Chapter 2.
Table of Contents

2.1. MATERIALS
2.1.1. Plant materials
2.1.2. Saccharomyces cerevisiae
2.1.3. Cell lines
2.1.4. Animals
2.1.5. Chemicals
2.1.6. Instruments
2.1.7. Reagents and stains

2.2. METHODS
2.2.1. Establishment of cultures
2.2.2. High performance liquid chromatography
2.2.3. Isolation of anthraquinones
2.2.4. Mass spectrometry and Nuclear magnetic spectrometry
2.2.5. Antioxidant assays
2.2.6. Determination of antitumor activity of anthraquinones
2.2.7. Cytotoxicity assay
2.2.8. DNA fragmentation analysis
2.2.9. Determination of O2. radicals in cells And medium
2.2.10. Determination of H2O2/OH radicals in cells
2.2.11. Caspase-3 expression study

2.3. STATISTICAL ANALYSIS
Chapter 2.
Materials and Methods

2.1. Materials

2.1.1. Plant materials

*Ophiorrhiza rugosa var. decumbens* was collected from outskirts of University of Calicut and *Ophiorrhiza pectinata* was collected from Pathanamthitta district, Kerala and authenticated by Dr. Sasidharan, Scientist, KFRI, Peechi, Kerala. The voucher specimens of the plants were identified and kept in the herbarium of Amala Ayurvedic Hospital and Research Centre (ACRH No. 97 & 98).

2.1.2. *Saccharomyces cerevisiae* (MTCC 463)

*S. cerevisiae* culture was purchased from M.T.C.C, Institute of Microbial Technology, IMTECH, Sector 39-A, Chandigarh, U.T., 160-036, India.

2.1.3. Cell lines

Ehrlich’s ascites carcinoma (EAC) cell line and Dalton’s Lymphoma Ascites (DLA) cell line were initially procured from Cancer Institute, Adyar and propagated as transplantable tumors in the peritoneal cavity of Swiss albino mice.

2.1.4. Animals

Swiss albino mice were obtained from Small Animal Breeding Station, College of Veterinary and Animal Sciences, Mannuthy. Animals were maintained in ventilated polypropylene cages and fed with normal chow (Sai Durga Feeds and Foods, Bangalore) and water *ad libitum*. The animal experiments were conducted after getting prior permission from Institutional Animal Ethics Committee (IAEC) and as per the instructions prescribed by the Committee for the Purpose of Control
and Supervision of Experiments on Animals (CPCSEA), Ministry of Environment and Forest, Government of India.

2.1.5. Chemicals

1. MS stock medium : Sigma-Aldrich, St. Louis, USA
2. Agar : Merck
3. Sucrose : Merck
4. N6-Benzyl adenine (BA) : Merck
5. Naphthalene acetic acid (NAA) : Merck
6. HPLC grade Acetonitrile : Merck
7. HPLC grade Water : Merck
8. Methyl Jasmonate : Sigma-Aldrich, St. Louis, USA
9. Tween 20 : Merck
10. Dimethyl sulfoxide (DMSO) : Merck
11. Chitin : A generous gift from CIFT, Kochi
12. Chitosan : A generous gift from CIFT, Kochi
13. Camptothecin : Sigma-Aldrich, St. Louis, USA
14. Silica Gel GR : Merck
15. Silica Gel for CC : Merck
16. Precoated TLC plates (20X20cm) : Merck
17. Chloroform : Merck
18. Diethylether : Merck
19. Methanol : Merck
20. Dulbecco’s Modified Eagle Medium (DMEM) : Hi-Media, Mumbai
21. Foetal calf serum : Biological industries, UK
22. Ethidium Bromide : Sigma-Aldrich, St. Louis,
22. Propidium iodide : Sigma-Aldrich, St. Louis, USA
23. Dichloroflourescein diacetate : Sigma-Aldrich, St. Louis, USA
24. HEPES buffer : Hi-Media, Mumbai
25. Tris Buffer : ---------do---------
26. Tris-HCl : ---------do---------
27. Sodium Azide : ---------do---------
28. Agarose : Genei, Bangalore
29. Riboflavin : Hi-Media, Mumbai
30. Nitroblue tetrazolium (NBT) : ---------do---------
31. Thiobarbituric acid (TBA) : ---------do---------
32. Hydrogen peroxide : ---------do---------
33. Sodium dodecyl sulfate : ---------do---------
34. Dimethyl sulfoxide (DMSO) : ---------do---------
35. DNA molecular weight marker : Genei, Bangalore
36. Taq polymerase : ---------do---------
37. RNase inhibitor (RNAsin) : ---------do---------
38. Bromophenol Blue : ---------do---------
39. dNTPs : ---------do---------
40. D-mercaptoethanol : BDH
41. Cell-to-cDNA™ II : Ambion Inc, Texas, USA
42 Nylone membrane filter : ---------do---------
43 pH paper : E-Merck, Germany

2.1.6.Instruments
1. Laminar Air Flow Chamber : Kemi Pvt. Ltd., India
2. Gyratory Shaker : Certomat SII, MO, B.Braun
   Biotech International
3. Freeze Dryer : Labconco, USA
4. UV Chamber : Commag
5. HPLC : Shimadzu, Japan
6. Upright research microscope : Meiji, Japan; Labex, Labovision
7. Deep freezers, -70°C, -20°C : Remi, India
8. High speed cooling centrifuge : ---------do---------
9. UV-Vis Spectrophotometer : Elico Ltd, Hyderabad
11. Fluorescent Microscope : Olympus, Japan
12. Thermo-cycler : MJ Research, USA
13. Electrophoresis unit : Genei, Bangalore
14. Gel documentation system : Vilber Lourmat, France

2.1.7. Reagents and stains

(a) Phosphate buffered saline (PBS)
NaCl - 8.00g
KCl - 0.20g
KH₂PO₄ - 0.20g
Na₂HPO₄·2H₂O - 1.44g
Dissolved the contents in distilled water, pH was adjusted to 7.2 with 1N NaOH/HCl and made up to 1000ml. Sterilized by autoclaving at 15lbs for 15min.

(b) 20mM Hydroxyl buffer
a) KOH - 0.1122g
b) KH₂PO₄ - 0.272g
Dissolve ‘a’ and ‘b’ separately in 100ml DDH₂O and ‘a’ is added to ‘b’ until pH reaches 7.4

(c) HBS buffer
NaCl - 818.16mg
MgCl₂ - 20.33mg
KCl - 14.702mg
HEPES - 238.3mg
Dextrose -180mg
Dissolve all the above chemicals in 100ml and pH adjusted to 7.4
(d) May-Grunwald stain
May-Grunwald powder - 250mg
Methanol - 100mL
The stain was dissolved in methanol by stirring and filtered through Whatmann No.1 filter paper and stored at 4°C.
(e) Giemsa stain
Giemsa powder - 800mg
Glycerol - 50mL
Methanol - 50mL
Giemsa powder was dissolved in glycerol at 60°C with shaking. The mixture was cooled to room temperature and methanol was added. Mixed well for 5 min and allowed to stand overnight. The solution was filtered through Whatmann No.1 filter paper and stored at 4°C.

2.2.Methods
2.2.1. Establishments of Cultures
a). Surface Sterilization of Explants- The mother plants were kept inside a green house. These were given prophylactic spray of 0.1% bavistin (fungicide) at weekly intervals. Young leaves and stem were taken as explants. They were kept under running tap water for 30 minutes to remove dust particles and phenolics. Inside the sterile laminar airflow, the explants were washed with Teepol for 2-3 minutes to remove fungal contamination. After the treatment, they were washed with sterile distilled water for 3-5 times to remove the traces of fungicide. Further surface sterilization was carried out by immersing the explants into 0.1% Mercuric Chloride (HgCl₂) for 3-5 minutes. The treated plant material was washed with sterilized distilled water for 4-5 times, blot dried and transferred to appropriate nutrient medium in culture tubes.
b). Preparation of nutrient medium- The cultures were inoculated in Murashige and Skoog Medium (MS)(Murashige and Skoog, 1962). For preparing the medium, all stock solutions were taken in appropriate proportions. Carbon source was added and made up to required volume with double distilled water.

The sucrose concentration was 3-5% (W/V). Different concentrations and combinations of BA and NAA were added to the medium, made up to required volume with double distilled water, pH was adjusted to 5.6- 5.8. For preparing solid medium, agar was used as the solidifying agent.

The media were then dispensed into culture tubes, plugged with non-adsorbent cotton, sterilized by autoclaving at 121°C, 15 lb pressure for 20 minutes.

c). Establishment of multiple shoot culture- After 4-5 washings with distilled water, explants were inoculated into Murashige and Skoog (MS) medium. The medium was supplemented with 1-5mg/l BA and 0.05-1mg/l NAA. The pH of the medium was adjusted to 5.8 before adding 0.7% agar. Culture tubes containing aliquots of medium were autoclaved at 121°C for 20 minutes. After inoculation the cultures were incubated. The cultures were given 12h photoperiod (94 μmol/m²/s-1). Medium without plant growth regulators was used as control. After one week, small shoot primordia appeared directly on the leaf explants. These shoot primordia were sub-cultured at the intervals of 28 days for the establishment of multiple shoots. Each hormone combination treatment consisted 18 explants and replicated 10 times. The number of shoots/explant was determined after 60 days.

d). Root initiation of regenerated shoots- For the induction of root, the isolated shoots (1cm or longer) were transferred to MS basal medium supplemented with NAA (0.05-5mg/l) and BA (0.01-0.05mg/l), pH 5.8. Each rooting treatment consisted of 9 explants, replicated 10 times representing a total of 90 observations. The
frequency of rooting, average number and length of roots / explant were determined after 60 days of culture.

**e). Preparation of shoot suspension culture for elicitation and treatment with elicitors** - Regenerated healthy shoots (=3g fresh weight) were inoculated into basal MS medium (25ml) in a 250ml Erlenmeyer flask and incubated on a gyratory shaker at 95 rpm, 25 ± 2°C, at 16 h photoperiod. The medium was changed at 7days' interval. To study the effect of different types of media, the cultures were kept in respective media for 5 passages before harvesting.

**Elicitation with Chitin** - Two concentrations of chitin were studied for elicitation for CPT production. Chitin was dissolved in minimum of formic acid and diluted with water. The final concentration of chitin in medium was 1mg/l and 5mg/l. An equal amount of formic acid was added to control culture medium for the comparison. On days 1st, 3rd and 7th, the cultures are harvested and CPT was quantified.

**Elicitation with Chitosan** – Chitosan was dissolved in formic acid and diluted with double distilled water (DDH2O). The final concentration of chitosan in medium was 1, 5 and 10mg/l. The CPT content in cultures was evaluated on 1st, 3rd and 7th days of culture.

**Elicitation with Methyl jasmonate (MJ)** - Methyl jasmonate was dissolved in ethanol and added to liquid medium for a final concentration of 1mM and 5mM. The same amount of ethanol was added to control cultures to eliminate the effect of ethanol. The CPT content in cultures and medium was analyzed on 1st, 3rd and 7th days of culture.

**Elicitation with Saccharomyces cerevisiae** - *S. cerevisiae* dried cultures was revived in the recommended medium at 25°C. After 24 hrs incubation, the single colonies of *S. cerevisiae* were transferred into liquid medium and kept on rotary shaker at 95rpm. After 48hrs the culture was autoclaved and filtered. The filtrate was used as the elicitor.
5ml and 10ml of filtrate were added per litre liquid medium. On days 1st, 3rd and 7th, the CPT content in cultures and medium was analyzed.

**f). Determination of fresh and dry weight**-The cultures were washed with distilled water to remove traces of media and blot dried. The cultures were transferred into a pre weighed sterile bottle and the weight of the culture was determined.

The cultures were washed with distilled water to remove traces of media and blot dried. The cultures were transferred into a pre weighed sterile bottle and freeze-dried until the water is completely removed and re-weighed.

**g). Determination of relative growth rate of cultures**- fresh weight of cultures was recorded at the time of initial subculture. At the occasion of next subculture after 28 days, the fresh masses were again recorded. The relative growth rate (RGR) over 28 days culture was calculate as,

\[ RGR = \frac{3(W_2 - W_1)}{T} \]

Where, \( W_1 \) = initial fresh weight

\( W_2 \) = fresh weight after 28 days

\( T \) = 28 days

**h). Bioconversion of Tryptophan and Permeabilization with Tween 20**- Three concentrations of Tween 20 were used such as 0.1, 0.2 and 0.3%. Tween 20 was added to the liquid cultures at the required concentrations and the permeabilization was evaluated on 1st, 3rd and 7th days. The CPT contents in cultures were also quantified.

**2.2.2. High performance liquid chromatography (HPLC)**

The chloroform extracts, redissolved in acetonitrile (ACN): water(H2O) solution(24:1) were subjected to HPLC analysis. The HPLC consist of binary pump system, reverse phase C-18 column of 250 mm length, 4 mm diameter. The UV absorbance detector was set at 256 nm for detection of CPT. The mobile phase used was acetonitrile: water (60:40 v/v) at a flow rate 1ml/ min. In some instances the mobile phase
was constituted by 40:60v/v ACN/H2O. The quantity of CPT was calculated by calibration with the standard.

2.2.4. Isolation of anthraquinones

The plant material was dried and weighed. 10% NaHCO3 was added to plant powder. This was extracted with CHCl3. The CHCl3 extract was evaporated under vacuum and redissolved in diethyl ether (Et2O). This was separated with 1M NaOH. The 1M NaOH fraction was acidulated with 1M HCL. This acidic solution was again extracted with Et2O. The Et2O extract contained crude anthraquinones. This was separated by column chromatography (silica gel-60-120mesh, column size: 2.75X35cm) and further purified by thin layer chromatography. The Rf of each band was calculated.

2.2.4. Mass Spectrometry and Nuclear Magnetic Spectrometry

The electrospray ionization mass spectra were recorded on a micromass Quattro II triple quadrapole mass spectrometer. The H1 and C13 NMR were recorded on a Varian-400Unity plus spectrometer.

2.2.5. Antioxidant assays

2.2.5.1. Superoxide radical

The reaction mixture contained 3mg NaCN dissolved in EDTA (6μM), riboflavin (2μM) NBT (50μM) various concentrations of the test material and phosphate buffer in a final volume of 3ml. The tubes containing the reaction mixture were uniformly illuminated with an incandescent lamp for 15min and the absorbance were measured at 530nm before and after the illumination (McCord and Fridovich, 1969).

2.2.5.2. Hydroxyl radical

Hydroxyl radical scavenging activity of the test material was measured by studying the competition between deoxyribose and test compounds for the hydroxyl radicals generated from Fe3+/ascorbate/EDTA/H2O2 system (Fenton reaction). The hydroxyl
radicals attack deoxyribose, which eventually results in the formation of thiobarbituric acid reacting substances (Elizabeth and Rao, 1990). The reaction mixture contained deoxyribose (2.8mM), ferric chloride (0.1mM) EDTA (0.1mM), H2O2 (1mM), ascorbate (0.1mM), KH2PO4-KOH (20mM, pH 7.4) and various concentrations of the sample in a volume of 1ml was incubated for 1hr at 37°C. Deoxyribose degradation was measured as thiobarbituric acid reactive substrate by the method of Ohkawa et al(1979). The inhibition produced by different concentration was calculated compared to control.

2.2.5.6. Nitric oxide

Nitric oxide, generated from sodium nitroprusside in aqueous solution at physiological pH, interacts with oxygen to produce nitrite ions, which were measured by Griess reaction (Green et al., 1982; Marcocci et al., 1994a). The reaction mixture (3ml) containing sodium nitroprusside (10mM) in phosphate buffered saline (PBS) and the compound (20-200μg/ml) was incubated at 25°C for 150min. after incubation, 0.5ml of the reaction mixture was removed and 0.5ml of Griess reagent (1% sulphanilamide, 2% H3PO4, 0.1% naphthylethylene diamine dihydrochloride) was added. The absorbance of the chromophore formed was measured at 546nm.

2.2.5.7. Lipid peroxidation assay

The level of lipid peroxidation was measured by the method of Ohkawa et al (1979). 20-200mg/ml of AQf was incubated with 0.1ml rat liver homogenate (25%) containing 30mM KCl, Tris-HCl buffer (0.04M, pH 7.0), ascorbic acid (0.06mM) and ferrous ion (0.16mM) in a total volume 0.5ml for 1hr. After incubation, 0.4ml of reaction mixture was treated with 0.2ml SDS (8.1%), 1.5ml TBA (0.8%) and 1.5ml acetic acid (20%, pH 3.5) distilled water were kept for 1hr in a boiling water bath at 100°C. After 1 h, the reaction mixture was removed from the water bath, cooled and added 5ml of butanol, mixed
thoroughly and centrifuged at 3000rpm for 10min. Absorbance of the clear supernatant was measured at 532 nm against butanol.

2.2.6. Determination of antitumor activity of anthraquinones

2.2.6.1. Solid tumor model

One million tumor cells (DLA) were injected into the right hind limb of mice (6 mice/group). Drug administration was started simultaneously (24h after tumor challenge) and 10 days after tumor challenge. Mode of administration of the drug was given intraperitoneally. Diameter of the tumor was measured on every fifth day using vernier calipers and volume was calculated using the formula, \( V = \frac{4}{3} \pi r_1 r_2^2 \).

2.2.6.2. Ascites tumor model

6X10^6 cells (EAC) were injected into the peritoneal cavity of the mice (6 mice/group). Drug administration (ip) was started simultaneously and after one week of tumor induction. Animals were observed for the development of ascites tumor and death due to tumor burden was recorded. The increase in life span (ILS) of treated group was calculated using the formula, \( \% \text{ ILS} = \frac{T-C}{C} \times 100 \), where T and C are mean survival of treated and control mice respectively (Mazumdar UK, 1997).

2.2.7. Cytotoxicity assay

The cytotoxicity of test compounds is determined by trypan blue exclusion method (Babu et al., 1995). The DLA and EAC cells aspirated from mice were counted to a density of 1 x 10^6 in 1ml PBS. Different concentrations of crude anthraquinone fraction (50-500\(\mu\)g/ml for DLA cells and 40-200\(\mu\)g/ml for EAC cells) and anthraquinones (AQs) (1-100\(\mu\)g/ml) were added to 100\(\mu\)l cell suspension. After 3hrs incubation at 37\(^\circ\)C, 100\(\mu\)l trypan blue dye was added and the cell death was determined using a hemocytometer.
2.2.8. DNA fragmentation analysis

DNA from the cells was isolated by phenol-chloroform method. Cells were centrifuged at 3000 rpm for 5 min and the cell pellet obtained was mixed with 1.0mL of cell lysis buffer (50mM Tris, 20mM EDTA, 2% SDS) for 1 hr at room temperature. The mixture was centrifuged again at 3000 rpm for 5 min and the supernatant was taken into a fresh vial. To this RNase (20µg/mL) was added and incubated at 37°C for 2 hr followed by proteinase K (100µg/mL) and the incubation continued at 50°C for 2hr. After incubation equal amount of buffer saturated phenol was added to each vial and mixed the contents thoroughly. The mixture was centrifuged at 3000rpm for 5 min and the aqueous phase was collected into a fresh vial. The aqueous phase was mixed with equal volume of chloroform-isoamyl alcohol mixture (49:1). The contents were mixed well and the vials were centrifuged again at 3000 rpm for 5 min. The aqueous phase was removed and treated with 2M sodium acetate and 2.5 vol. chilled ethanol and the vials were kept at -20°C for overnight. Next day, the vials were centrifuged at 12000rpm for 30 min at 4°C. The supernatant was removed and DNA was spooled out. The DNA was dissolved in TE buffer (10mM Tris HCl and 1mM EDTA, pH 10.0) and electrophoresis of the DNA (at 100v for 2 hr) was carried out on 1.5% agarose gels and visualized by staining with ethidium bromide.

2.2.9. Determination of O2⁻ radicals in cells and medium

The formation of O2⁻ was detected by measuring the reduction of NBT by O2⁻ generated within DLA and EAC cells when an oxidizer (AQs) is present. The nitroblue diformazan (reduced NBT) produced is proportional to the O2⁻ generation and it was measured by the increase in absorption at 560nm with regard to the basal situation (in the absence of AQs)(Montoya SC, et al., 2001). This procedure was performed at dark as well as illumination in order to evaluate photosensitization of AQs. 20µg/ml each of AQs were added to DLA
and EAC cell suspension. In the NBT assay, 0.1ml of cell suspension in PBS at pH 5 was incubated for with 0.1ml of AQs having aforementioned drug concentration and 0.5ml NBT at 35°C in darkness and a duplicate set was incubated under illumination of fluorescent lamp. The samples were centrifuged at 1500g for 10minutes after stopping the reaction with the addition of 0.1ml HCl (0.1M). The supernatant was removed and the cell pellet having nitroblue diformazan was extracted with 0.4ml of DMSO and 0.8ml of PBS. Absorbance was taken at 560nm.

2.2.10. Determination of H2O2/OH’ radicals in cells

DCFH-DA is a stable compound that readily diffuses into the cells and is hydrolyzed by intracellular esterase to yield DCFH, which is trapped within the cells. H2O2 or OH’ generated by the compounds oxidize DCFH to highly fluorescing DCF. After incubation of cells treated with test compounds for 12hrs, medium was removed and cells were treated with HBS buffer for 1hr. The cells were then treated with DCFH-DA dye at a final concentration of 10µM for 45minutes(LeBel CP et al., 1992). The green fluorescing cells indicating ROS generation was detected by fluorescent microscope.

2.2.10. Caspase-3 expression study

Caspase-3 expression was evaluated by the protocol provided by the manufacturer. The cells to cDNA was carried out by the method of Klebe et al(1996).

2.3. Statistical analysis of data

The data were analyzed by one-way ANOVA followed by Dunnet multiple comparison test. p<0.01 taken as significant. The statistical calculation was done using InStat GraphPad 3.00 software for Windows 95, San Diego, California, USA.