CHAPTER 3

MATERIALS AND
METHODS
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3.1 Collection and identification of the herb

Dried rhizomes of *C. rotundus* were collected from a local Ayurvedic pharmacy in Mangalore, Karnataka, India. The plant material was authenticated by Dr. Sunil Kumar, Senior Research officer, Department of Pharmacognosy, SDM Centre for Research in Ayurveda and Allied Sciences, Udupi and voucher specimen (No.11110101) was deposited in the plant repository of SDM Research Center. The shade dried rhizomes of the *C. rotundus* were coarsely powdered and preserved at -20ºC for further studies.

3.2 Analysis of physicochemical components

The various physicochemical parameters were determined according to the standard procedures prescribed in Ayurvedic Pharmacopeia of India [105].

3.2.1 Macroscopic study of rhizome

Dried whole rhizomes were used to study the external appearance, color, odor and taste. Coarse and fine powders were also prepared to record the organoleptic characters [106].

3.2.2 Microscopic study of rhizome

Few fully mature rhizomes were preserved in fixative solution FAA (5 ml Formalin+ 5 ml Acetic acid + 90 ml 70% Ethyl alcohol) for 48 h. The preserved specimens were cut into thin transverse sections using sharp blade [107]. The sections were stained with safranine as per standard methodology [108]. The selected diagnostic characters of the transverse sections were photographed under suitable magnification using the camera (Zeiss AxioCamERc 5s) attached to a trinocular microscope (Zeiss Axio Lab.A1) and the micro-measurements were taken using the pre-calibrated scale available in the software (AxioVision Rel. 4.8).
3.2.3 *Powder microscopy*

A pinch of the rhizome powder was warmed with drops of chloral hydrate on a microscopic slide and mounted in glycerin. Slides were observed under a microscope and diagnostic characters photographed using Zeiss AXIO trinocular microscope [109].

3.2.4 *Loss on drying*

10 g of the sample was placed in tarred evaporating dish. It was dried at 105 °C for 5 h in a hot air oven and weighed. The drying was continued until the difference between two successive weights were not more than 0.01g after cooling in a desiccator. Percentage of moisture was calculated with reference to the weight of the sample.

3.2.5 *Total ash*

2 g of the sample was incinerated in a tarred platinum crucible at a temperature not exceeding 450 °C until carbon-free ash is obtained. Percentage of ash was calculated with reference to the weight of the sample.

3.2.6 *Acid insoluble ash*

To the crucible containing total ash, 25 ml of dilute HCl was added. The insoluble matter was collected on ashless filter paper (Whatman No.41) and washed with hot water until the filtrate is neutral. The filter paper containing the insoluble matter was transferred to the original crucible, dried on a hot plate and ignited to constant weight. The residue was allowed to cool in suitable desiccators for 30 mins and weighed. The content of acid insoluble ash was calculated with reference to the air dried drug.

3.2.7 *Water soluble ash*

The ash was boiled for 5 min with 25 ml of water; the insoluble matter was collected on an ashless filter paper, washed with hot water and ignited for 15 min at a temperature not exceeding 450 °C. The weight of the insoluble matter was subtracted from the weight of
the ash; the difference in weight represents the water soluble ash with reference to the air-dried sample.

3.2.8 Alcohol soluble extractive

Accurately 4 g of the sample was weighed in a glass stoppered flask. 100 ml of distilled alcohol was added and shaken occasionally for 6 h. The mixture was allowed to stand for 18 h and filtered rapidly taking care not to lose any solvent. 25 ml of the filtrate was pipetted out in a pre-weighed 100 ml beaker and evaporated to dryness on a water bath. It was then kept in an oven at 105 °C for 6 h, cooled in a desiccator for 30 min and weighed. The percentage of alcohol extractable matter of the sample was calculated. The experiment was repeated twice and the average values were taken.

3.2.9 Water soluble extractive

Accurately 4 g of the sample was weighed in a glass stoppered flask. 100 ml of distilled water was added and shaken occasionally for 6 h. It was allowed to stand for 18 h and filtered rapidly taking care not to lose any solvent. 25 ml of the filtrate was pipetted out into a pre-weighed 100 ml beaker and evaporated to dryness on a water bath. It was then kept in an oven at 105 °C for 6 h, cooled in a desiccator for 30 min and weighed. The percentage of water extractable matter of the sample was calculated. The experiment was repeated twice and the average value was taken.

3.3 Preparation of extracts

Ethanol and aqueous extracts of *C. rotundus* were prepared as per the standard procedures [110].

3.4 Preliminary phytochemical screening

These tests are used to detect the presence of various organic functional groups, which are the indicative phytochemicals present in the plant. These tests specify the presence of the different class of constituents present in the extract. Tests were performed as per the standard methodology [111].
The following tests were carried out for both ethanol and aqueous extracts.

### 3.4.1 Tests for alkaloids

#### 3.4.1.1 Dragendroff’s test

To 5 mg of the extract dissolved in 2 ml of alcohol, two drops of acetic acid and Dragendroff’s reagent were added and shaken well. An orange-red precipitate formed indicates the presence of alkaloids.

#### 3.4.1.2 Wagner’s test

To 5 mg of extract dissolved in 1 ml of acetic acid, two drops of Wagner’s reagent was added. A reddish brown precipitate formed indicates the presence of alkaloids.

#### 3.4.1.3 Mayer’s test

To 5 mg of extract dissolved in 1 ml of acetic acid, two drops of Mayer’s reagent was added. A dull white precipitate formed indicates the presence of alkaloids.

#### 3.4.1.4 Hager’s test

To 5 mg of extract dissolved in 1 ml of acetic acid, 3 ml of Hager’s reagent was added. The formation of yellow precipitate indicates the presence of alkaloids.

### 3.4.2 Tests for carbohydrates

#### 3.4.2.1 Molisch’s test

To the extract, 1 ml of α-naphthol solution and conc. Sulphuric acid was added along the sides of the test tube. Violet color formed at the junction of the two liquids indicates the presence of carbohydrates.
3.4.2.2 *Fehling’s test*

10 mg of the extract was mixed with 3 ml of Fehling’s solution A and B. The mixture was warmed on a water bath. The formation of a brick red precipitate indicates the presence of carbohydrates.

3.4.2.3 *Benedict’s test*

To 5 ml of Benedict’s reagent, 10 mg of the extract was added, boiled for two minutes and cooled. Formation of a red precipitate indicates the presence of carbohydrates.

3.4.2.4 *Anthrone-sulphuric acid test*

10 mg of the extract was mixed with equal quantity of Anthrone and treated with two drops of conc. sulphuric acid. It was then heated gently on a water bath. Dark green color indicates the presence of glycosides.

3.4.3 *Test for steroids*

3.4.3.1 *Libermann-Burchard test*

To the extract dissolved in chloroform, 1 ml of acetic acid and 1 ml of acetic anhydridewere added, heated on a water bath and cooled. Few drops of conc. Sulphuric acid was added along the sides of the test tube. The appearance of bluish green color indicates the presence of steroids.

3.4.3.2 *Salkowski test*

The extract was dissolved in chloroform and an equal volume of conc.sulphuric acid was added. Formation of bluish red to cherry red color in Chloroform layer and green fluorescence in the acid layer indicates the presence of steroids.
3.4.4 Test for saponins

To 10 mg of extract, 5 ml of distilled water was added and shaken in a measuring cylinder. Stable froth formation indicates the presence of saponins.

3.4.5 Test for tannins

To the extract, a few drops of a dilute solution of ferric chloride were added. Formation of dark blue color shows the presence of tannins.

3.4.6 Test for flavonoids

3.4.6.1 Shinoda’s test

To the extract in alcohol, two magnesium turnings and few drops of conc. hydrochloric acid was added and heated on a water bath. Formation of red to pink color indicates the presence of flavonoids.

3.4.7 Test for phenolics

To the extract in alcohol, added two drops of alcoholic ferric chloride. Formation of blue to blue-black color indicates the presence of phenolics.

3.4.8 Test for coumarins

To the extract in alcohol, a few drops of 2 N sodium hydroxide solutions were added. Dark yellow color formation indicates the presence of coumarins.

3.4.9 Test for triterpenoids

The extract was warmed with tin bits and few drops of thionyl chloride. Formation of pink color indicates the presence of triterpenoids.

3.4.10 Test for carboxylic acid

Extract dissolved in water was treated with sodium bicarbonate. Brisk effervescence indicates the presence of carboxylic acid.
3.5 **HPTLC of *C. rotundus* rhizome extracts**

### 3.5.1 HPTLC of aqueous extract

1 g of the plant powder was extracted with 10 ml of distilled water by cold percolation for 48 h. 25 µl of the aqueous extract was applied on a pre-coated silica gel F254 on aluminum plates to a bandwidth of 8 mm using Linomat 5 TLC applicator. The plate was developed in ethyl acetate: formic acid: water (8.8:0.6:0.6) and the developed plates were visualized and scanned under UV 254 nm, 366 nm and after derivatisation in vanillin-sulphuric acid spray reagent. Rf value, the color of the spots and densitometric scan were recorded [112].

### 3.5.2 HPTLC of ethanol extract

1 g of the plant powder was extracted with 10 ml of ethanol by cold percolation for 48 h. 25 µl of the ethanol extract was applied on a pre-coated silica gel F254 on aluminum plates to a bandwidth of 8 mm using Linomat 5 TLC applicator. The plate was developed in Toluene – ethyl acetate (8:2) and developed plates were visualized and scanned under UV 254nm, 366nm an after derivatisation in vanillin-sulphuric acid spray reagent. Rf value, the color of the spots and densitometric scan were recorded [112].

### 3.6 Estimation of total phenolics

Total phenolic content of the crude drug, aqueous extract and ethanol extract were done according to the standard procedure [113].

### 3.6.1 Phenolic content of the crude extract

1 g of the sample was mixed well with 10 ml of aqueous acetone (70%) at room temperature for 20 min, centrifuged at 4 °C at 3000 rpm and the supernatant was made up to 15 ml with aqueous acetone.
3.6.2 Alcohol soluble extracts

50 g of sample was extracted with 175 ml of alcohol. The extract was concentrated and weight was recorded.

3.6.3 Water soluble extracts

50 g of sample was extracted with 175 ml of water. The extract was concentrated and weight was recorded. The extract was mixed well with 10 ml of aqueous acetone (70%) at room temperature for 20 min, centrifuged at 4 °C at 3000 rpm and the supernatant was made up to 15 ml with aqueous acetone.

1 ml of Folin’s reagent was diluted with 1 ml of distilled water. 20 g of Na₂CO₃ was dissolved in 100 ml of water at 70-80 °C and cooled overnight. The clear liquid was decanted before use. 10 mg of tannic acid was dissolved in 100 ml of water freshly. A standard curve was obtained by taking standard solution in 0.2, 0.4, 0.6, 0.8 and 1 ml volumes. After adding the reagents (phenols reagent followed by Na₂CO₃), the mixture was incubated at room temperature for 40 min in dark and blue color developed was read at 725 nm in a UV spectrophotometer (SYSTRONICS, 2201, India). The phenol content was estimated from the calibration curve of the standard tannic acid obtained by plotting concentration vs. absorbance.

3.7 Methodology of GC-MS Analysis [114]

One gram of the powdered sample was extracted with 10 ml ethanol and kept for cold percolation for 24 h, filtered; the filtrate was dried and subjected to GC-MS analysis.

GC-MS analysis was carried out on a Perkin-Elmer Turbo Mass Spectrophotometer (GC-MS-5975C [AGILENT]) which includes a Perkin-Elmer Autosampler XLGC. The column used was Perkin Elmer Elite - 5 capillary column measuring 30 m × 0.25 mm with a film thickness of 0.25 mm composed of 95% Dimethylpolysiloxane. The carrier gas used was Helium at a flow rate of 1.5ml /min. 1 μl of sample injection volume was utilized. The inlet temperature was maintained at 250 °C. The oven temperature was
programmed initially at 70 °C for 3 minutes and then programmed to increase to 300 °C at a rate of 10 °C ending with a duration of 9 min. Total run time was 35 minutes. The MS transfer line was maintained at a temperature of 240 °C. The source temperature was maintained at 230 °C. GC-MS was analyzed using electron impact ionization at 70 eV and data was evaluated using total ion count (TIC) for compound identification and quantification. The spectra of the components were compared with the database of the spectrum of known components stored in the GC-MS library (NIST-11). Measurement of peak areas and data processing were carried out by Turbo-Mass-OCPTVS-Demo SPL software.

3.8 Antioxidant Assays

3.8.1 DPPH scavenging assay [115]

Reagents

DPPH (1, 1-Diphenyl-2, Picryl-Hydrazyl) was purchased from Sigma, USA. All the other chemicals used were of analytical grade.

DPPH (1, 1-Diphenyl-2-picyrlyhydrazyl) is a stable free radical with purple color. If free radicals have been scavenged, DPPH will degenerate to yellow color. This assay uses the said principle to show free radical scavenging activity. 1 ml of the sample (25, 50, 100, 150, and 200 µg/ml) was mixed with 1 ml of 0.002% DPPH solution in methanol. Methanol was added to control tube. The contents were incubated at room temperature in dark for 30 mins. The absorbance of the sample at 517 nm was measured with a double beam UV-Visible Spectrophotometer (SYSTRONICS, 2201, India)

The percentage inhibition was calculated using the following formula:

\[
\% \text{ Inhibition} = \frac{A_0 - A_1}{A_0} \times 100
\]

\( A_0 \) = Absorbance of control
\( A_1 \) = Absorbance of sample
3.8.2 Reducing power assay [116]

Reagents

0.2 M phosphate buffer (pH 6.6) – Sodium chloride (8 g), potassium chloride (200 mg), disodium hydrogen phosphate (1.46 g) and potassium dihydrogen phosphate (240 mg) were dissolved in 800 ml of distilled water and pH was adjusted to 6.6 with HCl. The volume was made up to 1000 ml with distilled water.

1 % Potassium ferricyanide - 1 g of potassium ferricyanide was dissolved in 100 ml distilled water

10 % Trichloroacetic acid - 10 g of TCA was dissolved in 100 ml distilled water

0.1 % Ferric chloride – 0.1 g ferric chloride was dissolved in 100 ml distilled water

0.75 ml of 25, 50, 100, 150, and 200 µg/ml aqueous extract of *C. rotundus* were mixed with 0.75 ml of phosphate buffer (0.2 M pH 6.6) and 0.75 ml of potassium ferricyanide (1% v/v) was added. The contents were mixed and incubated at 50 °C for 20 min. The reaction was stopped by adding 0.75 ml of 10% Trichloroacetic acid and centrifuged at 800 rpm for 10 minutes. 1.5 ml of supernatant was mixed with 1.5 ml distilled water and 0.1 ml ferric chloride (0.1%), incubated at room temperature for 10 minutes and the absorbance was measured at 700 nm with double beam UV-Visible Spectrophotometer (SYSTRONICS 2201, India).

3.9 *In Vitro* anticancer activity of *C. rotundus* extracts

For the *in vitro* anticancer studies, MCF-7 (human breast cancer cell line) and HCT-116 (human colon cancer cell line) procured from NCCS Pune, India was used.
**Culture and maintenance of cell lines**

MCF-7 grown in 25 cm² tissue culture flasks containing Dulbecco’s modified eagles medium (DMEM) supplemented with 10% FBS, 1% L-glutamine and 50 μg/ml gentamycin sulphate at 37°C in CO₂ incubator in an atmosphere of humidified 5% CO₂ and 95% air. The cells were maintained by routine subculturing in 25 cm² tissue culture flasks.

For subculturing, culture media from the flasks containing monolayer culture was aspirated and washed with sterile phosphate buffered saline (PBS). To the flasks, 3 ml of 0.2% trypsin-EDTA solution was added and after few seconds it was aspirated and the flask was kept in an incubator for 2-3 min for detachment. The flasks were removed from the incubator and gently tapped to detach all the adhering cells. The cell detachment was confirmed by observing under an inverted microscope (Nikon Eclipse TE 2000-5, Japan). Once the cells were completely detached from the flasks, 2-3 ml of DMEM media containing 10% FBS was added and mixed well. Cell viability was checked with a small sample of the suspension by trypan blue dye exclusion test. From the stock cell suspension, 1 x 10⁴ viable cells/ml suspended in media were seeded in 25 cm² tissue culture flask containing about 4 ml of fresh media and incubated until the flasks attained 60-70% confluence. To obtain a single cell suspension from a monolayer culture, cells were dislodged from the culture flasks by trypsinization. From a 60-70% confluent flask, the culture media was aspirated out using a micropipette. Cells were washed with 3 ml of PBS to remove trace amount of media. To each culture flask, 3 ml of trypsin-EDTA was added and after few seconds it was aspirated and the flask was kept in the incubator for 3-4 min for cell detachment. Culture flasks were observed under an inverted microscope (Nikon Eclipse, Japan) to ensure that cells were completely dislodged. Trypsin activity was stopped by adding 2-3 ml media containing 10% FBS.
3.9.1 Sulphorhodamine-B (SRB) assay [117]

Reagents

1. 30 % w/v Trichloroacetic acid (TCA): 30 g of TCA was dissolved in 100 ml of milliQ water.
2. 1 % v/v Acetic acid: 1 ml of glacial acetic acid was made up to 100 ml with milliQ water.
3. 10 mM Tris base (pH 10.5): 121 mg of Trizma base was dissolved in 100 ml of milliQ water and pH was adjusted to 10.5.

Preparation of drug dilutions:

40 mg/ml stock solution was prepared using 100% DMSO. From this stock solution various final concentrations (viz. 25, 50, 100 and 200 µg/ml) of test compounds were prepared as follows:

Exponentially growing cell lines were harvested from 25 cm² tissue culture flask and a stock cell suspension (5x10⁴ cell/ml) was prepared. A 96-well flat bottom tissue culture plate was seeded with 5 x 10³ cells in 0.1 ml of DMEM medium supplemented with 10 % FBS and allowed to attach for 24 h. Test compounds were prepared just prior to the experiment and serially diluted with medium to get the working stock of 200, 100, 50 and 25 µg/ml solution. After 24 h of incubation, cells were treated with 100 µl of test solutions from respective stocks and the cells were incubated for 48 h. The cells in the control group received only the medium containing 0.5 ml of 0.25% DMSO. Each treatment was performed in triplicates. After the treatment, drug containing media was removed and washed with 200 µl of PBS. 50 µl of 30% TCA was added to fix the cells and was kept at 4 °C for 1 h. Each well was then gently washed with milliQ water to remove TCA, the medium and the dead cells. The plate was allowed to air dry and 50 µl of 0.05% SRB dye solution was added and placed in dark for 30 min. After 30 min, unbound SRB was removed by washing with 200 µl of 1% v/v acetic acid. The plate was air dried and 200 µl of trizma base buffer was added to each well to dissolve the cell
bound dye. The plate was shaken for 20 min on a shaker and the optical density was read at 540 nm with reference wavelength of 630 nm in an ELISA plate reader. Percentage cell viability was calculated. O.D of each well was read and expressed as % cell death: (absorbance of control wells - absorbance of test wells / absorbance of control wells) x 100. Results were expressed as Mean ± S.E. O.D values (proportional to cell death) was plotted against the tested drug concentrations.

3.9.2 Trypan blue exclusion assay [118]

In 100 ml of saline (0.9% NaCl), 100 mg of trypan blue was dissolved and stored at 4 ºC. The stock cell suspension of 1X10^7 cells was made in PBS from which 0.1 ml of suspension was taken in sterile test tubes. The cells were treated with 0.1 ml of test drugs of varying concentrations (not more than 0.1% DMSO) and 0.7 ml of PBS was added. The cells were incubated at 37 ºC for 3 h. After the exposure 0.1 ml of trypan blue was added and mixed well. The total numbers of dead and living cells in all the four corner squares of the chambers were counted using hemocytometer and the percentage viability/cytotoxicity was calculated as follows:

\[
\% \text{ viable cells} = \frac{\text{Number of unstained cells}}{\text{Total number of cells}} \times 100
\]

3.9.3 Hoechst staining [119]

100 µl of cell suspension containing the required number of cells (1000 cells/ml) was added to each well of a 96-well plate and the cells were allowed to attach for 24 h. 100 µl of extracts were added to the wells and the plate was incubated for 24 h. At the end of drug treatment, the media containing drugs was removed, 100 µl of chilled methanol (100%) was added and incubated for 30 min at room temperature. The solution was removed, the plate was dried and 50 µl of Hoechst reagent (2µg/ml in PBS) was added to each well. The plates were incubated at 37 ºC for 15 min. The plate was washed with PBS, dried and observed under Nikon eclipse TS100 inverted microscope with excitation filter 460/490 nm and emission filter 535/540 nm.
3.9.4 Acridine orange - ethidium bromide staining for apoptosis detection [120]

100 µl of cell suspension containing the required number of cells (1000 cells/ml) was added to each well of a 96-well plate and the cells were allowed to attach for 24 h. Extracts were added to the wells (100µg/mL & 200µg/mL) and the plate was incubated for 24 h. At the end of drug treatment, the media containing drug was removed, 100 µl of chilled methanol was added and incubated for 30 min at room temperature.

The solution was removed, the plate was dried and cells were then stained with a mixture of AO and EB (2µg/mL) for 10 min in a 37 °C CO₂ incubator. The plate was washed with PBS, dried and observed under Nikon eclipse TS100 inverted microscope with excitation filter 460/490 nm and emission filter 535/540 nm. AO - EB interpolates with DNA and preferentially stain the cell nuclei. AO is cell-permeant, allowing for visualization of nuclear structure in living cells. EB is excluded from living cells. When cells die and their plasma membrane ruptures, ethidium bromide reaches the nucleus and stains orange red. Therefore, in this assay, uniformly stained green nuclei indicate live cells and uniformly stained orange-red nuclei indicate necrotic cells, whereas a green or orange-red nucleus with condensed chromatin/fragmented DNA indicates apoptotic cells.

3.9.5 Apoptosis-DNA ladder assay [121]

Reagents

1. Lysis buffer (2 ml stock contains 10 % NP-40 [200 µl] + 200 mM EDTA [100 µl] + 0.2 M Tris-HCl [500µl; pH 7.5] + 2 mg Proteinase K + 1.2 ml millipore H₂O

2. RNAse solution (1mg/ml in millipore water).

3. TBE buffer [54g Trizma, 25.2 g boric acid, 0.5M EDTA (20ml)] in 1000 ml of milli-Q water) and adjusted to pH 8.0.

MCF-7 cells (1X10⁶) were seeded in 6 well plate with 2 ml of DMEM medium supplemented with 10% FBS and allowed to attach for 24 h. The cells were treated with
different concentrations of ethanol extract of *C. rotundus*. The plates containing cells were incubated for 48 h at 37 °C. Contents from plates containing dead cells were transferred to centrifuge tubes and centrifuged at 1000 rpm for 5 min to get the cell pellet. 300µl of lysis buffer was added to a T-25 flask containing attached monolayer cells. In this process, cells will be lysed and the DNA remains in the flask. DNA was added to the cell pellet and it was incubated at 37 °C for 1 h. 150µl of RNase was added to the above solution and kept at 50 °C for 1 h to destroy the RNA. A brief exposure at 65°C for 2 min was followed to destroy the un-reacted RNAse. The processed cell lysate was then cooled to room temperature, diluted with 30% glycerol and bromophenol blue in the ratio 1:1 and loaded into the wells of agarose gel (1.5% in TBE buffer). Electrophoresis was carried out at 60 V, 400 mA for 90 min using TBE buffer.

3.10 *In vivo* anticancer activity of *C. rotundus* extracts in Ehrlich’s ascites carcinoma cells

The EAC cells were obtained from Amala Cancer Research Center, Amala Nagar, Thrissur, Kerala, India. They were maintained and propagated by serial intraperitoneal inoculation (2 x10⁶ cells /mouse) in an aseptic environment. Cells propagated for 12-14 days were used in the experiments.

3.10.1 Animals

Eight to ten week old Swiss albino female mice, weighing 20 to 30 g, selected from an inbred colony maintained in the Central Animal Research Facility of Manipal University were selected for the study. The mice were housed in polypropylene cages (4 per cage) in an air conditioned room maintained at a comfortable temperature (23 ± 2 °C) with a 12 h light-dark cycle. They were fed with standard feed pellets and tap water *ad libitum*. The experiments on mice were approved by the Institutional Animal Ethical Committee (IAEC) and were conducted according to the guidelines of CPCSEA (No: YU-IAEC/4/25/8/2011).
3.10.2 Acute toxicity studies [122]

The *in vivo* acute oral toxicity studies of the extracts were carried out as per OECD guidelines - 425 to explore the acute dose lethal to 50% of the animals thereby establishing the therapeutic index. The animals were fasted overnight and then were administered orally with a starting dose of 2000 mg/kg body weight in a single dose. After dosing, the animals were observed for 3 h and monitored for 14 days for any mortality, behavioral changes, autonomic nervous system and central nervous system changes.

3.10.3 Antitumor activity in EAC model

Swiss albino mice were divided into 7 groups (*n* = 6). A known number of viable EAC cells (2.5 x 10⁶ cells/mice) were injected intraperitoneally into all the groups in an aseptic condition except for the normal group. The day of tumor inoculation was considered as day zero [123]. All the experiments on tumor bearing mice were conducted 24 h after EAC transplantation and that day were considered as day one.

Group 1 served as normal control where animals were fed with 5 ml/kg body weight of normal saline (0.9% NaCl w/v).

Group 2 animals received only tumor cells and served as tumor control.

Group 3 animals were injected with tumor cells and injected with standard drug Cisplatin (single dose of 3.5 mg/kg, i.p.) on day 1 which served as standard. This was calculated from the human dose using an appropriate conversion factor [124].

Group 4 tumor-bearing mice received ethanol extract of *C. rotundus* 250 mg/kg body weight. Group 5 tumor-bearing mice received ethanol extract of *C. rotundus* 500 mg/kg body weight. Group 6 tumor-bearing mice received aqueous extract of *C. rotundus* 250 mg/kg body weight. Group 7 tumor-bearing mice received an aqueous extract of *C. rotundus* 500 mg/kg body weight. The extracts were dissolved in CMC 0.25% (carboxymethyl cellulose) daily just prior to the dosage and administered orally on days

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1, 3, 5, 7, 9, 11 and 13 of tumor inoculation. All the experimental animals were observed for the development of ascitic tumor other than the normal group and on the 15\textsuperscript{th} day, 3 animals from all the groups were sacrificed by administering euthanizing agent thiopental sodium (300mg/kg). The remaining animals were observed up to 45 days with food and water \textit{ad libitum} to check the mean survival time (MST) and percentage increase in mean life span (\% IMLS). After sacrificing the animals, blood was withdrawn by cardiac puncture to evaluate the hematological parameters. Ascitic fluid was collected from the peritoneal cavity of each animal to observe the appearance of neoplastic cells. Liver and kidney were collected from all the animals for estimation of \textit{in vivo} antioxidant status and for histopathological studies.

The antitumor activity of the extracts was measured in EAC animals with respect to following parameters like Mean survival time (MST), the percentage increase in mean life span (\% IMLS), the percentage increase in the body weight, an average increase in the body weight and the tumor volume.

3.10.4 \textit{Mean survival time (MST) and percentage increase in mean life span (\% IMLS)} \[125\]

The total number of days an animal survived from the day of tumor inoculation was counted and MST was calculated as follows:

$$\text{MST} = \frac{\text{Day of first death + day of last death}}{2}$$

Subsequently, \% IMLS was calculated as follows:

\[\left(\frac{\text{Mean survival time of treated group}}{\text{mean survival time of control group}} - 1\right) \times 100\]

3.10.5 \textit{Percentage increase in weight as compared to day “0”} \[126\]

The animals were weighed on the day of tumor inoculation. During the post inoculation period, the animals were weighed once in three days till the completion of the experiments. The percentage increase in weight was calculated as follows:
\( \% \text{ increase in weight} = [(\text{Animal weight on respective day}/\text{animal weight on day 0})-1] \times 100 \)

The increase in the average body weights of the animals from these groups was also recorded.

3.10.6 Tumor Volume

The ascetic fluid was collected from the peritoneal cavity of tumor-bearing mice using graduated centrifuge tube and volume was noted.

3.10.7 Hematological parameters

On the 15\(^{th}\) day of the tumor inoculation, blood was withdrawn by cardiac puncture and hematological parameters like total WBC count, total RBC count and hemoglobin were assessed following the standard procedures [127].

3.10.8 Antioxidant assays

Preparation of tissue homogenate

Animals were sacrificed by cervical dislocation and were perfused transcardially with ice-cold saline. The whole liver was perfused in situ with ice-cold saline, dissected out, blotted dry and immediately weighed. 10\% liver homogenate was prepared in ice-cold KCl (150Mm) using Teflon – glass homogenizer (Yamato LSG LH-21, Japan). The homogenate was centrifuged at 10,000 rpm for 10 min and the pellet was discarded. The supernatant was again centrifuged at 20,000 rpm for 1 h at 4\(^{\circ}\)C. The supernatant obtained was used for the following the estimations.

- Lipid Peroxidation [128]
- Reduced Glutathione - GSH [129]
- Catalase - CAT [130]
3.10.8.1 Lipid Peroxidation

Reagents

Trichloroacetic acid (TCA, 10%): 10 g of TCA in 100 ml of distilled water

Thiobarbituric acid (TBA, 1.0%): 1g of TBA in 100 ml of distilled water

Normal saline (0.9% w/v) OR PBS (0.02M, 1X): 0.9 g of NaCl in 100 ml of PBS

Lipid Peroxidation (LPO) was assayed according to the standard method [129]. To 1 ml of tissue homogenate, 1 ml of normal saline and 2.0 ml of 10% TCA were added and mixed well. The mixture was kept at room temperature and then centrifuged at 3000 g for 10 min to separate proteins. 2 ml of supernatant was taken and 0.5 ml 1.0% TBA was added to it followed by heating at 95° C for 60 minutes to generate the pink colored MDA. OD of the samples was measured at 532 nm using Beckman DU 64 spectrophotometer. The levels of lipid peroxides were expressed as nM of MDA/mg wet tissue using extinction co-efficient of 1.56 x10^5 M^-1 cm^-1.

3.10.8.2 Reduced Glutathione (GSH)

Reagents

0.01 M DTNB - 0.099 g of DTNB was dissolved in absolute methanol to a final volume of 25 ml (prepared fresh before use)

0.2M Tris buffer - 48.458 g of Tris was dissolved in 800 ml of distilled water. 0.2 M EDTA solution was added and made up to final volume of 1 liter with distilled water. The pH was adjusted to 8.9 by the addition of 1 M HCl.

0.02 M EDTA - 37.224 g of EDTA was dissolved in a final volume of 500 ml with distilled water.

TCA (50% w/v) - 50 g of TCA was dissolved in a final volume of 100 ml of distilled water.
Standard reduced glutathione (1 mg GSH/ml) - Stock standard solution (0.001 M) was prepared by dissolving 0.0307 g of GSH in a final volume of 100 ml of (0.2 M) EDTA solution. Dilutions are made in EDTA solution to 25, 50, 100, 150 and 250 µM.

To 0.1 ml of different tissue samples, 2.4 ml of 0.02 M EDTA solution was added and kept on ice bath for 10 min. 2 ml of distilled water and 0.5 ml of 50% TCA were added. This mixture was kept on ice for 10-15 min and then centrifuged at 3000 g for 15 min. To 1 ml of supernatant, 2.0 ml of Tris buffer was added. Then 0.05 ml of DTNB solution (Ellman’s reagent) was added and vortexed thoroughly. OD was read (within 2-3 min after the addition of DTNB) at 412 nm in a spectrophotometer against a reagent blank. Appropriate standards were run simultaneously.

3.10.8.3 Catalase (CAT)

Reagents

M/15 phosphate buffer (pH-7.0) – It was prepared by mixing 61.1 ml of M/15 Na₂HPO₄ with 38.9ml of M/15 KH₂PO₄

M/150 phosphate buffer (pH-7.0) – 3.522 g KH₂PO₄ and 7.268g Na₂HPO₄2H₂O were dissolved in distilled water and volume was made up to 1 liter. This assay buffer was diluted 10 times and used.

H₂O₂ phosphate buffer: 0.16 ml H₂O₂ (30% w/v) was diluted to 100 ml with phosphate buffer. H₂O₂ at a concentration of 14.2 mM had an absorbance of approximately 0.50 at 240 nm with the 1cm light path.

Catalase activity was measured based on the ability of the enzyme to break down H₂O₂. 10 µl samples were taken in tubes containing 3.0 ml of H₂O₂ in phosphate buffer. The time required for 0.05 optical density changes was observed at 240 nm against a blank containing the enzyme source in H₂O₂ free phosphate buffer (0.16 ml H₂O₂ is 30% w/v) diluted to 100 ml of phosphate buffer. The absorbance was recorded at 240 nm and after the addition of enzyme; Δt was noted till OD was 0.45. If Δt was longer the 60 seconds,
the procedure was repeated with more concentrated enzyme sample. Reading was taken at every 3 second interval. A unit catalase activity is the amount of enzyme that liberates half the peroxide oxygen from the $\text{H}_2\text{O}_2$ solution of any concentration in 100 seconds at $25^\circ\text{C}$.

CAT activity was expressed as follows:

Moles of $\text{H}_2\text{O}_2$ consumed/min (Units/mg) = $\frac{2.3}{\Delta t} \times \log \left( \frac{E_{\text{initial}}}{E_{\text{final}}} \right) \times 1.63 \times 10^{-3}$

$E$ = optical density at 240 nm

2.3 = factor to convert into log.

$\Delta t$ = time required for decrease in the absorbance

3.10.9 **Histopathological studies**

3.10.9.1 **Pathology of the ascitic tumor [131]**

A small amount of the ascitic fluid was withdrawn aseptically from the peritoneum of the mice on the 15$^{\text{th}}$ day of tumor inoculation. A drop of the fluid was placed on the slide and made into a smear. The slide was kept aside and the smear was fixed with methanol for half an hour. Then a few drops of Leishman’s reagent were added to the smear. The slide was allowed to stain for three minutes and was washed with an excess of distilled water. Then the slide was allowed to stand for half an hour. It was fixed with xylene and examined under the microscope at 100 X magnification. Photographs were taken focusing the appropriate regions in the smear.

3.10.9.2 **Histopathology of the Liver and kidney [132]**

At the end of treatment, both control and experimental mice were sacrificed by cervical dislocation. The liver and kidney tissues from all groups were collected, fixed in 10% formalin in saline, dehydrated in ascending grades of ethyl alcohol, cleared in xylol and mounted in molten paraffin at 58-62 °C. 5 µ sections were obtained, stained with Harris
Hematoxylin & Eosin and evaluated for any structural changes under a bright field microscope.

**Statistical analysis**

Data were expressed as Mean ± SEM. Statistical analysis was carried out using one way ANOVA (Graph Pad Version 5.02, Instat Software) followed by Dennett’s post hoc test. The values *$p<0.05$* was considered as statistically significant, **$p<0.01$** as highly significant and ***$p<0.001$*** as extremely significant. Here all experimental groups were compared with tumor control group.