71±0.65$^{e\#B}$	19.04±0.04$^{e\#B}$	46.04±0.12$^{e\#B}$	196.38±0.04$^{e\#B}$
EAC+ethyl acetate extract (200 mg/kg bwt)	27.10±0.03$^{f\#C}$	20.20±0.02$^{f\#C}$	24.04±0.03$^{f\#C}$	38.04±0.07$^{f\#C}$	170.50±0.06$^{f\#C}$

Values are represented as mean±SD (n=3). bwt, body weight; EAC, Ehrlich ascites carcinoma; Hb, hemoglobin. Significance difference between the untreated control and experimental groups is represented in lower case and between the standard fluorouracil and experimental groups in hashes. Significance difference between the concentrations is represented in upper case. Those not sharing the same letter are significantly different at P value less than 0.05.

superoxide dismutase (30.43±0.07 U/mg protein), catalase (25.42±1.08 μmol H_2O_2 consumed/min/mg protein), and reduced glutathione (27.11±0.03 U/mg protein), whereas decreased glutathione peroxidase (35.11±0.02 U/g Hb) and MDA (160.09±0.07 nmol/mg protein) in EAC mice significantly (P=0.05) (Table 5).

Discussion

Previous reports by the same authors demonstrated that extracts of *C. cupreum* are rich in various phytochemicals such as flavonoids, carbohydrates, saponins, tannins, glycosides, phytosterol, phenolic terpenoids, azaphilones, and coumarins compounds [17]. Another study reported that extracts of *C. cupreum* have antioxidant potential because of various phytochemicals present [31]. Based on these observations, anticancer activity of ethyl acetate extract of *C. cupreum* extract against EAC cells was evaluated. The hematological parameters, serum biochemical parameters, and antioxidant enzymes are considered as important tools for the evaluation of physiological and health status of the organism as well as diagnosis and disease treatment. These parameters indicate the changes in the metabolism of the organism [32]. The parameters such as red blood cells and white blood cells and Hb content are used to analyze blood diagnosis. Owing to the clinical importance of various biochemical parameters such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphate, total cholesterol, and triglycerides, they are used for monitoring and are used as diagnostic tools in patients with diseases in liver, pancreas, kidney, and blood. All these biochemical parameters are used for disease detection all over the world, as well as by International Federation of Clinical Chemistry (IFCC) and the Scandinavian Committee on Enzymes (SCE) [33–35]. Moreover, during overproduction of reactive oxygen species, the antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase, and reduced glutathione provide the measurement free radicals as well as about the oxidative damage caused by the free radical in the cell [36,37].

The toxicity report of the present study proved that ethyl acetate extract of *C. cupreum* did not provoke any pharmacological and behavioral changes. Thus, ethyl acetate extract of *C. cupreum* appeared safe for preclinical trials. At 50 mg/kg bwt, ethyl acetate extract of *C. cupreum* decreased cancer volume, cancer weight, and cancer cell viability. At 50 mg/kg bwt, ethyl acetate extract showed 21.92% cell death whereas 5-fluorouracil showed 96.04% cell death. The hemolysis results in red blood cell or Hb count reduction and causes anemia [38]. However, ethyl acetate extract treatment replenishes red blood cells and Hb count toward normal level in a dose-dependent manner. Thus, it indicates that ethyl acetate extract of *C. cupreum* protects cell surface and thus helps in maintaining normal hemopoietic system. The elevated levels of biochemical parameters such as aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, cholesterol, and triglycerides indicate impaired liver function owing to injection of EAC cells [39]. The treatment of EAC-bearing mice with ethyl acetate extract decreased the level of biochemical parameters in ascitic cancer in a dose-dependent manner, which suggests that phytochemicals present in *C. cupreum* have the capacity to stabilize the plasma membrane of a cell.

The reduced function of enzymatic antioxidants will result in overproduction of free radicals which is detrimental to cell membrane integrity. The superoxide dismutase and catalase catalyze the dismutation of superoxide anion radical to hydrogen peroxide. Then catalase converts hydrogen peroxide into oxygen and water [40]. Previous studies have presented evidence that reduced glutathione has chemoprotective action, and its deficiency is a risk to cells to oxidative damage [41]. Another study demonstrated that *C. cupreum* extracts contain high levels of different types of phytochemicals [17]. These phytochemicals donate electrons or atoms to free radicals and thus also repair the enzymatic antioxidants of a cell. Similarly, treatment of EAC-bearing mice with ethyl acetate extract to different doses increases the enzymatic antioxidants to normal level in a dose-dependent manner. Oxidative stress damages lipids and induce lipid peroxidation. MDA is a product produced owing to lipid peroxidation and is used as a marker of cell membrane injury [42]. The MDA is a potentially important contributor to DNA damage and mutations, which it generates endogenously through lipid peroxidation [43]. Thus, MDA can be used as main marker for diagnosis for tumors. The level of lipid peroxides in EAC cell-bearing mice was increased and was decreased with ethyl acetate extract treatment in treated groups. The results from this study indicate that ethyl acetate extract of *C. cupreum* exhibited antitumor activity against EAC cells and can be a candidate drug for screening in an animal model.

Conclusion

The evaluation of *in vivo* anticancer activity of ethyl acetate extract of *C. cupreum* displayed significant anticancer activity against EAC cells in treated animals. In this study, the work was aimed to explore the toxicity study and the anticancer activity of *C. cupreum* extract. In this study, analyses of blood parameters, biochemical parameters, enzymatic antioxidants, and marker of oxidative stress were examined at the tolerated concentrations of 50, 100, 200 mg/kg bwt in the control and experimental animals, respectively. The results showed that treatment with ethyl acetate extract of *C. cupreum* inhibited anticancer activity in a dose-dependent manner but reactive oxygen species in an independent manner against EAC cells. The results showed that *C. cupreum* extracts contain potent phytochemicals that can be used for therapeutic application after their purification and characterization.

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Conflicts of interest

There are no conflicts of interest.

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