4.1 AREA OF RESEARCH

The present study was approved by the Institutional Ethics Committee, Sri Ramachandra University. REF: IEC-NI/11/APR/22/20

The present study was conducted in the Department of Pharmacognosy, Faculty of Pharmacy, Department of Oral Pathology, Faculty of Dental Sciences, Center for Stem Cells and Regeneration, Herbal and Indian Medicinal Research Laboratory, V Clin Bio, Sri Ramachandra University, Asthagiri Herbal Research Foundation; University of Madras.

4.2 RESEARCH DESIGN

This study has an *in-vitro* design. *Streptococcus mutans* and *Candida albicans* used for the antimicrobial and antimycotic assays were procured from Microbial Type Culture Collection and Gene Bank (MTCC), Chandigarh. Oral squamous cell carcinoma cell line was procured from American Type Culture Collection (ATCC), USA.

4.3 SOURCES OF DATA

Data collection was done using secondary source such as standard textbooks and journals.

4.4 COLLECTION, AUTHENTICATION, PREPARATION OF EXTRACTS AND *IN-VITRO* PRELIMINARY PHYTOCHEMICAL ANALYSIS

Armamentarium

- Rotary microtome
- Water bath
- Microscopic glass slides
- Slide warmer
- Slide holders
- Stainless steel staining bath
- Glass Beakers
- Bell Jars
- Round bottom flask
- Amber colored screw capped bottles
- Whatman filter paper 1
- Glass funnels
- Aluminum Foil
- Cotton rolls
- Mortar and pestle
- Cutter miller
- Mixer cum blender
- Measuring jar
- Weighing machine
- Refrigerator

- Staining bath
- Tissue paper
- Cover slip
- Light microscope
- Rotary flash
- Air desiccator
- Vacuum desiccator
- Test tubes
- Test tube racks
- Test tube holders
- Glass rod
- Watch glass
- UV Spectrophotometer

Reagents Used

- Prepared extracts of Fresh leaves of CO. 2 Strain *Carica papaya* L. (male and female plant), *Trigonella foenum graecum* L. (seeds), CO. 2 Strain *Carica papaya* L. (seeds), *Cinnamomum verum* J. Presl (bark)
- Ethanol (60%, 70%)
- Distilled Water
- Acetone
- Xylene
- Propanol (30%, 50%, 70%, 80%, 90%, 100%)

- Formaldehyde Acetic acid Alcohol mixture
- 10% Formalin
- Haupt's Adhesive
- Paraffin Wax
- Toluidine Blue
- Safranin
- Sodium nitroprusside (10mM)
- Phosphate buffered saline
- Griess Reagent
- 1% Potassium Ferricyanide
- 10% Trichloroacetic acid
- 0.1% Ferric Chloride
- Dimethyl sulphoxide
- Sulphuric acid (0.6M)
- Sodium Phosphate (28mM)
- Ammonium molybdate (4mM)
- 1,1 Diphenyl 2 picryl hydrazyl
- Tris. Hcl buffer pH 8, 16mM
- Nitro blue tetrazolium 0.3mM
- Nicotinamide Adenine Dinucleotide Reduced 0.936mM
- Phenazine Methosulphate 0.12mM
- DPX mounting media
- Tin

- Thionyl Chloride
- Magnesium turnings
- Concentrated hydrochloric acid
- 10% Sodium hydroxide
- Ammonia
- Chloroform
- Acetic anhydride
- Glacial acetic acid
- Concentrated Sulphuric acid
- Aqueous ammonia
- Sodium hydroxide
- Anthrone
- Fehling solution I and II
- Dragendorff's reagent
- Dilute hydrochloric acid
- Mayer's reagent
- Ferric Chloride
- Lead acetate
- Copper sulphate
- Ninhydrin's Reagent

PROCEDURE

4.4.1 Collection of Plant Material and Authentication

Trigonella foenum-graecum L. (seeds) were collected from a reputed organic store in Chennai. *Cinnamomum verum* J. Presl (bark) was collected from a reputed spice market in Coimbatore. *Carica papaya* L. CO.2 strain (leaves of male and female plant) and *Carica papaya* L. CO.2 strain (seeds) were collected from Tamil Nadu Agricultural University Coimbatore. The collected plant specimens were authenticated by Professor P. Jayaraman, Plant Anatomy Research Center, Chennai, Tamil Nadu, India.

4.4.2 Microscopic Characterization of the Plant Material

Fixation

The required samples were cut and removed from the collected leaves and seeds of selected plants. Fixation of the specimens was done using FAA (Formalin-5ml+ Acetic acid-5ml + 70% Ethyl alcohol-90ml).

Processing

- Following 24h of fixation dehydration of plant samples was carried out in different gradations of tertiary –Butyl alcohol according to the schedule given by Sass, (1940).
- The specimens were subjected to dehydration in different gradations of alcohol (30%, 50%, 70%, 80%, 90%, 100% alcohol) in each for 24h.

- The specimens were kept in safranin staining solution for 24 h.
- The specimens were kept in alcohol/xylene (1:1) for 24h.
- The specimens were cleared in two changes of xylene each for 24h.

Infiltration

- Infiltration of the specimens was carried out with three changes of paraffin wax each for 24h paraffin wax (melting point 58-60 C) so that TBA solution attained super saturation
- The specimens were embedded in paraffin wax.

Microtomy

Sections of 10-12µm were made from the paraffin embedded specimens was obtained using a Rotary Microtome.

Staining protocol

Deparaffinization

Dewaxing of the sections was by customary procedure of **Johansen, (1940).**

- The sections were kept on the slide warmer at 58˚C for 15 minutes.
- The tissue sections were deparaffinized by immersing the slides in fresh xylene for 5 minutes.

- The tissue sections were dehydrated by immersing the slides in different gradations of alcohol (70%, 80%, 90%, 100%) each for 30 seconds.

Toluidine blue staining

The sections were stained with **Toluidine blue** as per the method published by **O'Brien *et al*., (1964)**

The tissue sections were subjected to the following changes.

- Immersed in toluidine blue stain for 1 minute. (Toluidine blue was prepared by dissolving 0.25g benzoic acid, 0.29g sodium benzoate in 200ml distilled water to which 0.05% (50mg) Toluidine blue was added and shaken well).
- Immersed in distilled water for 30 seconds
- Immersed in two changes of 100% alcohol each for 1 second
- Immersed in alcohol/ xylene (1:1) solution for 1 second.
- Cleared in 2 changes of xylene each for 1 second.
- Air dried and mounted with DPX

Paradermal sections

To assess morphology of stomata, pattern of veins and distribution of trichome, paradermal sections (sections that taken parallel to the leaf surface) and clearing of leaf with 5% NaOH (sodium hydroxide) or peeling of epidermis by

partial maceration with Jeffrey's maceration fluid **(Sass, 1940)** were prepared. Mounting was done with glycerine to obtain temporary preparations were made.

Powder Microscopy

Powdered materials of the leaves were cleared using NaoH and mounted in glycerine medium after staining. Different components of the cell were studied and measured.

Photomicrographs

Photographs were taken with Nikon lab-photo 2 microscopic Unit at different magnifications and field of view. Bright field was used for normal observations. Polarized light was used to crystals, starch grains and lignified cells. These structures with bi-refringent property appear bright against dark background under polarized light. Scale-bars indicate magnifications of the figures. The microscopic features were analyzed and anatomical features were described. (**Easu, 1964; Easu, 1979; Gamble, 1935; Henry *et al*., 1987; Gamopetala, 1983; Metcalfe and Chalk, 1979; Wallis, 1985 and Yoga Narasimhan, 2000).**

4.4.3 Preparation of Herbal Extracts

- *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), fresh leaves of male and female plant, and seeds of *Carica papaya* L. (CO. 2 Strain) were washed, weighed and shade dried.

- Dried *Trigonella foenum-graecum* L. (seeds) were weighed and powdered using electronic mixer. Dried *Cinnamomum verum* J. Presl

(bark) were weighed, hand crushed, and ground using cutter miller and powdered using mortar and pestle. Dried leaves of male and female plant of *Carica papaya* L. (CO. 2 Strain) was weighed and crushed using a mortar and pestle. Dried seeds of *Carica papaya* L. (CO. 2 Strain) were weighed and crushed using mortar and pestle.

- Powdered form of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), leaves of male and female plant and seeds of *Carica papaya* L. (CO. 2 Strain) were weighed.

- The samples of *Trigonella foenum-graecum* L. (seeds), leaves of male and female plant and seeds of *Carica papaya* L. (CO. 2 Strain), were subjected to maceration with 60% ethanol and samples of *Cinnamomum verum* J. Presl (bark) were subjected to 70% ethanol for 72 h followed by 48 and 24 h respectively.

- The filtrates were collected in a round bottom flask at 72h, 48 h and 24 h respectively using Whatman filter paper number 1 and stored in refrigerator at 2-8ºC.

- The pooled extracts were concentrated using rotary flash and placed on a water bath at a temperature of $50^{o}C$ – $60^{o}C$ to evaporate the moisture content of the extract. The extracts were further dried using vacuum desiccator and stored in refrigerator at 2-8ºC.

4.4.4 Preliminary Phytochemical Analysis

Tests to assess the presence of phytochemicals were performed according to **Harborne, (1998)**

Test for Terpenoid Noller's Test: The test substance was taken in a test tube and warmed with tin and thionyl chloride. Formation of pink color indicates the presence of triterpenoids. However Libermann Burchard test has to be performed to assess the presence of bound terpenoid.

Test for Flavonoids: 10% Sodium hydroxide solution or ammonia was added to the test substance in alcohol. Formation of dark yellow color indicates the presence of flavonoids.

Test for Steroids and Bound Terpenoids: Libermann Burchard Test: 3ml of acetic anhydride was added to the test substance and dissolved. Following this 3ml glacial acetic acid was added. Warming of reaction mixture was done followed by cooling under running tap water. Following this concentrated sulfuric acid was added drop by drop along the walls of the test tubes. Bluish green color indicates presence of steroids and formation of brown layer indicates the presence of bound terpenoids.

Test for Glycosides: The test substance was taken in a watch glass and mixed with a small quantity of anthrone. To this a drop of concentrated sulphuric acid was added and a paste of the reaction components was made. The reaction mixture was warmed gently on a water bath. Formation of dark green color indicates the presence of glycosides.

Test for Carbohydrates: To the test substance Fehling solution I and II was added and warmed. Formation of blue, green or red color indicates the presence of carbohydrates.

Test for Alkaloids: To the test substance addition of a few drops of acetic acid was done, following which Dragendorff's reagent was added and shaken well. Orange red precipitate formation indicates the presence of alkaloids.

Test for Polyphenols: Test substance was taken in a test tube to which few drops of alcohol and ferric chloride solution was added. Formation of bluish green or red color indicates the presence of phenols.

Test for Tannins: The test substance was taken in a test tube to which basic lead acetate solution was added and mixed. White precipitate formation indicates the presence of tannins.

Test for Saponins: The test substance was taken in a test tube to which water was added and shaken. Formation of copious lather indicates the presence of saponins.

Test for Amino acids: The test substance was taken in a test tube to which ninhydrin reagent was added and warmed. Appearance of purple color indicates the presence of free amino acids.

4.5 *IN-VITRO* ANTIOXIDANT ACTIVITY

Antioxidant activity of various concentration of extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), leaves of male and female plant, and seeds of CO. 2 Strain *Carica papaya* L. ranging from 10µg/ml to 10000µg/ml were assessed by determination of Total Antioxidant Activity, Reducing Power, Nitric oxide scavenging activity assay, 1,1 di phenyl 2 picryl hydrazyl (DPPH) scavenging assay, Superoxide radical scavenging activity assay.

Reagent Preparation

Phosphate buffered Saline

Phosphate buffered saline was prepared by dissolving 2.5g of sodium dihydrogen phosphate, 2.525g of disodium hydrogen phosphate, 8.2g of sodium chloride in 1000ml of distilled water.

Reagent for total antioxidant activity assay

The reagent was prepared by dissolving 0.6M sulphuric acid, 28mM sodium phosphate and 4mM Ammonium molybdate in 1000ml of distilled water.

Griess Reagent

Griess Reagent was prepared by dissolving 1% sulphanilamide, 2% phosphoric acid and 0.1% Naphthyl ethylene diaminedihydrochloride in 1000ml of distilled water.

Tris. Hcl Buffer

252.16mg of Tris. Hcl was dissolved in 100 ml of water and pH was adjusted to 8 by addition of dilute Hcl and dilute NaOH for the preparation of 16mM of Tris. Hcl.

Preparation of stock solution of extract for Antioxidant Assays

10mg of the extract was dissolved in 1 ml of dimethyl sulphoxide and 9ml of water and shaken well. The serial dilutions of the extracts (1000µg, 800µg. 400µg, 200µg, 100µg, 50µg, 10µg) was prepared by dissolving the extracts in distilled water.

4.5.1 Total antioxidant activity Assay (Prieto *et al*., 1999)

Principle

This assay is based on the principle of reduction of molybdenum by the test substance and formation of a green phosphate molybdenum complex at acidic pH.

Procedure

0.2ml of the test substance at various concentrations was taken in a test tube to which 2ml of the reagent (0.6M sulfuric acid, 28mM sodium phosphate, 4mM Ammonium molybdate in 1000ml of water) was added and incubation was done for a duration of 90 minutes at 95ºC. Quercetin was used as standard and

distilled water was used as reagent blank. The readings were recorded at 695nm using UV spectrophotometer.

4.5.2 Reducing power Assay (Oyaizu, 1986)

Principle

This assay is based on the principle of colored complex formed by the sample with Potassium ferricyanide, Trichloroacetic acid, Ferric Chloride.

Procedure

1 ml of different concentrations of the extracts ranging from 1000µg/ml to 10 µg/ml was taken in labeled test tubes to which 2.5ml Phosphate buffer (0.2M, pH 6.6) and 2.5ml of 1% Potassium ferricyanide were added. The reaction mixture was incubated at 50ºC for 20 minutes. Following this 2.5ml of 10% Tricarboxyllic acid was added and centrifuged at 3000rpm for 10 minutes. 2.5ml of the supernatant was taken in separate labeled test tubes to which 2.5ml of distilled water and 0.1% Ferric Chloride was added. Absorbance was taken at 700nm. Distilled water was used as reagent blank and quercetin was used as standard.

4.5.3 DPPH radical scavenging assay (Yokozawa *et al*., 1998)

Principle

This assay is based on the reduction of absorbance of methanol solution of DPPH by the free radical scavenger. The antioxidant activity (free radical

scavenging activity) of the extracts on the stable radical 2,2-diphenyl-1-picryl hydrazyl (DPPH) was determined by the method of *Yohozowa et al.*

Procedure

0.1 ml of the test substance at various concentrations were taken in labeled test tubes to which 1.9ml of 200µM of DPPH in ethanol was added. Following this incubation was done in the dark for 20 minutes. Readings were recorded at 517nm using UV spectrophotometer using ethanol as reagent blank. 0.1ml of dimethylsulfoxide (DMSO) in distilled water (1:9) and 1.9ml of 200µM of DPPH in ethanol was used as control. Ascorbic acid was used as standard. Calculation of percentage inhibition was done using the formula:

$$\{1 - (AB_{sample} / ABS_{control})\} \text{ X } 100.$$

4.5.4 Nitric oxide scavenging activity Assay (Alderton *et al*., 2001)

Principle

Aqueous solution of sodium nitro prusside spontaneously generates nitric oxide (NO) at a physiological pH, which interacts with oxygen to produce nitrate ions. The principle behind this method is the inhibition of nitric oxide radical generated from sodium nitro prusside in buffered saline and measured by Griess reagent.

Procedure

1 ml of the test substance at various concentrations was taken in labeled test tubes to which 2ml of 10mM of Sodium Nitro prusside in Phosphate Buffered Saline was added to and mixed well using vortex. Incubated was done for 4h at 37℃. Following this 0.5ml of Griess Reagent was added and the absorbance was recorded at 546nm using ascorbic acid as standard and dimethylsulfoxide in distilled water (1:9) as control. Percentage inhibition was calculated using the formula:

$$\{1 - (AB_{sample} / ABS_{control})\} X 100$$

4.5.5 Superoxide radical scavenging activity assay (Robak and Gryglewski, 1988)

Principle

This assay is based on the principle of reduction of superoxide radicals generated by Nitrobluetetrazolium, Nicotinamide Adenine Dinucleotide Reduced (NADH), Phenazine Methosulphate (PMS) in Tris Hydrochloride buffer.

Procedure

1ml of the test substance at various concentrations were taken in labeled test tubes to which 0.5ml of 16mM Tris. Hcl buffer (pH 8), 0.5ml of 0.3mM Nitrobluetetrazolium, and 0.5ml of 0.936mM of Nicotinamide Adenine Dinucleotide Reduced, 0.5ml of 0.12mM Phenazine Methosulphate were added and mixed well with in a vortex. Incubation was done in dark for 5 minutes and absorbance at 560 nm was recorded using distilled water as blank. Quercetin was used as standard and

dimethylsulfoxide in distilled water (1:9) was used as control. Percentage inhibition was calculated using the formula:

$$\{1 - (AB_{sample} / ABS_{control})\} X 100$$

Statistical analysis

All the experiments were performed in triplicates. Mean and standard deviation were calculated. The results of each antioxidant assay were subjected to statistical analysis using SPSS software version 16. Kruskall Wallis test was used to analyze variations in antioxidant activities between the different herbal extracts. Mann Whitney test was used to analyze the differences in activities of each extract at different concentrations.

4.6 *IN-VITRO* ANTIMICROBIAL ACTIVITY

Reagents required

Cryovial of microorganisms MTCC 890, fungi MTCC 227, Muller Hinton Broth, Potato Dextrose Broth culture dish, incubator, chemicals, glassware, micro-tips, micro pipettes, standard fluconazole and kanamycin

4.6.1 *In-vitro* Antimycotic Activity against fluconazole resistant *Candida albicans*

Preparation of *Candia albicans* Culture

- *Candida albicans* MTTC 227 was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh.

- Thawing of the ampoule was done using water bath at 25°C for 2 minutes. 70% ethanol was used wipe the ampoule. The content was transferred to potato dextrose agar and incubated at 28°C.

Induction of drug resistance

Induction of drug resistance according to the standard protocol of **Yan *et al.*, (2008)** with some modifications.

- *Candida albicans* culture was transferred into potato dextrose broth. Incubation was done overnight at 30°C in an orbit shaker at 200 rpm (revolutions per minute).

- Treatment with fluconazole at two times greater than the concentration of recently measured minimum inhibitory concentration was performed to an aliquot of 10^6 cells. Incubation was done at 30ºC at in orbit shaker at 200 rpm.

- The cultures were incubated until they attained a density of 10^8 cells from which an aliquot containing 10^6 cells was taken and repetition of the procedure was done.

- In order to confirm fluconazole resistance antibiotic sensitivity test for the last passage was performed and the strain was used for the study.

- Colonies of fluconazole resistant strain of *C. albicans* were inoculated in potato dextrose broth and incubation was done at 37ºC for 24 h in an orbit shaker at 200 rpm.

- Standard inoculum of the microorganism of 1.5×10^6 colony forming units (CFU/ ml) was diluted to 1:100. Adjustment of turbidity to match McFarland standard was done.

Antimycotic activity

The antimycotic activity of the extracts were assessed by agar well diffusion method according to the protocol of **Perez *et al*., (1990)**

- *Candida albicans* was swabbed onto potato dextrose agar using sterile swab sticks.

- Sterile cork borer was used to cut 9mm diameter wells. The cultures were treated with hydro alcoholic extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J.Presl (bark), *Carica papaya* CO.2 strain (leaves of male and female plant and seeds) at different concentrations 250, 500, 1000 µg/ml using sterile microtips and incubation was done at 37ºC for 24 h.

- The zone of clearance was measured.

- Three independent experiments were performed in triplicates and calculation of mean and standard deviation was done.

Minimum Inhibitory Concentration (MIC)

Extracts that exhibited significant zones of inhibition were selected for the assay. MIC was determined according to the protocol of **Wariso and Ebong, (1996)** with slight modifications**.**

- Sterile Potato Dextrose broth was prepared. 100µl of the prepared broth was dispensed into the labeled tubes (numbered 1-12) with sterile micropipette.
- A stock solution containing 10mg/ml of the extract was prepared. Then 100 µl of the solution was dispensed into the tube numbered 1.
- Subsequently, from tube 1, serial dilution was carried out upto tube 10. To make a final concentration of 500 µg/ml 250 µg/ml; 125 µg/ml; 62.5 µg/ml; 31.25 µg/ml; 15.625 µg/ml; 7.812 µg/ml; 3.90 µg/ml; 1.953 µg/ml and 0.976 µg/ml. Well 11, and 12 were used as organism control.
- An overnight culture of *C. albicans* isolates was prepared in sterile nutrient broth. 100 µl of the inoculum was transferred into each tube from well 1 to 12.
- Incubation was done at 37°C for 24h and examined for growth. Subsequently, to each tube 20 µl of Blue tetrazolium (5 mg/ml) was added and incubated for 30 min. Following incubation, formation of blue color indicates viable organisms and presence of yellow color indicates complete inhibition of organisms.

The antimycotic activity was classified based on MIC as follows:

<100 µg/ml – good

100-500 µg/ml - moderate

500-1000 µg/ml- weak

>1000 µg/ml- inactive

4.6.2 *In-vitro* Antimicrobial Activity against *Streptococcus mutans*

Preparation of *Streptococcus mutans*

- *Streptococcus mutans* MTTC 890 was procured from Microbial Type Culture Collection and Gene Bank, Chandigarh.

- Thawing of the ampoule was done using water bath at 25°C for 2 minutes and 70% ethanol was used to wipe the ampoule. The content was transferred to Muller Hinton broth and incubation was done at 28°C.

- The colonies of the strains were inoculated into Muller Hinton broth. Incubation was done at 37ºC for 24 h in orbit shaker at 200 rpm.

- Standard inoculum 1.5×10^6 colony forming units (CFU/ml) of *Streptococcus mutans* was diluted to 1:100. The turbidity was adjusted to match McFarland standard (0.5 ml of 1.175% w/v (0.048 M) $BaCl_2.H_2O$ and 99.5 ml of 1% w/v (0.36 M) sulphuric acid)

Antimicrobial activity: Agar well diffusion method

Procedure

The anti-microbial activity of the extracts were assessed by agar well diffusion method according to the protocol of **Perez *et al*., (1990)**

- The stock solution was prepared by dissolving 10 mg of the extract in 2 ml of dimethylsulfoxide (DMSO). From the stock solution 50 µl, 100 µl, 200 µl were used for the study to obtain a final concentration of 250, 500 and 1000 µg/ml.
- *Streptococcus mutans* was swabbed onto potato dextrose agar with sterile swab sticks.
- Sterile cork borer was used to cut 9mm diameter wells.
- The bacterial culture was treated with hydro alcoholic extracts of *T. foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO.2 strain (leaves of male and female plant and seeds) at different concentrations 250, 500, 1000 µg/ml and 30 µg/ml kanamycin using sterile microtips and incubation at 37ºC for 24 h was done.
- The zone of clearance was measured.
- Three independent experiments were performed in triplicates and mean and standard deviation was calculated.

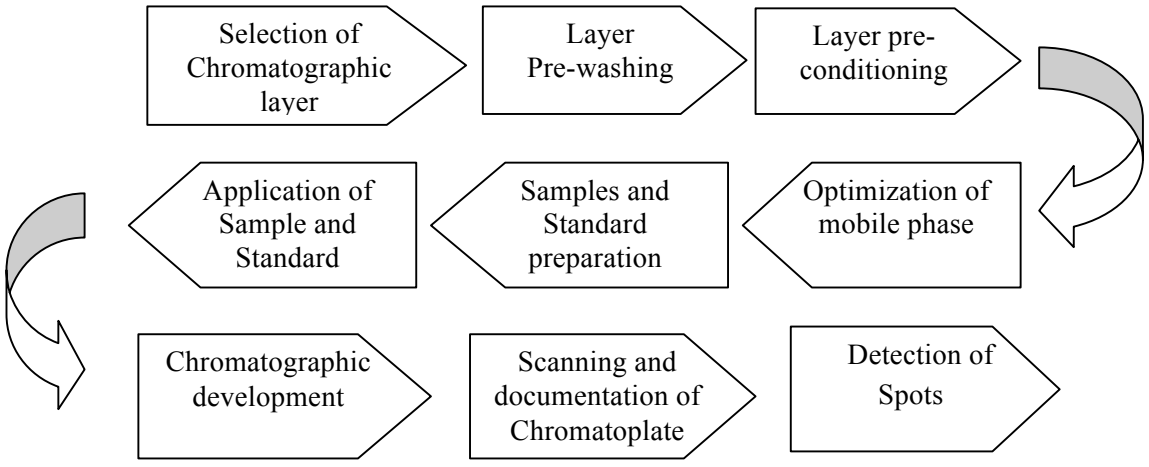
4.7 QUANTIFICATION OF ACTIVE INGREDIENTS AND SELECTION OF THE EXTRACTS BY HIGH PERFORMANCE THIN LAYER CHROMATOGRAPHY

4.7.1 Introduction

High performance thin layer chromatography (HPTLC) is a modern, sophisticated, analytical and instrumental technique with an excellent potential of optimization, automation and multidimensional applications. It is a powerful tool to determine chromatographic information of complex mixtures of organic, inorganic and biomolecules.

Modern HPTLC technique is sensitive and suitable for both qualitative and quantitative analysis due to the combination of automated sample application and densitometric scanning. The chromatographic fingerprints obtained from the instrument can be visualized and stored as electronic images. Advantages of HPTLC include low cost per analysis, high sample throughput, standards and multiple samples can be separated simultaneously and minimal sample preparation required as the stationary phase is disposable.

Schematic representation for HPTLC method development



The extent of separation of various components in a formulation is largely dependent on various factors that are also important for ideal quantitative analysis

Type of stationary phase, type of pre-coated plates, layer thickness, binder in the layer, mobile phase, solvent purity, size of the developing chamber, saturation of chamber, samples volume to be spotted, size of the initial spot, solvent level in the chamber, gradient, relative humidity, temperature, flow-rate of solvent, separation distance, mode of development.

Theoretical Consideration

- Separation Efficiency
- Partition coefficient
- Retention/ Retardation factor
- Capacity Factor
- Spot Capacity
- Plate Height
- Resolution
- Selectivity

4.7.2 Armamentarium

a. Reagents required

Ethanol, Methanol, Propanol, Toluene Ethyl acetate, Formic acid, trigonelline hydrochloride, cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, benzyl isothiocyanate aqueous, ethanol and ethanol water extracts.

b. Equipment required

HPTLC Applicator: CAMAG LINOMAT IV Automatic sample spotter; CAMAG (Muttenz Switzerland)

Syringe: 100ml Hamilton (Bonadug, Switzerland)

TLC chamber: AMD II

Densitometer: TLC scanner II with Wincats 4 software, CAMAG

Sample amplification: CAMAG Automatic TLC scanner 2 (ATS4)

TLC silica gel 60F254, Merck

c. Experiment

- Quantification of trigonelline hydrochloride in aqueous, ethanol and hydroalcoholic (60 ethanol: 40 water; v/v) extracts of *Trigonella foenum-graecum* L. (seeds) by HPTLC **(Scott *et al*., 2013 and Chopra *et al.,* 2006)**
- Quantification of cinnamaldehyde, 4 hydroxy cinnamic acid and eugenol in aqueous, ethanol and hydroalcoholic extracts (70 ethanol: 30 water; v/v) of *Cinnamomum verum* J. Presl (bark) by HPTLC **(Scott *et al*., 2013; Charde *et al*., 2014 and Gopu *et al*., 2008)**
- 5.5.4 Quantification of benzyl isothiocyanate in aqueous, ethanol and hydroalcoholic (60 ethanol: 40 water; v/v) extracts of *Carica papaya* L. (seeds) by HPTLC **(Scott *et al*., 2013 and Sofrata *et al.,* 2011)**

4.7.3 HPTLC Profile

CHROMATOGRAPHIC CONDITIONS			
Sample	*Trigonella foenum-graecum* L. (seeds)	*Cinamomum verum* J. Presl (bark)	*Carica papaya* L. (seeds)
Standard	Trigonelline hydrochloride	Cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol	Benzyl isothiocyanate
Standard prepared in	Methanol	Methanol	Methanol
Stationary phase	TLC silica gel $60F_{254,}$ Merck	TLC silica gel $60F_{254,}$ Merck	TLC silica gel $60F_{254,}$ Merck
Mobile phase	Propanol:water:Ethyl acetate: Methanol(3:3:2:1)	Toluene: ethylacetate:hexane:formic acid::2:2:5.5:0.5	Toluene: ethylacetate: acetic acid::5:4:1
Scanning wavelength	Deuterium (254nm)	Deuterium (254nm)	Deuterium (254nm)
Sample concentration	Track 1: Trigonelline hydrochloride (6.1mg in 1ml methanol) Track 2 Ethanol extract (8.9mg in 1 ml methanol), Track 3 hydroalcoholic extracts (60:40) (10.8mg in 0.9ml methanol and 0.1ml water) Track 4 Aqueous extract of Trigonella *foenum-graecum* L. (seeds) (9.7mg in 0.9ml methanol and 0.1ml water)	Track 1: cinnamaldehyde (12.5mg in 1 ml chloroform) Track 2: 4 hydroxy cinnamic acid (9.6mg in 1ml methanol) Track 3 Eugenol (11.5mg in 1ml chloroform) Track 4 Ethanol extract (10.2mg in 9.5ml ethanol and 0.5ml water) Track 5 Hydroalcoholic extract (70:30) (10.5mg in 0.5ml ethanol and 0.5ml water) Track 6 Aqueous Extract of *Cinnamomum verum J.* Presl (bark) (10.3mg in 0.5ml water and 0.5ml ethanol)	Track A Benzyl isothiocyanate (11.8mg in 1ml dimethylsulfoxide) Track B Ethanol extract (9.6mg in 1ml ethanol) Track C hydroalcoholic extracts (60:40) (7.7mg in 1ml dimethylsulfoxide) Track D Aqueous extract of Carica *papaya* L. (seeds) (8.7mg in 1ml water)
Applied volume	10µl	10µl	10µl
Development mode	Ascending	Ascending	Ascending

4.7.4 Procedure

- Quantification of the respective active compounds using the photo-densitometric HPTLC method was developed.
- 10µl of the samples and standard were spotted on HPTLC plates pre-coated with Si-gel Si60F254 (E. Merck) of band length 5 mm,
- Chromatograms were developed using respective mobile phase.
- The plate was dried and scanned at 254nm.

Calculations

- Standard purity:

  % of purity = Area of standard / Total area

- Sample percentage:

  Sample area / Standard Area X Standard Weight / Sample Weight X Sample dilution/ Standard dilution X % of standard purity.

4.8 ISOLATION AND QUANTIFICATION OF ALKALOID FRACTION

4.8.1 Isolation of Alkaloid Fraction by Acid base Precipitation Method

Reagents Required

Carica papaya L. CO. 2 strain (leaves of male and female plant), methanol, chloroform, hydrochloric acid, ammonia, Dragendorff's reagent.

Instruments Required

Rotary flash, glassware, separator, vaccum desiccator

Reagent preparation

Dragendorff Reagent was prepared by mixing 0.8 g bismuth nitrate pentahydrate in 40 ml distilled water and 10 ml glacial acetic acid with 8.0 g potassium iodide in 20 ml distilled water.

Procedure

1. Collected plant material (*Carica papaya* L. *Carica papaya* L. CO. 2 strain leaves of male and female plant) was weighed, impurities were removed and further weighing was done.
2. The plant material was shade dried and weighed.
3. The plant material was crushed with mortar and pestle and maceration was done with methanol for 72h, 48h and 24h and pooled extracts were collected.
4. The pooled extracts were concentrated using rotary flash and dried with vaccum desiccator.
5. The extract obtained was dissolved with minimum quantity of methanol and the pH was adjusted to 2 by adding Hcl.
6. The extract obtained was macerated with chloroform twice and the pooled extract was placed in a separator.

7. The chloroform non-alkaloid layer and the aqueous alkaloid layer were collected separately.
8. pH of the obtained alkaloid fraction was adjusted to 8 by addition of ammonia.
9. The extract was macerated with chloroform twice and pooled extract was obtained.
10. The pooled extract was concentrated with rotary flash and dried with vaccum desiccator.
11. Presence of alkaloids was confirmed by using Dragendorff's reagent.

4.8.2 Estimation of Alkaloids (Narasimhan *et al*., 2003)

Reagent preparation

(a) Dragendorff Reagent was prepared by mixing 0.8 g bismuth nitrate pentahydrate in 40 ml distilled water and 10 ml glacial acetic acid with 8.0 g potassium iodide in 20 ml distilled water.

(b) Standard bismuth nitrate solution: Bismuth nitrate stock solution was made by dissolving 10 mg Bi (NO3) $3{\cdot}5H_2O$ in 5 ml concentrated nitric acid. The volume was made up to 100 ml with distilled water.

(c) Thiourea, 3% was prepared by dissolving 3 g in 100ml distilled water.

(d) Disodium sulfide, 1% was prepared by dissolving 1 g in 100ml distilled water.

Procedure for Calibration Curve

The calibration curve was obtained with bismuth nitrate pentahydrate stock solution. Serial dilutions of the stock solution was made by pipetting out 1, 2, 3, 4, 5, 6, 7, 8, and 9 ml stock solution into separate 10ml standard flasks and the volume was made up to 10ml with distilled water. 1 ml of each solution was taken separately and 5 ml thiourea solution was added. The absorbance value of the yellow solution was measured at 435 nm against colorless reagent blank.

Procedure for Assay of Alkaloids and Plant Extracts

5 ml of the extract was taken and maintenance of pH at 2–2.5 was done with dilute Hcl, to which 2 ml of Dragendorff Reagent was added. The precipitate formed was centrifuged following which the supernatant was removed. The precipitate was washed with alcohol. The residue was then treated with 2 ml disodium sulfide solution and the filtrate was discarded. The brownish black precipitate formed was then centrifuged. Completion of precipitation was checked by addition of 2 drops of disodium sulfide. The residue was dissolved in 2 ml concentrated nitric acid, with warming if necessary following which dilution to 10ml in a standard flask was done using distilled water. To 1 ml of the solution taken in a test tube 5 ml thiourea solution was added. The absorbance was measured at 435 nm.

Calculation of result

The absorbance of the unknown solution (Au) is then used with the slope and intercept from the calibration curve to calculate the concentration of the unknown solution, (Cu).

Cu = Au – intercept / slope

Concentration of the unknown directly correlates to the concentration of the Alkaloid present since only the total alkaloids precipitate with Dragondorff reagent.

4.9 ASSESSMENT OF *IN-VITRO* ANTICANCER ACTIVITY

4.9.1 SCC 25 ATCC CRL 1628 cell line maintenance

Reagents required

Product	Catalogue number	Stock solution	Dilution for 50ml	Dilution for 100ml	Dilution for 500ml
Dulbecco's Modified Eagle Medium and Hams F12 nutrient mixture	High Media AL140s	1x	39ml	80 ml	400ml
2.5nM Gluta XL	High Media TCL030	100x	0.5ml	1ml	5ml
15% Fetal Bovine serum	**1**0270-106	1x	7.5ml	15ml	75ml
Antibiotic P/S/A	High Media A002	100x	0.5ml	1ml	5ml
1000ng Hydrocortisone	High Media TC344	1µg/ml	50µl	To be added just before adding to media as it degrades	
Essential amino acids	ACL002	100x	0.5ml	1ml	5 ml
Non essential amino acids	ACL006	100x	0.5ml	1ml	5 ml
MEM vitamin	VA002	100x	0.5ml	1ml	5 ml

Procedure

1. SCC 25 ATCC CRL 1628 were removed from liquid nitrogen, rubbed with palm for about thirty seconds, washed with media Dulbecco's modified eagle medium F12 1:1 containing 1.2g/L sodium bicarbonate, 2.5mM sodium pyruvate, 15mM HEPES and

supplemented with 400ng/ml hydrocortisone and 10% Fetal bovine serum) and suspended in 50 ml tubes containing 20ml of medium. Cells were re-suspended in 10 ml centrifuge tubes and were centrifuged at setting 3 for 4 minutes. Supernatant was discarded and remaining 10ml of medium containing cells was re suspended in 1ml centrifuge tube and the procedure was repeated. Clear supernatant was discarded and pellet of cells were washed with fresh media and transferred to T 75 flask (15ml medium). The cells were viewed under microscope and incubated at 37 degree and 5% CO2.

2. Medium was changed after 4h, 18 h, 36 h, and 96 h.
3. Since many cells had vacuoles the culture medium was supplemented with 15% fetal bovine serum and 1000ng/ml hydrocortisone, essential amino acids non-essential amino acids and vitamin solution.

Passaging

All the reagents were brought to 37 ºC before use.

1. The T25 flask containing monolayer of cells was rinsed twice with 1x PBS.
2. Sufficient amount of Trypsin EDTA (2 ml for T25 flask) solution was added to cover the monolayer and incubated at 37 ºC in the CO_2 incubator for 10 minutes.

3. The flask was gently shaken to allow the detachment of the cells from the surface, 5ml of DMEM F12 1:1 containing 15% FBS was added to the flask to neutralize the action of Trypsin EDTA.
4. Repetitive pipetting was carried out to breakdown the cell clumps so as to permit complete revival of detached cells.
5. The medium containing detached cells was centrifuged at 100rpm for 5 minutes.
6. The supernatant was discarded and the pellet was suspended with fresh complete growth medium.

Trypan blue assay

To 20µl of the medium containing cells, 20µl of trypan blue was added and mixed well and loaded on to a haemocytometer. Cells in the four peripheral squares of the haemocytometer (Neubauer chamber) were counted and total cell count was calculated.

Interpretation: Cells that have a break in their cell membrane take up the blue dye and are considered non -viable cells. Cells that do not take up the blue dye have their cell membrane intact and are considered viable.

Formula applied **(Abcam.com, n.d.)**

$$\frac{\text{Number of cells}}{4} \text{ X dilution factor x } 10^{4-x} \text{ amount of fluid in centrifuge tube}$$

Total number of cells was calculated; medium was added according to the cell population needed. Required amount of medium containing the required number of cells (0.5 - 1.0 x 10^5 cells/ml) were transferred into 15ml falcon tube. Based on the cell count, the volume was made up with 5ml of growth medium for T25 flask. The flasks were incubated at 37ºC and the cells were checked periodically for changes in cellular morphology and contamination. On formation of monolayer, the cells were further utilized for the assays.

4.9.2 Cytokeratin 8 Stain Staining Protocol

Materials required & preparation

1. 10X Phosphate Buffer Saline (PH 7.6)

   Salts

   Potassium Chloride (KCl; MW: 74.56): 2g

   Sodium Chloride (Nacl; MW: 58.44 : 80g

   Potassium Dihydrogen Orthophosphate (KH_2PO_4; MW: 136.09): 2g

   Sodium dihydrogen Orthophosphate (NaH_2PO_4; MW: 156.01): 14.4g

   Milli Q – 80ml

2. 3.7% Formaldehyde or Methanol

   Working solution: 9 ml PBS was added to 1ml formaldehyde; it was freshly prepared in a fume hood and brought down to 1X concentration. Methanol was used for fixation at – 20 ºC.

3. Blocking solution was prepared and stored at -20° C

3% Goat serum

1% BSA in PBS

16.3 M glycine

4. Dako N1560 cytokeratin 8 antimouse (ready to use monoclonal)

5. Goat antimouse FITC conjugated FTC3 (Lot no 031030) dilution 1:50 in blocking buffer.

Working solution: 10µl of secondary antibody was added to 490µl of blocking buffer.

6. DAPI was used as counterstain

Stock solution: 5mg/ml

Working solution: 1µg/ml (4µl of DAPI was added to 20 ml PBS)

7. DABCO was used as mounting media (1µg/ml)

- 1.5ml of PBS 10x was diluted with 13.5ml of ultra pure water to make 1x PBS
- 1.225g DABCO was dissolved in 15ml 1xPBS and pH was adjusted to 7.4
- To this 3.5ml 10xPBS was added
- The volume was made up to to 50ml glycerol aliquot and stored at -20^{o} C

Procedure

1. 1x 10^4 cells suspended in complete growth media (Dulbecco's modified eagle medium with Ham's F12 1:1 containing 1.2g/L sodium bicarbonate, 2.5mM sodium pyruvate, 15mM HEPES and supplemented with 1000ng/ml hydrocortisone and 15% Fetal bovine serum supplemented with essential amino acids, non essential amino acids and vitamin were seeded onto a coverslip placed in 12 well plates and allowed to attain 40% - 50% confluency.

2. Cells were observed every 24h; on attaining the required confluency, the growth media was discarded and the cells on the coverslip were washed thrice in 1X PBS at room temperature.

3. Two samples were fixed with absolute ice-cold absolute methanol and two were fixed with ice-cold absolute methanol followed by 3.7% formalin.

4. The samples were washed twice with Phosphate buffered saline and incubated with 200µl blocking buffer (3% goat serum in PBS, 1% BSA in PBS) for one hour.

5. The samples were washed twice with Phosphate buffered saline.

6. One methanol fixed and one formalin fixed sample was incubated with primary antibody (Dako N1560 cytokeratin 8 antimouse ready to use monoclonal) overnight. Control samples both formalin and

methanol-fixed were incubated with negative control (Dako N1560 cytokeratin 8) overnight.

7. The samples were washed thrice with phosphate buffered saline and incubated with 200**µl** secondary antibody (goat antimouse FITC conjugated FTC3 Lot no 031030 dilution 1:50 in blocking buffer) for 1 h.

8. The samples were washed thrice with Phosphate buffered saline and incubated with 300µl DAPI (stock 5mg/ml working concentration 1µg/ml →300nM or 1 µl of stock in 50 microlitre Phosphate buffered saline and stored at 4 degree) for 30 minutes.

9. The samples were washed with Phosphate buffered saline and distilled water.

10. The samples were mounted on glass slides cleaned with methanol using DABCO Sigma 2522 mountant (Glycerol Merck No. 104095 and Phosphate buffered saline). 1 drop of the working solution was placed over the coverslip, onto which a small circular coverslip was placed.

11. The circular coverslip was sealed by applying nail polish varnish on the rim.

12. The samples were viewed under fluorescent microscope (exposure time 10 seconds).

4.9.3 Cytotoxicity Screening

Determination of Mitochondrial Synthesis by Micro culture Tetrazolium MTT Assay (Mosmann, 1983 and Carmichael *et al*., 1988)

The principle behind most of the cytotoxicity assays is the ability of the cells to survive when exposed to a toxic insult. The present assay is based on the principle that dead cells or their products cannot reduce tetrazolium. Live cells cleave MTT leading to the formation of blue formazan derivative. The assay is dependent on the total number of cells present as well as the mitochondrial activity per cell.

Principle

The principle of this assay is the cleavage of tetrazolium salt 3-(4, 5 dimethyl thiazole-2 yl) -2, 5-diphenyl tetrazolium bromide (MTT) into a blue colored product (formazan) by the enzyme mitochondrial enzyme succinate dehydrogenase present in live cells.

Procedure

1. The monolayer culture of SCC25 cells was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using medium containing 15% FBS.
2. 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added to each well of the 96 well microtitre plate.

3. On completion of 24 h, when a partial monolayer was formed, the supernatant was discarded, monolayer was washed once with 1X PBS; 100µl of different drug concentrations of (hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO.2 strain (seeds) and alkaloid fraction of CO.2 strain *Carica papaya* L. (leaves of male and female plant); their respective active compounds trigonelline hydrochloride, cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, benzyl isothiocyanate and standard cisplatin in serum free media were added to the cells in microtitre plates. The plates were then incubated at 37 ºC for 48 h in 5% CO2 atmosphere; microscopic examination was carried out and observations recorded every 24 h.

4. On completion of 48h, the drug solutions in the wells were discarded and 20 µl of MTT was added to each well. (MTT solution was prepared in 5 mg/ml of PBS just before use and filtered through a 0.22-µm filter).

5. The plates were gently shaken and incubation was done for 4 h at 37 ºC in 5% CO2 atmosphere.

6. The supernatant was discarded; 100 µl of DMSO was added and the plates were incubated for 1 h at 37 ºC in 5% CO2 atmosphere to facilitate solubilization of the formed formazan crystals.

7. The absorbance was measured using a micro plate reader at 590nm.

8. The percentage growth inhibition was calculated using the formula below

% Growth Inhibition = 100 - **(**Mean OD of Individual Test Group/Mean OD of Control Group) x100

9. The experiment was performed in triplicates. IC 50 was calculated by non-linear regression of the concentration- response using Graph Pad prism 5.0 software.

4.9.4 Acridine Orange and Ethidium Bromide Staining (Kasibhatla *et al*., 2006)

Principle

Assessment of apoptosis is an integral part to estimate anticancer effects. Acridine orange, a cell-permeative dye, intercalates into double-strand DNA, and emits green fluorescence thereby aiding visualization of the nuclear chromatin pattern. Ethidium bromide stains DNA orange but does not stain live cells. This dual staining by AO/EB was used to visualize (i) live cells (uniformly green stain), (ii) cells in early apoptosis (yellow stain), (iii) cells in late apoptosis (orange nuclei with condensed or fragmented chromatin or reddish stain)

Reagents required:

1. 10X Phosphate Buffer Saline (PH 7.6)

   Salts

   Potassium Chloride (KCl; MW: 74.56): 2g

   Sodium Chloride (Nacl; MW: 58.44 : 80g

Potassium Dihydrogen Orthophosphate (KH_2PO_4; MW: 136.09) : 2g

Sodium dihydrogen Orthophosphate (NaH_2PO_4; MW: 156.01): 14.4g

Milli Q – 80ml

2. Acridine orange and Ethidium bromide staining solution

   100 µg/ml Acridine orange and 100 µg/ml Ethidium bromide in Phosphate buffered saline

Procedure

1. The monolayer culture of SCC25 cells was trypsinized and the cell count was adjusted to $1.0x10^5$ cells/ml using medium containing 15% FBS.

2. 0.1ml of the diluted cell suspension (approximately 10,000 cells) was added to each well of the 96 well microtitre plate.

3. On completion of 24 h, when a partial monolayer was formed, the supernatant was discarded, monolayer was washed once with 1X PBS; 100 µl of IC_{50} and half of IC_{50} concentrations of (hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO.2 strain (seeds) and alkaloid fraction of CO.2 strain *Carica papaya* L. (leaves of male and female plant); their respective active compounds trigonelline, cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, benzyl isothiocyanate and standard cisplatin in serum free media) were added to the cells in microtitre plates. Incubation of the plates

was done at 37 ºC for 48 h in 5% CO2 atmosphere. The cells were observed using a microscope every 24 h.

4. On completion of treatment period the spent media was collected in separate tubes and the cells were washed with 1xPBS and the detached cells were also pooled.

5. Following this 20 µl of Trypsin was added and incubated at 37 ºC and 5 % CO_2 for 10 minutes.

6. The reaction was ceased by adding complete media containing Fetal Bovine serum and the detached cells were pooled and centrifuged at 1000rpm for 5 minutes.

7. The cells were re-suspended in 25 µl of Phosphate buffered saline.

8. To this 1ml of acridine orange and Ethidium bromide staining solution was added and mixed gently before analysis.

9. 10 µl of the stained cells were placed on a clean microscope slide and covered with glass coverslip.

10. The cells were viewed under a fluorescent microscope using excitation filter of 480/30nm.

4.9.5 DNA Fragmentation Assay (Kotamraju *et al*., 2000)

Principle

Apoptosis or programmed cell death is characterized by several morphological changes that include chromatin condensation, loss of cell volume, membrane blebbing. The important biochemical hallmark of apoptosis is

endogenous Ca^{++} Mg^{++} dependent endonucleases that cause fragmentation of DNA at intranucleosomal sites. It results in ologonucleosomal sized (180-200bp) fragments (Wyille, 1980). DNA fragmentation can be visualized by gel electrophoresis using 200bp as control that can be used to detect apoptosis.

Reagents Required

Solution	***Preparation***	***Storage***
Tris EDTA buffer	10 mM Tris. Hcl pH 7.4 (was prepared by diluting stock solution), 1 mM EDTA.	Room temperature
Tris. Hcl stock solution (1 M)	121 g Tris base was dissolved in 800 ml H_2O, to desired pH was adjusted with concentrated Hcl, mix and add H_2O to 1L.	Room temperature
Loading buffer	▪ 3ml glycerol (30%) ▪ 25mg bromophenol blue (0.25%) dH_2O to 10ml	Room temperature
Tris-borate-EDTA (TBE)	Dissolve in 800 ml of H_2O, 108 g Tris base (89 mM), 55 g boric acid (89 mM), 40 ml 0.5M EDTA, pH 8.0 (2mM); bring to 1Lwith H_2O.	Room temperature
Ethidium bromide stock solution	50 mg of Ethidium bromide was dissolved in 100 ml of H_2O. Use diluted 1:1000.	4°C Protect from light.
Agarose gel	1% agarose was dissolved in 1x TBE buffer (in the presence of 0.5g/ml Ethidium bromide) by heating until melted.	Prepare just before use.

Procedure

1. The monolayer culture of SCC25 cells was trypsinized and suspended in complete growth medium containing 15% Fetal Bovine serum.

2. The cells were seeded onto six well plates at a density of 4 x 10^6 cells per well and incubated at 37**°C** and 5% CO_2 for 24 h.
3. The cells were observed using phase contrast microscope.
4. The supernatant was discarded and the monolayer was washed with 1X PBS.
5. Following this the cells were treated with IC_{50} concentration of hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO.2 strain (seeds) and alkaloid fraction of CO.2 strain *Carica papaya* L. (leaves of male and female plant); their respective active compounds trigonelline, cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, benzyl isothiocyanate and standard cisplatin in serum free media and untreated control.
6. The cells were incubated at 37**°C** and 5% CO_2 for 48 h and the cells were observed at an interval 24 h.
7. On completion of treatment period the culture medium was removed and centrifuged at 3000g for 5 minutes.
8. The remaining adherent cells were lysed using a hypotonic lies buffer (10mM Tris. Hcl pH8) containing 10mM EDTA and Triton-X 100 (0.5%) and pooled with pellets made of detached cells.
9. RNA present in the samples was digested using Raze (0.1mg/ml) at 37°C for 1 hour followed by Proteinase K for 2 h at 50°C.

10. DNA extraction was performed by addition of phenol: chloroform: isoamylalcohol (25:24:1). Following this equal volume of isopropyl alcohol was added and stored overnight at 20°C to precipitate DNA.
11. The mixture was centrifuged at 12000xg for 15 minutes at 4°C.
12. The pellet obtained was air dried and suspended in 20µl of Tris acetate EDTA buffer supplemented with loading buffer containing 0.25% bromophenol blue and 30 % glycerol.

Electrophoresis

1. 1% Agarose gel in Tris Acetate EDTA buffer containing 0.5mg/ml Ethidium Bromide was prepared and poured gently onto the mould and allowed to solidify.
2. 20**µ**l of the sample was loaded onto the wells and 200bp was used as standard.
3. Electrophoresis was performed in Tris-borate-EDTA buffer at 4 V/cm for about 4 h and DNA fragments were observed under UV light. Images were captured using gel documentation system.

4.9.6 Cell Cycle Analysis by Flowcytometry (Axel, 2004)

Principle

Dysregulation of cell cycle is one of the important features of carcinoma. Assessment of effect of the drugs on the cell cycle on cancer cells aids to determine their mechanism of action.

Flow cytometry is a rapid method to measure the DNA content of cells. It is a convenient research tool to monitor cell cycle status and regulation. Exponentially dividing cells will exhibit a DNA content distribution containing an initial peak of G0/G1 cells, a valley of S Phase cells, and a second peak containing G2/M cells. Cells in the G2/M Phase have twice the DNA content as cells in the G0/G1 Phase The flowcytometer computes the data and presents the cell cycle phases such as GI (Go), S and G2/M in the form of an integrated histogram from which percentage of DNA content in each phase of cell cycle was calculated.

Reagents

Phosphate-buffered Saline (PBS) [1X; pH 7.4]

Phosphate-buffered saline (1X) was prepared by mixing 1 volume of 10X PBS (pH 7.4) with 9 volumes autoclaved Milli Q water.

Triton X-100 (0.5%)

Triton X-100 (0.5%) was prepared by mixing 500 µl of Triton X-100 with 99.5 ml of 1X PBS (pH 7.4) thoroughly and stored at 4°C for use.

Ribonuclease A (RNase A) (1 mg/ml)

RNase (1 mg/ml) was prepared by mixing 0.1 ml from 50 mg/ml stock with 4.9 ml of RNase free water and stored at 4°C for use.

Propidium Iodide (PI) (1 mg/ml)

Five milligram (5 mg) of PI was dissolved in 5 ml of distilled water, wrapped with aluminium foil and stored at 4°C for use.

Procedure

- The SCC 25 cells were trypsinized when they were at 80% confluent and seeded at a density of 0.5 x 10^6 cells / well in 6-well plates.
- The cells were incubated in a CO_2 incubator at 37°C and 5% CO_2 under controlled humidified atmosphere overnight to allow them for attachment.
- After overnight incubation, the cells were treated with IC_{50} concentration and a concentration below IC_{50} with hydroalcoholic extracts of *Trigonella foenum-graecum* L. (seeds), *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO. 2 strain (seeds) and alkaloid fraction of *Carica papaya* L. CO. 2 strain (male and female leaves), standard cisplatin, and active compounds trigonelline hydrochloride, cinnamaldehyde, 4 hydroxy cinnamic acid, eugenol, benzyl isothiocyanate and incubated at 37ºC and 5% CO_2 under controlled humidified atmosphere for 48 h.
- At the end of 48 h, the cells were washed with 1X PBS (pH 7.4), trypsinized, washed with media and centrifuged at 1200 rpm for 5 min at room temperature.

- The supernatant was discarded, cell pellet was re-suspended in 5 ml of 1X PBS, washed and centrifuged at 1200 rpm for 5 min at room temperature.
- The supernatant was discarded and cell pellet was re-suspended in 300 µl of 1X PBS.
- This cell suspension was added drop wise into 700 µl of absolute alcohol with constant mixing and stored at 4°C for 24 h.
- On the day of cell cycle analysis, ethanol-fixed cells were centrifuged at 1500 rpm for 10 min at room temperature to remove ethanol. The cells were re-suspended in 5 ml of 1X PBS, washed and centrifuged at 1500 rpm for 10 min at room temperature.
- The supernatant was discarded completely, cells were re-suspended in 556 µl of 0.5% triton X-100, 20µl of RNase (1 mg/ml) were added, mixed well and incubated for 1 hour at room temperature.
- After incubation, 24µl of PI (1 mg/ml) was added, incubated for 45 min and acquired in a flow cytometer (BD FACS Calibur), equipped with an air-cooled argon laser providing 15 mW at 488 nm with standard filter setup.
- Ten thousand (10,000) events were acquired and the percentage of DNA content in each cell cycle phase was analyzed using CellQuest Pro software (Becton Dickinson, USA).

4.9.7 Assessment of Mitochondrial Membrane Potential using JC1 (5,5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethylbenzimidazolyl

carbocyanine iodide) stain by Confocal Microscopy (Salido *et al*., 2007)

Principle

Mitochondrial membrane potential is a vital parameter of mitochondrial function and hence loss of mitochondrial membrane potential indicates cell death. Cationic dyes such as Rhodamine has been used to assess variations in mitochondrial membrane potential. However the 5,5′, 6,6′-tetrachloro-1, 1′, 3,3′-tetraethylbenzimidazolylcarbocyanine iodide (JC-1), a cytoflourimetric cationic dye that enters the mitochondria and changes its fluorescent properties based on the mitochondrial membrane potential in the cell is more specific. In healthy cells the dye forms J aggregates that emits red fluorescence and in cells with low potential the dye remains monomeric emitting green fluorescence.

Reagents

Phosphate-buffered Saline (PBS) [1X; pH 7.4]

Phosphate-buffered saline (1X) was prepared by mixing 1 volume of 10X PBS (pH 7.4) with 9 volumes autoclaved MilliQ water.

JC-1 (1 mg/ml)

One milligram (1 mg) of JC-1was dissolved in 1 ml of DMSO and stored at 4°C in an amber colored vial, until use. From this, 10 µl aliquot was made up to 1 ml with 1X PBS to prepare a working stock of 1:100 dilution of JC1.

Procedure

- The monolayer culture of SCC25 cells at 80% confluence was trypsinized and suspended in complete growth medium containing 15% Fetal Bovine serum.
- The cells were seeded on a cover slip (22 x 22 mm) placed inside 6-well plate at the density of 0.2 x 10^6 cells / coverslip.
- The cells were incubated in a CO_2 incubator at 37°C and 5% CO_2 under controlled humidified atmosphere overnight to allow them for attachment.
- After overnight incubation, the cells were exposed to IC_{50} concentration of Cisplatin, Benzyl isothiocyanate, *Cinnamomum verum* J. Presl (bark), *Carica papaya* L. CO. 2 strain (seeds) and alkaloid fraction of *Carica papaya* L. CO. 2 strain (male and female leaves) respectively based on cell cycle data and again incubated for 48 h at 37°C and 5% CO_2 under controlled humidified atmosphere. Ionomycin was used as positive control.
- At the end of 48 h, cells were washed with 1X PBS (pH 7.4), 100 µl (1:100 dilution) of JC-1 was added to cells and incubated for 20 min at room temperature in dark.
- At the end of incubation, cells were washed with 1X PBS (pH 7.4). The cover slips were removed from 6-well plate and placed over another cover slip (40 x 22 mm).

- The cover slips were viewed under LSM 510 Meta confocal laser scanning microscope (Carl Zeiss, Germany) at 20 X magnification excitation/emission = 590/610 nm for red fluorescence and excitation/ emission = 485/535 nm for green fluorescence and cell images were captured from different fields.

4.10 QUANTIFICATION OF SAPONINS, POLYPHENOLS AND TANNINS IN SELECTED EXTRACT

4.10.1 Estimation of Saponins by High Performance Liquid Chromatography

Introduction

High performance liquid chromatography or high-pressure liquid chromatography is a process by which separation; identification and quantification of each component in a mixture can be performed. The basic principle of the technique is passing a pressurized liquid solvent containing the sample mixture through a stationary column filled with a solid adsorbent material. HPLC is more versatile as it has the advantages of high resolution, rapid separation, continuous monitoring of column effluent, accurate quantification, rapid durability, automated analysis and wider choices for stationery and mobile phase.

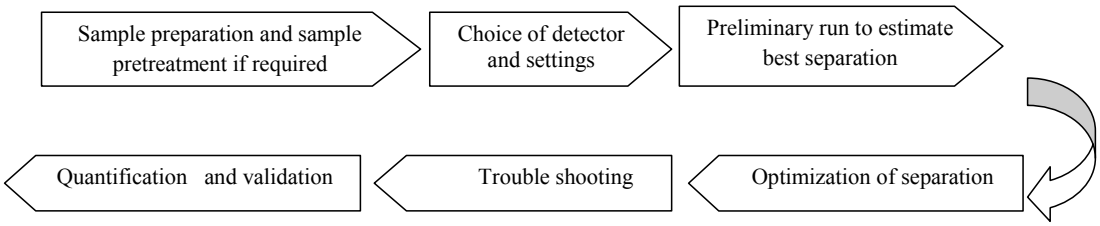
Components

Mobile phase delivery system, high-pressure pump, sample injection port, column, detector, data processor, sample collector.

Reverse Phase Chromatography

The use of non- polar stationary phase and polar mobile phase is called reverse phase chromatography. Silica modified with C 18 carbon atom to produce octodecylsilane is used as stationary phase for the technique.

Schematic representation of High Performance Liquid Chromatography



Reagents required

- Tetra butyl ammonium hydrogen phosphate extra pure (AR)
- Potassium dihydrogen phosphate buffer salt (Ranken Delhi)
- HPLC grade water (Ranken Delhi)
- Acetonitrile HPLC- Qualigens
- Methanol
- 6N Hcl, NaCl, ethyl acetate
- Centrifuge eppendorf.

Instrumentation

Instrument: SHIMADZU SPD-20A (PDA Detector)

Column: Enable C18 column, 4.6 x250mm (5 micron)

Standard Preparation

25mg of Digoxin was dissolved in 10ml of methanol

Sample preparation

10mg of the extract was dissolved each in 10ml of methanol and shaken well.

CHROMATOGRAPHIC CONDITIONS	
Sample	*Cinnamomum verum* J. Presl (bark) hydroalcoholic extract
Standard	Digoxin
Stationary phase	Enable C 18 column, 4.6x250mm (5 micron)
Mobile phase	Acetonitrile: Methanol: Water (30:30:40)
Detector wavelength	220nm
Injection volume	20 µl
Total flow rate	1ml/min

Methodology (Ceyhun Sezgin and Art Pk, 2010)

Chromatography was performed using reverse phase C18 column as stationary phase and Acetonitrile: Methanol: Water (30:30:40) as mobile phase.

- Mobile phases was filtered through 0.2µm cellulose acetate filter and degassed by ultrasonification at a flow rate of 1ml/minute.
- Detection and quantification was performed using UV detector at 220nm and PC computer was used to record the data.

Calculation

- Saponin was detected by matching the peak height of the sample with standard.
- Area under the curve was measured to quantify saponins

4.10.2 Estimation of Total Phenol Content (Ainsworth and Gillespie, 2007)

Principle

Polyphenols of plant extract react with Folin Ciocalteu reagent forming complex with phosphomolybdic /phosphotungstic acid giving rise to blue color. The intensity of blue color is directly proportional to the quantity of phenols.

Reagent Preparation

Folin Ciocalteu reagent preparation

10 g sodium tungstate and 2.5 g sodium molybdate was dissolved in 70 ml water to which 5 ml 85% phosphoric acid and 10 ml concentrated hydrochloric acid was added. The mixture was refluxed for 10 h. Following this 15 g lithium sulfate, 5 ml water and 1 drop bromine was added and refluxed for 15 min. The mixture was cooled to room temperature and the volume was made up to 100 ml with water.

7.5% w/v sodium carbonate solution

75 g of Sodium carbonate was dissolved in 1L water at a temperature of 70^{o}C. The solution was incubated oversight and filtered through glass wool.

Stock gallic acid solution

10mg of gallic acid was dissolved in methanol and the volume was made up to 10 ml in a standard flask (Concentration: 1 mg/ml).

Working standard: 1 ml of the stock solution was diluted with 9 ml methanol in a standard flask, to make a final concentration of 100 µg/ml of gallic acid.

Preparation of standard curve

- 0.05ml (5µg/ml), 0.1ml (10µg.ml), 0.2ml (20µg/ml), 0.3ml (30µg/ml), 0.4ml (40µg/ml) and 0.5 ml (50µg/ml) of the working standard was taken into a series of labeled test tubes.
- The volume was made up to 1 ml with methanol, 1 ml of water serves as blank
- To this 5 ml Folin Ciocalteu reagent (1:2) followed by 4 ml of 7.5% w/v sodium carbonate were added and kept at room temperature for 1.5 h.
- Blue color was read at 725 nm.
- A standard graph of gallic acid was plotted to determine polyphenol content present in the extract.

Estimation of polyphenol content in extract

Preparation of stock solution of test substance for assay

1 mg of the extract was weighed and dissolved in 10 ml of ethanol-water (70:30), from which 1 ml was used for experiment.

Procedure

- 1 ml of the extract in duplicates was added to labeled test tubes
- To this 5 ml Folin Ciocalteu reagent (1:2) followed by 4 ml of 7.5% w/v sodium carbonate were added and kept at room temperature for 1.5 hrs.
- Blue color was read at 725 nm. The total polyphenol content in the extract was determined from the standard graph of gallic acid.

4.14 Estimation of Tannins: (Ainsworth and Gillespie, 2007)

Principle

Tannins cause reduction of phosphotungstomolybdic acid in alkaline solution giving rise to blue solution. The intensity of color can be measured using a spectrophotometer that is directly proportional to the amount of tannins present in the test substance.

Reagent Preparation

Preparation of Folin-Denis reagent

100g of Sodium tungstate and 20 g of phosphomolybdic acid were dissolved in 750 ml distilled water to which 50 ml phosphoric acid was added and refluxed for 2h. Following this, distilled water was added to constitute a final volume of 1000ml.

Preparation of carbonate solution

350 g of Sodium carbonate was dissolved in 1L water at a temperature of 70^{o}C. The solution was incubated oversight and filtered through glass-wool

Preparation of tannic acid solution

Stock solution

100 mg Tannic acid was dissolved in 100 ml distilled water.

Working solution

5 ml stock solution was diluted with distilled water to give rise to final concentration of 50µg/ml.

Preparation of standard curve

- 0.1ml (5µg/ml), 0.2ml (10µg.ml), 0.3ml (15µg/ml), 0.4ml (20µg/ml) and 0.5ml (50µg/ml) of the working standard were added into labeled test tubes.

- The volume was made up to 1ml with distilled water; 1ml of water was used as the blank.
- 0.5ml of Folin-Denis reagent and 5 ml of 35% sodium carbonate solution was added to each tube.
- All the reagents in each tube were mixed well and kept undisturbed for about 10 minutes and read at 725 nm against reagent blank.
- A standard graph of tannic acid was plotted to determine polyphenol content present in the extract.

Procedure for estimation of tannins in extracts

Preparation of stock solution of test substance for assay

1 mg of the extract was weighed and dissolved in 10 ml of methanol-water (70:30), from this 1 ml was used for estimation of tannins.

Procedure

- 1ml of the plant extract in duplicates was taken in labeled test tubes to which 0.5 ml of Folin Denis Reagent was added.
- 5 ml of 35 % Na_2CO_3 solution was added.
- The mixture was shaken well and kept at room temperature for 10 min.
- The absorbance was measured at 725nm using UV spectrophotometer. Tannin content of the samples was estimated from the standard graph of tannic acid.