Chapter – 3

Extraction, characterization of phytocomponents from crude extract of *Bauhinia variegata* L. and evaluation of its *invitro* anti cancerous activity on lung cancer cell lines

3.1 Introduction

Bauhinia variegata Linn (Mountain ebony), is a species of flowering plant, belonging to legume family Fabaceae, indigenous to the Indian subcontinent, Southeast Asia, and China has been chosen in the present study. The plant is generally utilised by ethnical people in India and is well accepted in numerous different traditional medical systems like Ayurveda, Unani, and homoeopathy. The medicinal and beneficial properties of *Bauhinia variegata* parts, including the flowers, stem, bark, root, and seeds, are well proved. These plants are among the most generally utilised plants for traditional medical reasons globally and have conceivably the longest history of proved medicinal use.

3.1.1 Classification of *Bauhinia variegata*

Kingdom: Plantae

Division: Magnoliophyta

Class: Magnoliopsida

Order: Fabales

Family: Fabaceae

Genus: Bauhinia

Species: variegata

3.1.2. Vernacular names of *Bauhinia variegata*

Common name: Mountain Ebony; Hindi: Kachnar, Kaniar; Sanskrit: Kovidara; English: - Mountain ebony; Marathi: Raktakanchan; Gujarati: Kovindara; Punjabi:-Kanchan; Kannada: Kempumandara; Telgu: Devakanchanum; Tamil: Sigappu mandaraii; Urdu: Kachnal; Oria : Kosonaro; Kashmiri: Kanchana; Bengali: Raktakanchana; Assami : Shonapushpaka; Malayam: Kovidaram.

3.1.3. Part of use: Stem bark, flower, leaves, seed.

3.1.4. Distribution of *Bauhinia variegata*

The trees are indigenous to numerous nations, including India, China, Pakistan, Burma, North Thailand, North Vietnam, the People's Democratic Republic of Lao, Cambodia, and Laos, and

are generally set up in tropical and subtropical regions[1]. In India, they are found in most states except Jammu and Kashmir, Himachal Pradesh, Sikkim.

3.1.5. Morphology of *Bauhinia variegata*

Bauhinia variegata is a small to medium- sized evanescent tree with a height of over to 15 m and periphery of around 50 cm. The bark is scaled, smooth to slightly fissured, and light brownish grey in colour[2]. Inner bark is Bitter, fibrous, and pinkish. The leaves have small stipules that are 1-2 mm long and early caducous. The petiole is 3-4 cm long and puberulous to rough. The lamella is astronomically elliptical to indirect, constantly wider than long, and has 11–13 nerves. The light green, relatively hairy calyx forms a pointed 5-angled bud, splits open on one side, and remains attached. The many flowers have five slightly inadequate petals with crimpy perimeters and narrowed bases, five twisted stamens, and a veritably slender, stalked, twisted pistil with a narrow, green, 1-celled ovary, style, and dot-like stigma[3]. The tree is deciduous in its native niche in India, going without leaves from January to April and dropping them in November and December. Flowering takes place when the plant is without leaves. Trees begin to bloom between two and three years old. Morphology of flower, leaves and bark of *Bauhinia variegata* is shown in figure 1.

Figure 3.1: a) Flower b) Leaf c) Bark

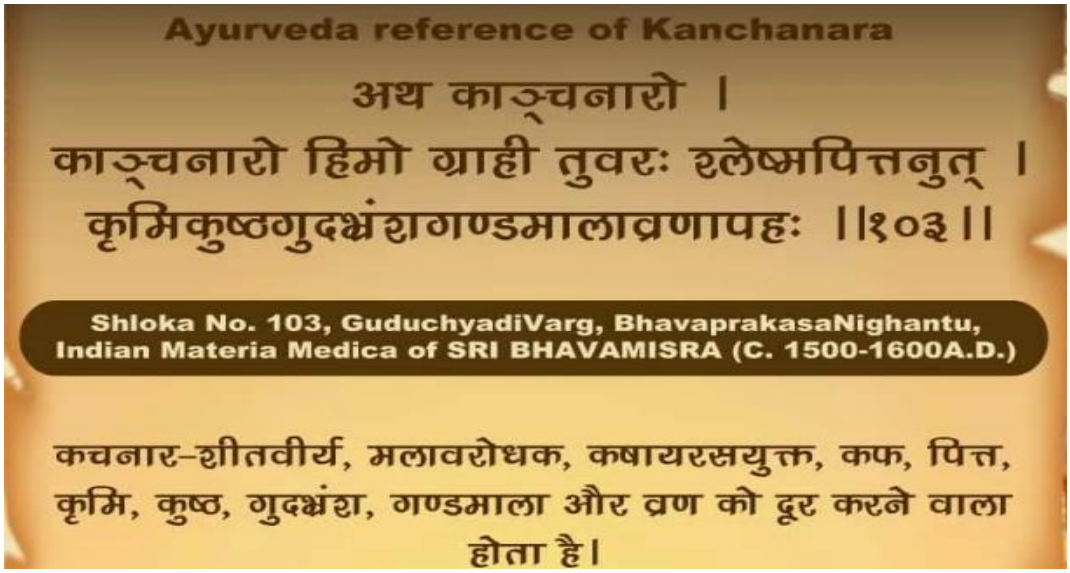
3.1.6. Traditional Uses of *Bauhinia variegata*

3.1.6.1. Folk medicine

Ethnical people use the plant's bark, which has advanced medicinal value, to treat a range of conditions. The bark's uses include treating fever, acting as a tonic and astringent[4], being antileprotic, treating skin conditions and injuries and acting as an antitumor[5].

3.1.6.2. Ayurveda

Bauhinia variegata is veritably well described in ancient Indian science i e., Ayurveda and its Stem bark and flowers are extensively used as drug against various diseases. **Kachnar bark** is used in diseases like Gandamala (Lymphadenopathy), Galaganda (Goitre), Arbuda (Tumor), Ashthila (BPH)[6]. Ayurvedic doctors have employed *Bauhinia variegata* bark powder in concurrence with other medicines to treat a variety of ailments in conjunction with turmeric (*Curcuma domestica* Linn.) and ashoka (*Saraca indica* Linn.)[7]**.**



The above shloka shows the **Ayurvedic reference to use of *Bauhinia variegata*** and explains the medicinal properties of kanchanara. It removes Pitta and Kapha doshas. It help to cure different conditions like Skin disorders, Goitre, ulcers etc.

3.1.6.3. Unani

The plant's bark is characterised as an astringent for the intestine and a tonic for the liver in the Unani medical system. Leucoderma, leprosy, menorrhagia, asthma, injuries, and ulcers are said to be treated with it.

3.1.7. Phytochemicals present in *Bauhinia variegata*

All plants have variety of phytochemicals. These in concert/ individually have been used for their therapeutic effect. Leaves, bark and flowers from individual *Bauhinia variegata* plants within the same species may have varying bioactive phytochemicals. The presence of varied compounds in *Bauhinia variegata* flower, leaves as well as bark extracts showing different pharmacological activities has been reported[8,9,10,11,12]. The difference in the concentration of these phytocomponents depends on various factors like the part of plant used for the extraction,

the extraction method, the solvent used and the place of plant collection. Different phytocomponents reported in *Bauhinia variegata* are β-sitosterol, kaempferol-3-glucoside, tannins, carbohydrates, crude protein, fibers, calcium, phosphorus, quercetin, rutin,, apigenin, apigenin- 7-O-glucoside, heptatriacontan-12, 13-diol and dotetracontan-15-en-9-ol[13]. Phytochemicals produced by plants which have good antioxidant activity are helpful in treating diseases[14,15].

Phytocomponents of *Bauhinia variegata* have showed anti- hyperglycaemic[16] and anti-depressant activity[17]**.** These reports indicate that the phenolic compounds and other phytochemicals present in the *Bauhinia variegata* bark have an eventuality to manage metabolic conditions like diabetes.

Despite the significant medical benefits indicated in ancient literature and a thorough and regular analysis, there are no published studies on the phytochemical and pharmacological effects of *Bauhinia variegata* Linn stem 's bark, hence the focus of the current study is on evaluating the anticancerous effect of bark of *Bauhinia variegata*.

This chapter has been divided into two sections:

Section I – Extraction and Characterization of phytocomponents from crude *Bauhinia variegata* L bark extracts.

Section II - Evaluation of *invitro* anti cancerous activity of *Bauhinia variegata* L bark extracts on lung cancer cell lines.

Section I – Extraction and Characterization of phytocomponents from crude *Bauhinia variegata* L bark extracts.

3.2 Materials and methods

3.2.1. Collection of plant material

Bauhinia variegata bark was collected from Waghai botanical garden, Dang, Gujarat during December-January each year. The plant material was identified and authenticated based on the morphological characteristics by Prof. Nagar of Department of Botany, The Maharaja Sayajirao University of Baroda, India. Samples were stored in herbarium for future reference (IIRS Biodiversity Project 09-10).

3.2.2 Preparation of extracts

The extraction from the *Bauhinia variegata* L. Bark was carried out by Soxhlet extraction method. In this method different solvents were used, in order of increase order of polarity. The temperature used for each solvent was based on the boiling temperature of the solvent. Plant material was subjected to 8-12 hours for extraction with each solvent. The extracts were filtered and concentrated to dryness under reduced pressure and controlled temperature in a rotavapor apparatus. Extract yield and its nature was noted. The extract was stored in a refrigerator at 4°C till further use.

Bauhinia variegata Bark (950g) was collected from the Waghai botanical garden, Dang

Bark was authenticated by experts from Botany Department of MSU.

Specimen were stored for the future reference in the herbarium

Followed by washing, surface sterilization, rinsing, shade-drying of the plant material and crushing using heavy mixer grinder

Crushed material was packed in a thimble and placed into Soxhlet extractor

Extraction was carried out using each solvent in order of increasing polarity for 8-12 hrs each.

3.2.3. Preliminary Phytochemical Analysis

Preliminary tests to identify the phytocomponents was done using standard procedure. The results for this is reported in Table 3.1.

Table 3.1: Qualitative Analysis

Phytocomponents	**Test**	**Reagents and assay procedure**	**Inference**
Alkaloids	**Wagner's Test:**	1.27g Iodine and 2g KI were added to 5 ml distilled water. The volume was made up to 100 ml with distilled water. This reagent	Reddish brown precipitate

		was added by side of the test tube to 1 ml of extract.	
Carbohydrate	**Molish Test**	To 2ml of extract, few drops of alcoholic solution of α-naphthol (Shake well) + 1ml of concentrated H2SO4 in a drop-wise manner (allow to stand).	violet ring formation at the intersection of two layers
Protein	**Biuret Test**	2ml filtrate+2% CuSO4+add 1ml 90% ethanol+ excess of KOH pellets	Pink colour in ethanol
Phenol	**Ferric Chloride Test**	500mg extract+ 5ml d/w+ few drops of neutral 5% $FeCl_3$	Dark green phenolic compounds Blue colour: Tannins
Flavonoid	**Alkaline reagent Test:**	10% $(NH4)_3OH$+ aqueous solution of extract	Yellow Fluorescence
Steroids	**Lieberman-Buchard test:**	Extract in chloroform+1ml of acetic acid & 1ml of acetic anhydride (heated in water bath) + few drops of H2SO4	Blue green colour
Saponins	**Foam Test**	To 1 mg of extract, 1ml of distilled water was added and warmed.	Formation of stable foam in the form of honeycomb.
Coumarins	**Sodium hydroxide Test**	To 2ml of extract, add 3ml of 10% NaOH solution	Yellow colour was observed
Quinones	**Sodium hydroxide Test**	1ml crude extract + Dilute NaOH	Blue green or red colour
Cardiac Glycosides	**Keller Killani Test:**	1ml of extract was mixed with 2ml of Glacial acetic acid containing 1-2 drops of FeCl3 solution.	Formation of a brown ring during transition stage.

		Concentrated H2SO4 was added along the sides of the test tubes.	

3.2.4. Antioxidant activity determination

The most important property a phytocomponent should possess to show therapeutic properties is its ability to be a good antioxidant. The antioxidant potential can be determined using various assays.

3.2.4.1 DPPH (2, 2-diphenyl-1-picrylhydrazyl) assay

The free radical scavenging activity of purified extract was measured by DPPH method[18].

Principle:

2, 2-Diphenyl-1-picrylhydrazyl (DPPH) is a violet colour compound which is considered as a stable radical. Anti-oxidant reacts with DPPH, which tend to capture hydrogen ion and convert into a yellow colour diamagnetic compound which gives absorbance at 517nm. The degree of discolouration reveals the extracts capacity for scavenging.

Reagents

1. The DPPH solution was made as a 0.1 mM solution in methanol.

2. Standard (L-ascorbic acid - 1%).

Procedure

The reaction mixture (3.0 ml) consisting 1.0 ml DPPH in methanol (0.1 mM), 1.0 ml methanol and 1.0 ml different concentrations of the petroleum ether, n-hexane, chloroform, ethyl acetate, methanol and aqueous extracts (0, 50,100, 150 µg.ml) was incubated in dark for 10 min., and the OD was measured at 517 nm against blank. For control, 1.0 ml of methanol was used in place extract. L-ascorbic acid as a positive control.

The following formula was used to compute the percentage inhibition of DPPH:

$$\text{Inhibition (\%)} = \frac{\text{OD of Control} - \text{OD of Experiment}}{\text{OD of Control}} \times 100$$

3.3 Results:

3.3.1 Phytochemical Analysis

The weight of bark after drying was 887.3g. The powder of the bark after grinding was 864.01g. This powder was subjected to Soxhlet extraction method. Extract yield is shown in table 3.2.

Table 3.2 Percentage yield obtained from 42.8g of powdered bark.

Solvent	% Yield (w/w)	Dried Extract (g)
Petroleum Ether	1.56%	0.67g
N. Hexane	0.87%	0.37g
Chloroform	2.28%	0.98g
Ethyl acetate	0.35%	0.15g
Methanol	9.4%	4.02g
Water	4.45%	1.90g

The % yield was calculated after solvent was recovered from extract.

Extraction yield (%) = W1/W2×100; Where W1 is the mass of crude extract and W2 is the mass of the sample

The identification of compounds was done by biochemical assay for each extract obtained from Soxhlet extraction.

Table 3.3 Phytochemicals present in bark extracts of *Bauhinia variegata*

Component	Petroleum ether	N-hexane	Chloroform	Ethyl acetate	Methanol	Aqueous
Carbohydrates	+	-	-	+	+	-
Fats and Oil	+	+	+	-	-	-
Steroid	+	+	+	-	-	-
Cardiac glycosides	+	-	+	+	+	+
Saponin	+	+	-	+	+	+
Coumarin	-	-	+	+	-	-

Flavanoids	-	-	-	-	+	+
Alkaloids	+	-	+	-	+	+
Phenols	-	+	+	-	+	+
Tannins	-	+	+	-	-	-

3.3.2 Antioxidant activity of whole plant extracts of *Bauhinia variegata*

The antioxidant potential of the *Bauhinia variegata* extracts was determined by DPPH assay. For this study Ascorbic acid was taken as a standard. It was observed that n-hexane and petroleum ether extracts showed lesser antioxidant activity as compared to other extracts. The methanolic and water extracts showed maximum antioxidant activity with the strongest DPPH radical scavenging activity among all the extracts (Figure 3.2).

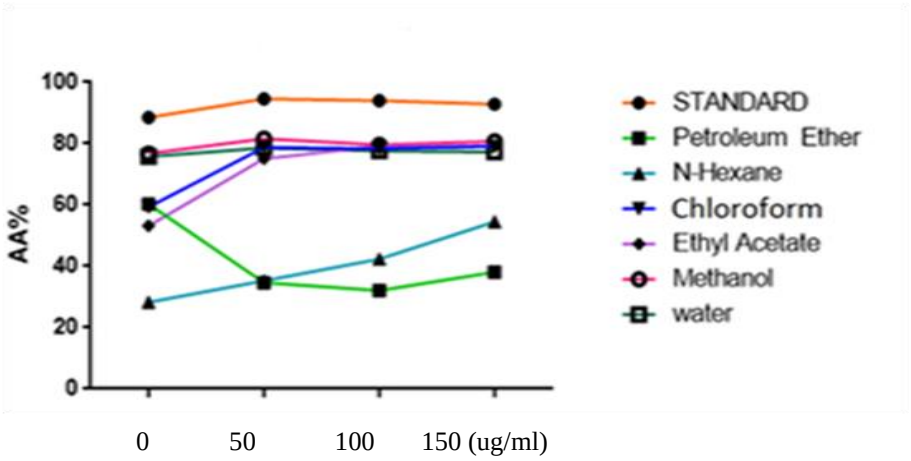


Figure 3.2 Percentage scavenging of DPPH free radical with different concentrations of *Bauhinia variegata* bark extracts.

Section II - Evaluation of *in vitro* anti cancerous activity of *Bauhinia variegata* L bark extracts on lung cancer cell lines.

3.4 Introduction

The impact of an exponential rise in cancer cases is felt worldwide. Every year India reports about 70,275 lung cancer cases (fourth among all cancers) with 50 % mortality within a year and 5-year survival has remained at 11-17 % for these lung cancer patients[19,20]. Non-small cell lung cancer (NSCLC) contributes for about 85 % of the lung cancer cases while 15 % cases

are SCLC[20]. The most typical NSCLC is adenocarcinoma (40 %), followed by large cell carcinoma (15%)[21]. Hence, in this study A549 (adenocarcinoma) and H460 (large cell carcinoma) cell lines were used. The 5-year relative survival rate of lung cancer has increased with time, but less than 21 %[21]. The poor survival rate along with low efficacy and side effects of chemotherapy (20-30 %) are major causes of concern in lung cancer[22]. So, Scientists are looking for anticancer substances from natural sources, such as plant phytochemicals, due to the adverse effects associated with current medications. Phytocomponents have lower toxicity providing an attractive alternative in cancer therapy[23,24]. Plant used in the study is ***Bauhinia variegata*** which has multiple pharmacological activities explained in section I of this chapter.

Cancer cells has shown to have increased ROS levels as compared to their normal counterparts which are detoxified by complex antioxidative mechanisms[25,26]. An imbalance between the system that produce and remove ROS causes an increase in an oxidative stress. Cancer cells have the ability to circumvent apoptosis, making proteins involved in the apoptotic cascades which can be used as ideal targets for cancer therapy[27]. Reestablishing apoptotic programming in malignant cells selectively kills tumor cells and caspases as primary inducers of apoptosis provide an ideal platform to develop effective therapeutic strategies for cancer[28,29]. With this background, the present chapter (divided into two sections) aimed to extract and characterize the crude extracts of bark of *Bauhinia variegata* L. and evaluation of *invitro* anti cancerous activity of crude extracts of bark of *Bauhinia variegata* L on lung cancer cell lines.

3.5 Materials and methods

3.5.1 *In Vitro* Culturing of Human Lung Cancer Cell Lines

Human lung carcinoma cells (A549; H460) procured from (NCCS, Pune, India) were grown in (DMEM, Himedia) supplemented with 10% fetal bovine serum (Gibco-Invitrogen) and 1% antibiotic (PSN, Himedia). Cell lines were maintained at 37°C in a 5% (v/v) CO_2 atmosphere with 95% (v/v) humidity in a humidified incubator[30]. A stock solution of 1 mg/ml of PEBE and CBE was prepared in 0.5% dimethyl sulfoxide (DMSO) and diluted for further use (DMSO concentration did not exceed 0.1 %).

3.5.1.1 Cytotoxic Assay by MTT method

The stock solutions of *Bauhinia variegata* bark extracts were prepared in 0.5 % Dimethyl Sulfoxide (DMSO) and diluted for further use (DMSO concentration) did not exceed 0.1 %).

Cytotoxic potential of extract was determined by MTT assay[30]. Cells were grown in 96-well plates overnight and treated with different concentrations of bark extracts for 24 h, 48 h and 72 h time points. Untreated cells were taken as control. A549 and H460 cells grown in medium served as control. After 24, 48 and 72 h incubation, MTT (5 mg/ ml) solution was added followed by 3 h incubation, medium with MTT was removed and therefore the formazan crystals were solubilized in 100ul DMSO. At 575 nm, the absorbance of each well was determined. IC50 value of extracts was calculated by determining percentage cell growth inhibition using Graph Pad Prism 6.0 software.

3.5.2 Soft Agar Colony Formation to Evaluate Cellular Transformation

For Colonogenic assay, the cells were plated in 6-well tissue culture plate with 5,000 viable cells (per well) as determined by the Trypan blue staining. A549 and H460 cells were allowed to grow overnight and after 24 h the fresh media modified with different concentrations of the different extracts were added for 48 h and 24 h respectively. Using a Neubauer Chamber, the quantity of A549 and H460 cells were counted after treatment with the corresponding extracts. 2 % Agar was melted and cooled to 40°C in a water bath and media containing serum was added in equal volume to give 1 % base agar solution[31]. 500µl of the base agar solution was pipetted into each well of a 24-well plate. 0.7 % agar was melted and cooled to 40 °C and mixed with media containing A549 cells treated with PEBE for 48 h.

For H460, 0.7 % agar was mixed with media containing H460 cells treated with CBE for 24 h. 500µl of this solution was pipetted onto the top agar. Cell number was maintained at 1250-1500 cells per well. The cells could grow for 13 -15 days for both cell line and in every 3-4 days, 200 µl of fresh media was added above the top layer as a feeder. After 15 days, the medium above the cells was removed and rinsed carefully with PBS. 2-3 ml of a mix of 6.0 % glutaraldehyde and 0.5 % crystal violet was added on cells and left for a minimum 20 min. The glutaraldehyde crystal violet mixture was removed and rinsed with water. Software called Image J was used to measure the colony size.

3.5.3 Wound Healing Assay/Scratch Test

Cancer cells undergo epithelial to mesenchymal transition during metastasis[32]. Cells were grown and starved in low serum media (1.5 ml; 2 % serum in DMEM) overnight. A549 and

H460 cells monolayers were scraped during a line to make a "scratch" with a pipette 200 µl tip. Cells were washed with PBS and low- serum media (2 % serum to prevent cell proliferation) was replaced with media containing different concentrations of extracts. A549 cells were treated with PEBE extract for 48 h and H460 cells with CBE for 24 h. Plates were placed in an incubator at 37 °C for 0-36h. Cells were stained with crystal violet and images were captured at different time-points from 0 to 36 h respectively. The pictures acquired for every sample was further analyzed quantitatively by using computing software.

3.5.4 DNA Fragmentation Examination

A549 and H460 cells (5 x 106) were grown for 24 h and treated with various concentrations of PEBE and CBE for 48 h and 24 h, respectively. Cells were collected, washed with 1× PBS and centrifuged at 300 g. Cell pellet was collected and resuspended in 0.5 ml lysis buffer and incubated for 1.5 h at 37 °C followed by centrifugation at 10 K rpm for 15 min (at room temperature). Pellet was discarded and supernatant was mixed with equal volume of isopropanol followed by an addition of 25 µl of 4M NaCl and incubated at -20 ºC overnight. Mixture was centrifuged again at 10 K rpm for 20 min and the pellet was dissolved in 40 µl ddH_2O. 5 µl of RNase A (10 mg/ml) was added to the lysate and further incubated for 1 h at 37 ºC. The DNA was then electrophoresed in a 1.8% agarose gel in TAE (triacetate-EDTA) buffer (pH 8.0). After electrophoresis, ethidium bromide was used to stain the DNA and visualized using a gel-doc system (BIO-RAD).

3.5.5 Analysis of Intracellular Reactive Oxygen Species by DCHF-DA

The 2′, 7′-dichlorodihydrofluorescein diacetate (H2DCF-DA) fluorescent probe is commonly employed that enabled the monitoring of intracellular accumulation of ROS. A549 and H460 cells were grown overnight in 6 well plates. Cells were treated with PEBE (A549) and CBE (H460) respectively and incubated for various time intervals (0 to 10h). At the end of the incubation period, the media was removed and 5 µM of DCHF-DA which is diluted in media was added to the cells and incubated for 40 min at 37 °C. Remove the dye, trypsinize the cells and add media to stop its action. Centrifuge it at 1500 rpm for 3 min. The cells were then washed thrice with PBS and fluorescence intensity (excitation = 485 nm and emission = 530 nm) was measured by fluorimeter[24,33].

3.5.6 Fluorescent Microscopy Analysis by DAPI Staining and Acridine Orange/Ethidium Bromide Staining

Cell nuclear morphology was checked by fluorescence microscopy using DAPI[34] and AO/EtBr[35] staining. A549 cells were treated with the PEBE for 48 h and H460 cells with CBE for 24 h followed by DAPI and AO/EB dye mix. For DAPI staining, the cells were then permeabilized with 0.1% Triton X-100, fixed with 4% paraformaldehyde, and stained for 10 minutes with 1 mg/ml of DAPI solution. The fluorescent images of the samples were examined under a Nikon Eclipse Ti fluorescence microscope. For AO/EB staining, AO/EB staining dye mixture containing (100µg/ml AO and 100µg/ml) of 1µl was added on cells after respective treatments and examined under a Nikon Eclipse Ti fluorescence microscope.

3.5.7 Determination of Caspase-3 Activity in Cell Lines

Bio Vision Caspase-3 Colorimetric assay kit (K-106) (for measuring the activity of caspases) was used to determine caspase-3 levels in A549 and H460 cell lines as per the protocol mentioned in the kit instruction manual.

3.5.8 TMRM Staining for Mitochondrial Membrane Potential Measurement

A549 and H460 cells were grown in 6-well plates and treated with PEBE (IC50, 48 h) and CBE (IC50, 24 h) respectively. The cells were stained with tetramethyl rhodamine, methyl ester and perchlorate (TMRM, 100 nM) at 37 °C for 30 minutes[35]. After PBS wash, the cells were imaged using a fluorescence microscope.

3.5.9 Statistical Analysis

All experiments were done in triplicate, and data expressed as mean ± standard error of mean (SEM). Statistical differences between control and treated cells with a p value < 0.05 were considered to be statistically significant and were assessed by the one-way ANOVA test using GraphPad Prism 6.0 software. The IC50 values were calculated from the curves constructed by plotting cell viability (%) vs concentration (mg/ml), using GraphPad Prism 6.0 software.

3.6 Results and Discussion

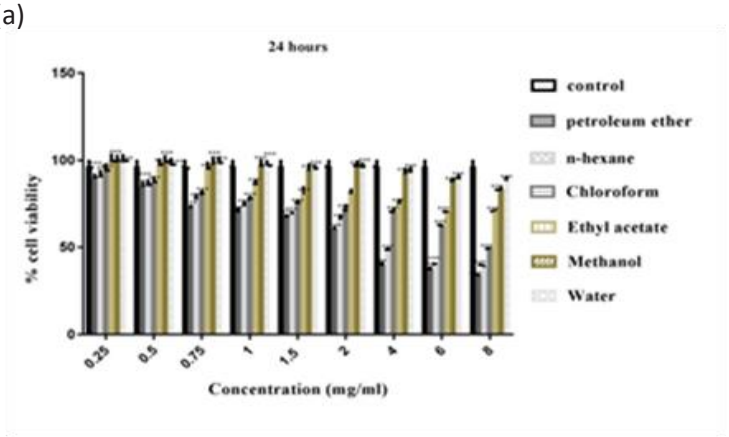
3.6.1 MTT Assay: Potential for Cytotoxicity

3.6.1.1 Screening for cytotoxic effect of Extracts on A549 Cells

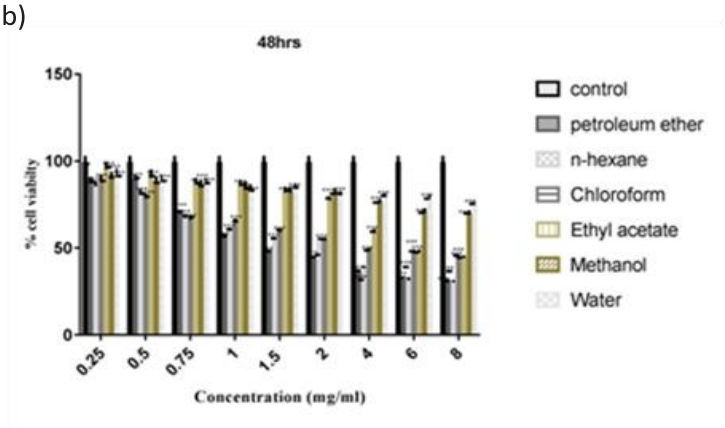
The effect of the various extracts was evaluated on A549 cell line. Cytotoxic activities against A549 cells growth were measured after treating with phytochemical extracts of PE, N-hexane, Chloroform, Ethyl acetate, Methanol and Water of *Bauhinia variegata* at concentrations of 0.25 mg/ml to 8mg/ml for 24 h, 48 h and 72 h respectively. The results showed that A549 cells responded to the cytotoxic effects of the plant extracts in a dose and time-dependent manner.

Petroleum ether and n-Hexane extracts showed the foremost cytotoxic effect on A549 cell line as compared to other extracts at 48 h treatment. The polarities of Petroleum ether and n-hexane are almost similar, and both the solvents have shown almost similar composition. Hence, it's likely that the cytotoxic agent (s) in both the solvents are identical. Therefore, Petroleum ether bark extract (PEBE) has been selected for the further study due to lesser yield of n-hexane extract. The cytotoxicity of PEBE on A549 cells increased from 33 % to 30 % to 26 % after 24, 48 and 72 h respectively. The cytotoxicity of PEBE on A549 cells is shown in Figure 3.3 a, b, c.

(a)



(b)



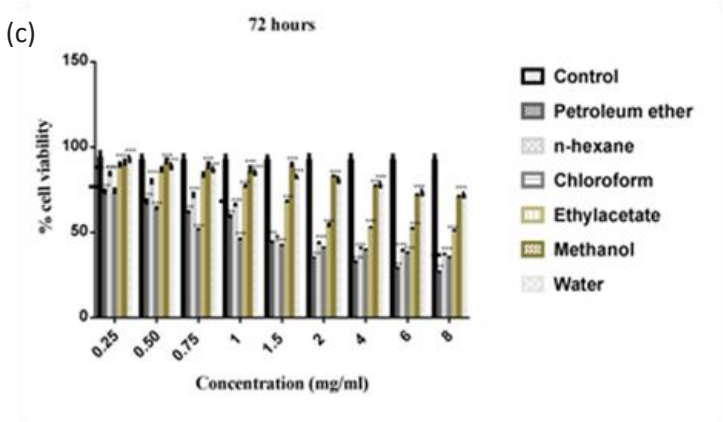


Figure 3.3 The effect of *Bauhinia variegata* bark extracts against A549 cell line for 24 h, 48 h and 72 h. Percentage growth proliferation of A549 cells was assayed at 100 mg/ ml concentration of extracts using MTT assay as described in the Methods section **(a)** Effect of different concentrations of different bark extracts on A549 cells for 24 h **(b)** Effect of different concentrations of different bark extracts on A549 cells for 48 h **(c)** Effect of different concentrations of different bark extracts on A549 cells for 72 h respectively. Date represented as the mean± standard deviation of at least three experiments. ***P<0.001.

3.6.1.2 PEBE Inhibit Growth and Proliferation of A549 Cells

Examination of A549 cell morphology was done at 48 h treatment of PEBE and it was observed that from 0.5, 1, 1.5, 2 and 4 mg/ml concentrations cells had altered morphologically and started to shrink showing the symptoms of the cell death. The morphological examination of the cells after PEBE treatment showed cell shrinkage and rounding up of the cells which are typical features of cell death as shown in Figure 3.4.

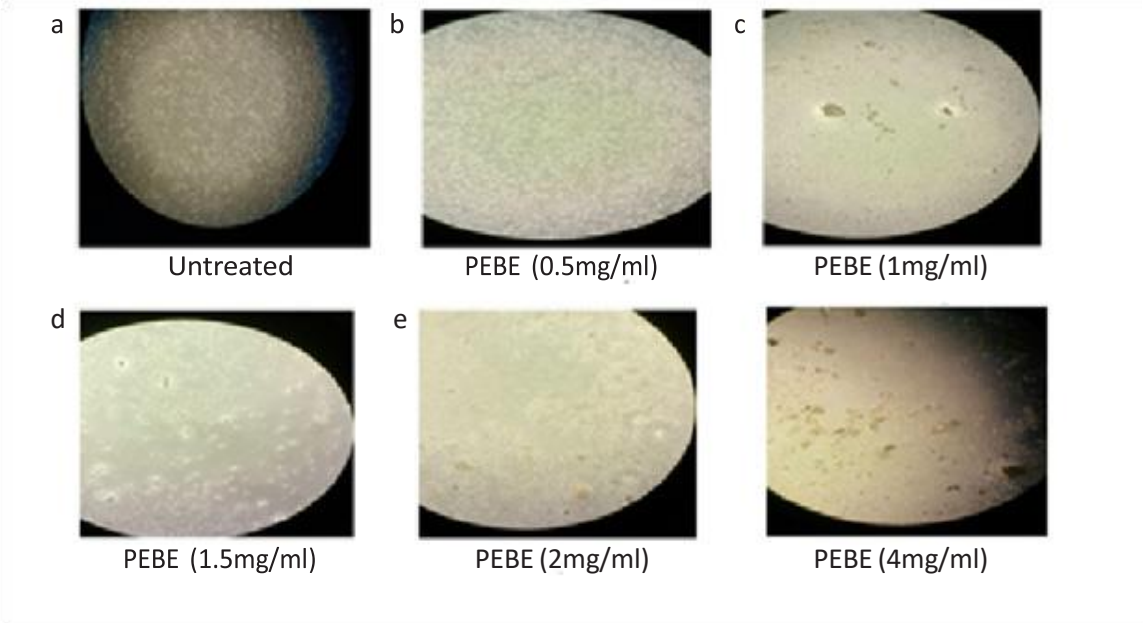


Figure 3.4 Morphological effects of A549 untreated cells **(a)** without treatment and cells exposed to **(b)** PEBE (0.5 mg/ml), **(c)** PEBE (1 mg/ml), **(d)** PEBE (1.5 mg/ml), **(e)** PEBE (2 mg/ml), **(f)** PEBE (4 mg/ml) of *Bauhinia variegata*.

3.6.1.3 Determination of IC50 Value of PEBE Extract

Effect of different concentrations of PEBE of *Bauhinia variegata* on the viability of A549 cells for different time measure (at 24 h, 48 h and 72 h) was assessed. The IC50 values were 2.8 mg/ml for 24 h, 1.6 mg/ml for 48 h and 1.5 mg/ml for 72 h as shown in Figure 3.5. The extract was more potent after 48 h treatment; hence this time point was selected for further experiments.

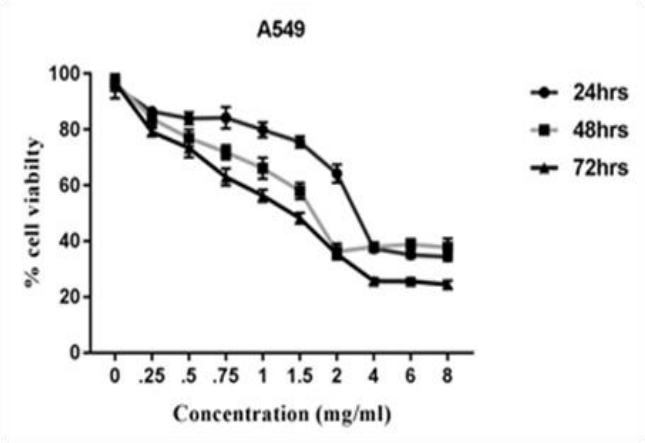
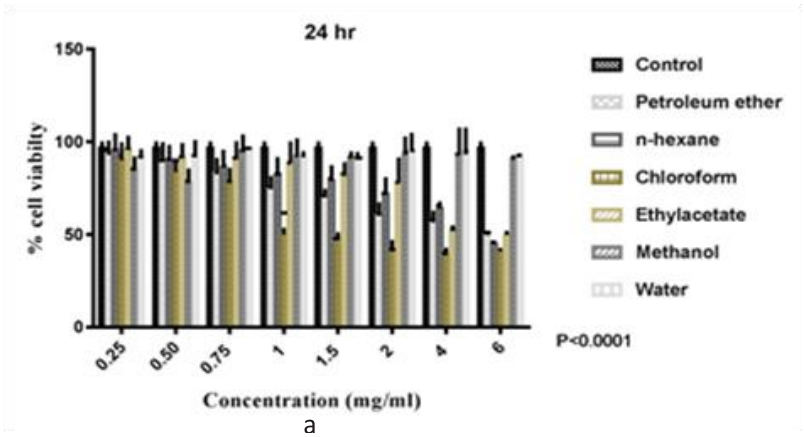


Figure 3.5. IC50 values of crude petroleum ether bark extract of *Bauhinia variegata* on A549 cells for 24 h, 48 h, and 72 h (n=3).

3.6.2 MTT Assay - H460 Cells

3.6.2.1 Screening for cytotoxic effect of Extracts on H460 cells

The effect of the extracts on H460 cell line showed that the chloroform bark extract (CBE) had a greater cytotoxicity at 24 h treatment. The cytotoxicity of CBE on H460 cells was time dependent demonstrating a potent and definite growth inhibitory effect as shown in Figure 3.6. The cytotoxicity of CBE on H460 cells increased from 30.9 % to 27.9 % to 26 % after 24, 48 and 72 h respectively. The difference in the behavior of both cell lines may be due to their different molecular characteristics which are targeted by the different phytochemicals present in the two bark extracts.



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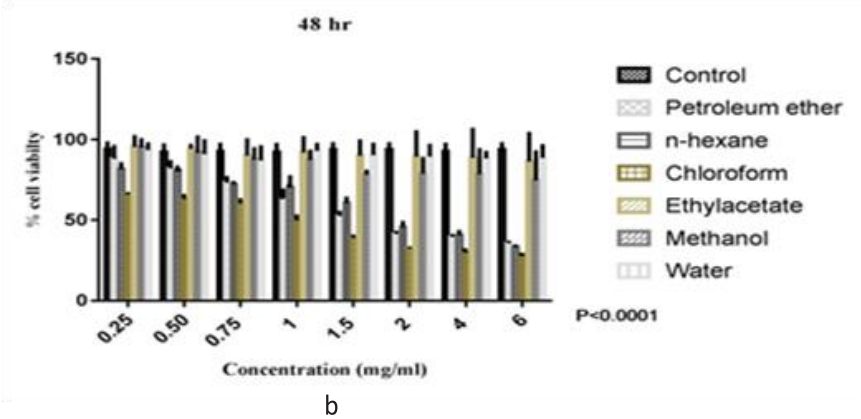
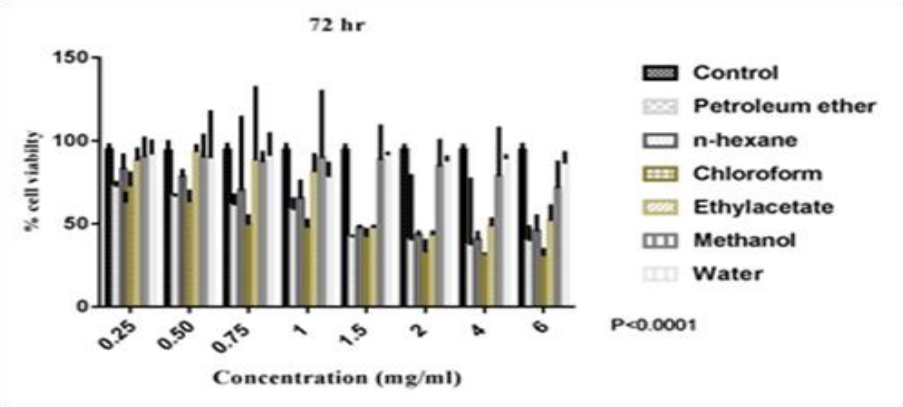


b



c

Figure 3.6 The effect of *Bauhinia variegata* bark extracts against H460 cell line for 24 h, 48 h, and 72 h. Percentage growth proliferation of H460 cells was assayed at 100 mg/ ml concentration of extracts using MTT assay as described in the Methods section. a) Effect of different concentrations of different bark extracts on H460 cells for 24 h b) Effect of different concentrations of different bark extracts on H460 cells for 48 h c) Effect of different concentrations of different bark extracts on H460 cells for 72 h respectively. Date represented as the mean ± standard deviation of at least three experiments ****P<0.0001.

3.6.2.2 CBE Inhibits Growth and Proliferation of H460 Cells

Examination of H460 cell morphology was done at 24 h treatment of CBE and it was observed that from 0.5, 1, 1.5, 2 mg/ml concentrations, cells had changed morphologically and started to shrink showing the symptoms of the cell death. The morphological examination of the cells after CBE treatment showed as cell shrinkage and rounding up of the cells which are typical feature of cell death as shown in Figure 3.7.

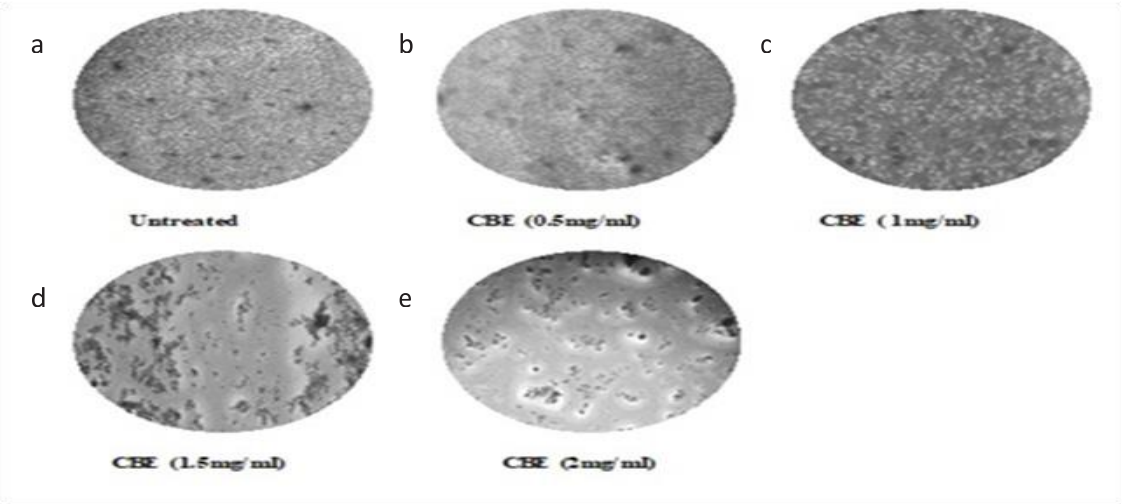


Figure 3.7 Morphological effects of H460 untreated cells a) without treatment, and cells exposed to b) CBE (0.5 mg/ml), c) CBE (1 mg/ml), d) CBE (1.5 mg/ml), e) CBE (2 mg/ml) of *Bauhinia* variegata.

3.6.2.3 Determination of IC50 Value of CBE Extract

Effect of different concentrations of CBE of *Bauhinia variegata* on the viability of H460 cells for different time interval (at 24 h, 48 h and 72 h) was assessed. The IC50 values were 1.0 mg/ml for 24 h, 0.78 mg/ml for 48 h and 0.74 mg/ml for 72 h as shown in Figure 3.8. The extract was more potent after 24 h than after 48 h treatment. The inhibitory concentrations for 24 and 48 h do not show much difference. So, the 24 h treatment was selected for further experiments on H460 cells.

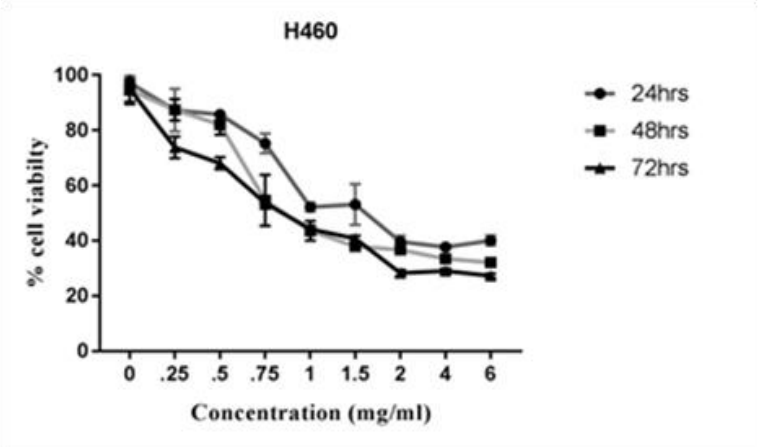
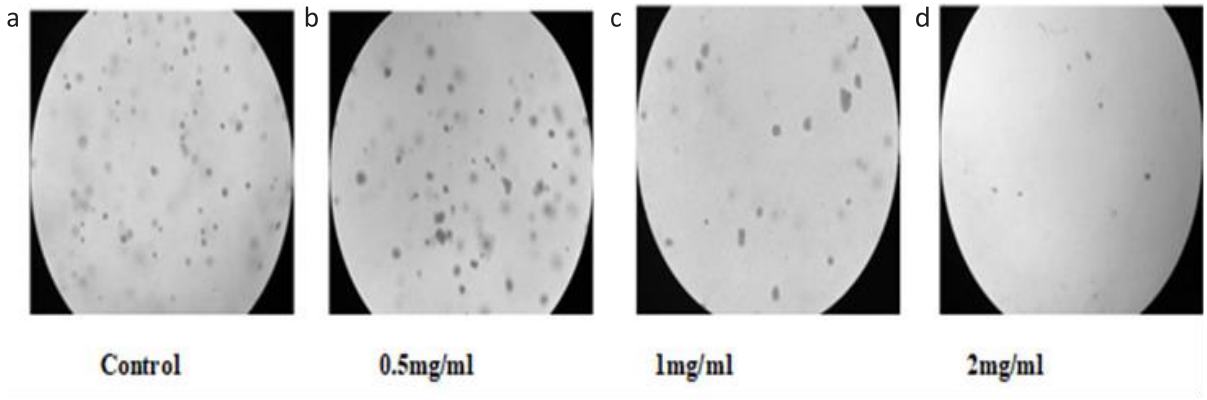


Figure 3.8 IC50 values of crude chloroform bark extract of *Bauhinia variegata* on H460 cells for 24 h, 48 h, and 72h (n=3).

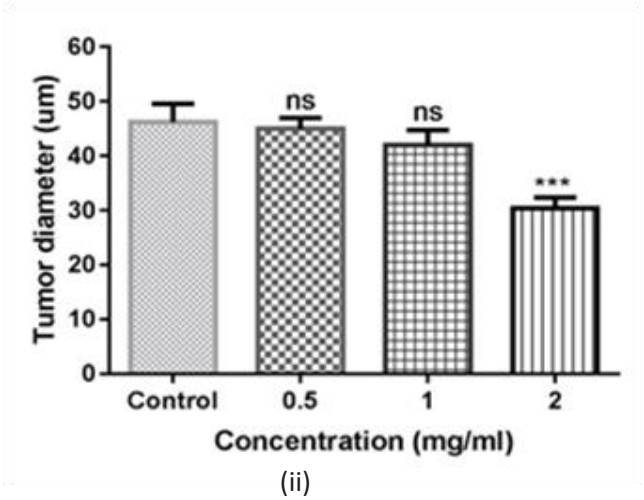
3.6.3 Colony Growth Inhibition Studies - Soft Agar Assay

3.6.3.1 PEBE Inhibits A549 Colony Growth

The ability of PEBE to inhibit the expansion of tumors (cell colonies) and therefore the spread of cancer cells was assayed *in vitro*. PEBE significantly decreased A549 colony growth in a concentration-dependent manner showing significant antitumorigenic activity at 2 mg/ ml as shown in Figure 3.9. The mean tumor diameter in the control (untreated) was 46.2 µm, while at 2 mg/ml treatment the diameter was reduced to 30.4 µm as shown in Figure 3.9(i). Quantitative analysis of these results is shown in Figure 3.9(ii).



i



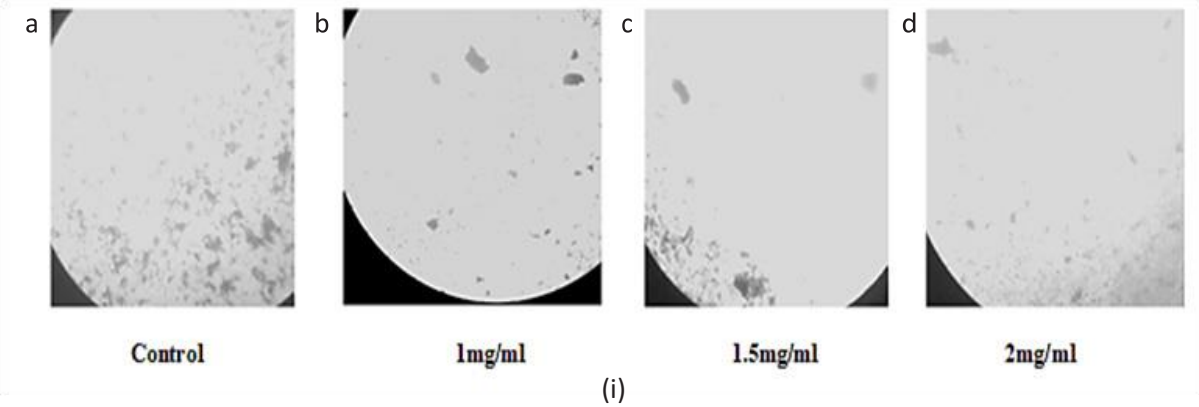
(ii)

Figure 3.9. Representative images for *in vitro* assay to assess the anti- tumorigenic activity of the PEBE on A549 cells. Cell colonies in soft agar in **(i)** a) Untreated cells, b) 0.5 mg/ml PEBE treatment, c) 1 mg/ml PEBE treatment, d) 2 mg/ml PEBE treatment; n = 3. Quantitative analysis of mean tumor (colony) diameter of different concentrations of PEBE is shown in **(ii)**

Values are the means ± SD of at least three independent experiments; ***P<0.001, ns = non-significant.

3.6.3.2 CBE Inhibits H460 Colony Growth

CBE significantly decreased colony growth of H460 cells in a concentration-dependent manner with a significant antitumorigenic activity at a concentration of 2 mg/ml Figure 3.10. The mean tumor diameter for control (untreated) is 63.38 µm which decreased to 27.93 µm in 2 mg/ml concentration treatment and showed a reduction in size of around 33.27 µm in diameter as shown in Figure 3.10(i). Lower concentrations of 1 and 1.5 mg/ml showed no significant difference in mean tumor diameter as compared to control cells. Quantitative analysis of these results is shown in Figure 3.10(ii).



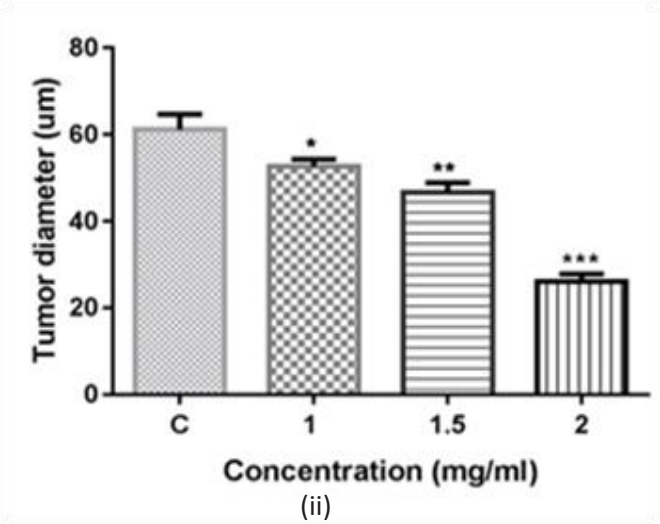


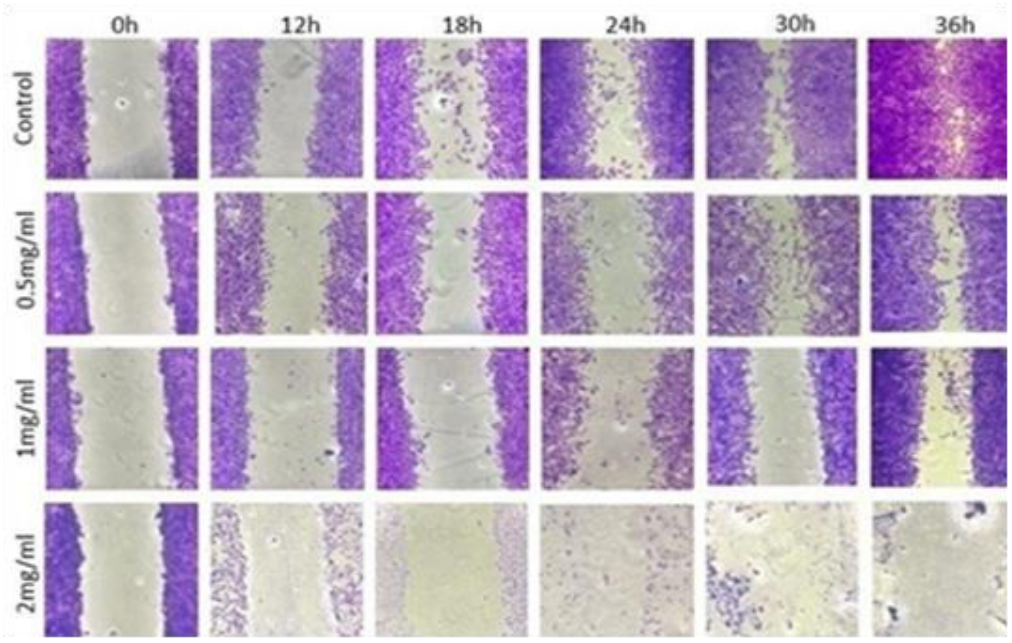
Figure 3.10 Representative images for the *in vitro* assay to assess the anti-tumorigenic activity of the CBE on H460 cells. Cell colonies in soft agar in (i) a) Untreated cells, b) 1mg/ml CBE treatment, c)1.5 mg/ml CBE treatment, d) 2mg/ml CBE treatment; n = 3. Quantitative analysis

of mean tumor (colony) diameter of different concentrations of CBE is shown in (ii) Values are the means ± SD of at least three independent experiment: **P<0.01., ***P<0.001.

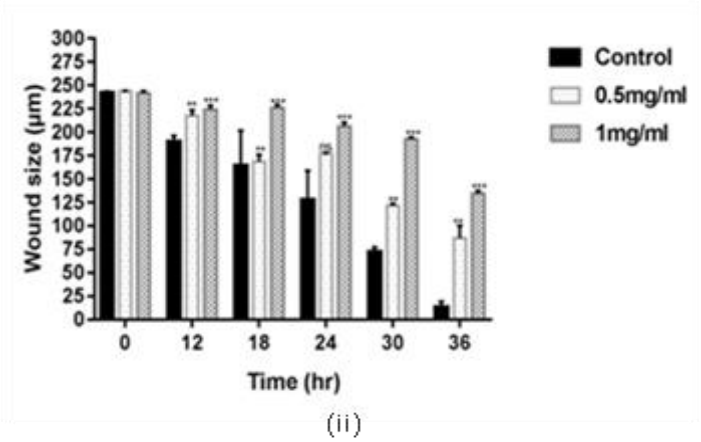
3.6.4 Wound Healing Assay/ Scratch Test

3.6.4.1 PEBE Showed Slower Migration in A549 Treated Cells

To assess the effect of the extracts on cell migration a cell scratch assay was performed. In A549 cells, as compared with the control group, gradual reduction was noticed within the number and rate of migrated cells with PEBE treatment. A slower rate of migration was observed with 0.5 and 1 mg/ml PEBE treatment while at 2 mg/ ml the cells were unable to survive. Hence, this dose was excluded from the study. The distance was more between the edges of the wound when A549 cells were treated with PEBE for 12 to 36 h, demonstrating the reduced migration of A549 cells as shown in Figure 3.11(i). Quantitative analysis of the scratch diameter is shown in Figure 3.11(ii).



(i)

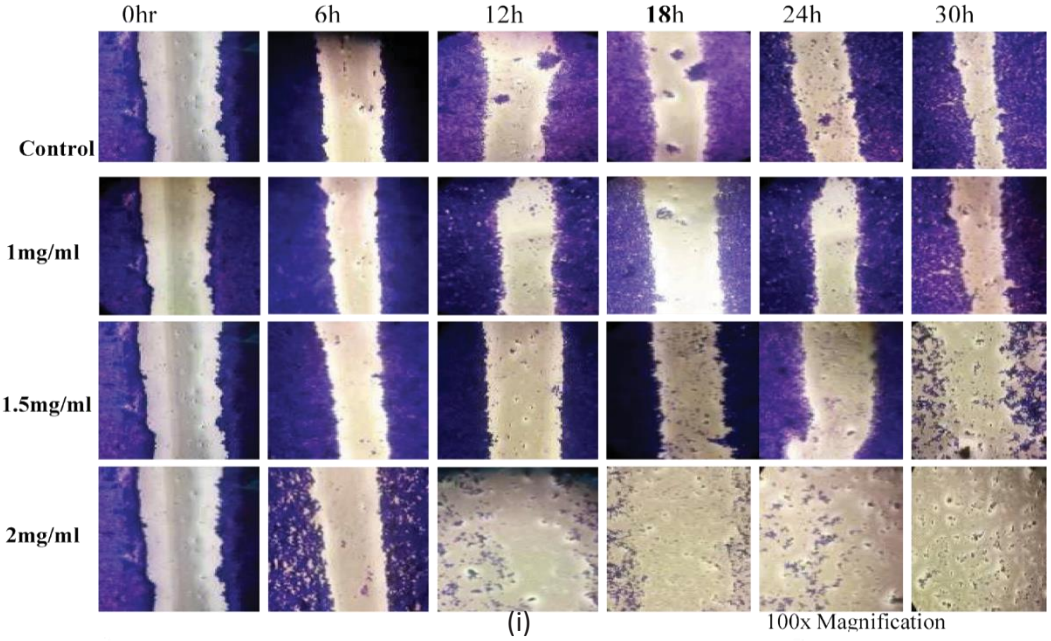


(ii)

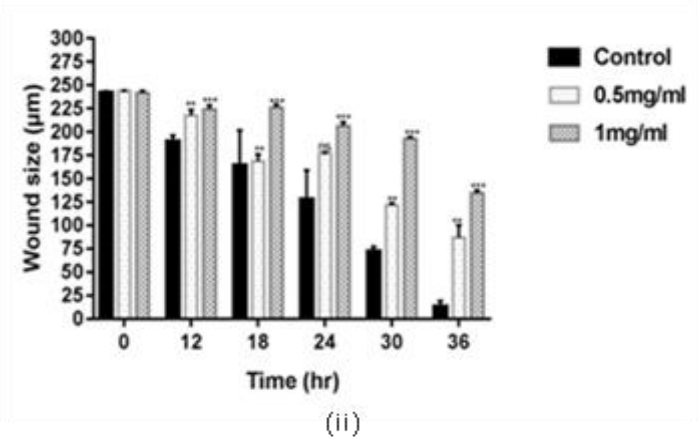
Figure 3.11 Representative images are the results of *in vitro* scratch test to assess effect of PEBE on cell migration of A549 cells; n = 3. PEBE inhibits migration of A549 cells as shown in (i). Wound healing assay to determine the effect of PEBE on A549 cell migration at 0.5, 1, 1.5 and 2mg/ml concentrations for 36h.Quantitative analysis of wound size (µm) within 36h. is represented in (ii). Error bars indicates the standard error of the mean of three independent experiments. **P<0.01, ***P<0.001.

3.6.4.2 CBE Showed Slower Migration in H460 Treated cells

The effect of different concentrations of CBE extract on H460 cells after 24 h treatment indicated a dose- dependent decrease in viability of cells in comparison to control cells. H460 cells also showed slower migration and wound healing with 1mg/ml and 1.5 mg/ml CBE treatment. CBE impaired cell migration for 12 to 30 h as shown in. The cells lost their viability at 2 mg/ml treatment, and hence this dose was not included for the comparison as shown in Figure 3.12(i). Quantitative analysis of the scratch diameter is shown in Figure 3.12(ii).



(i)



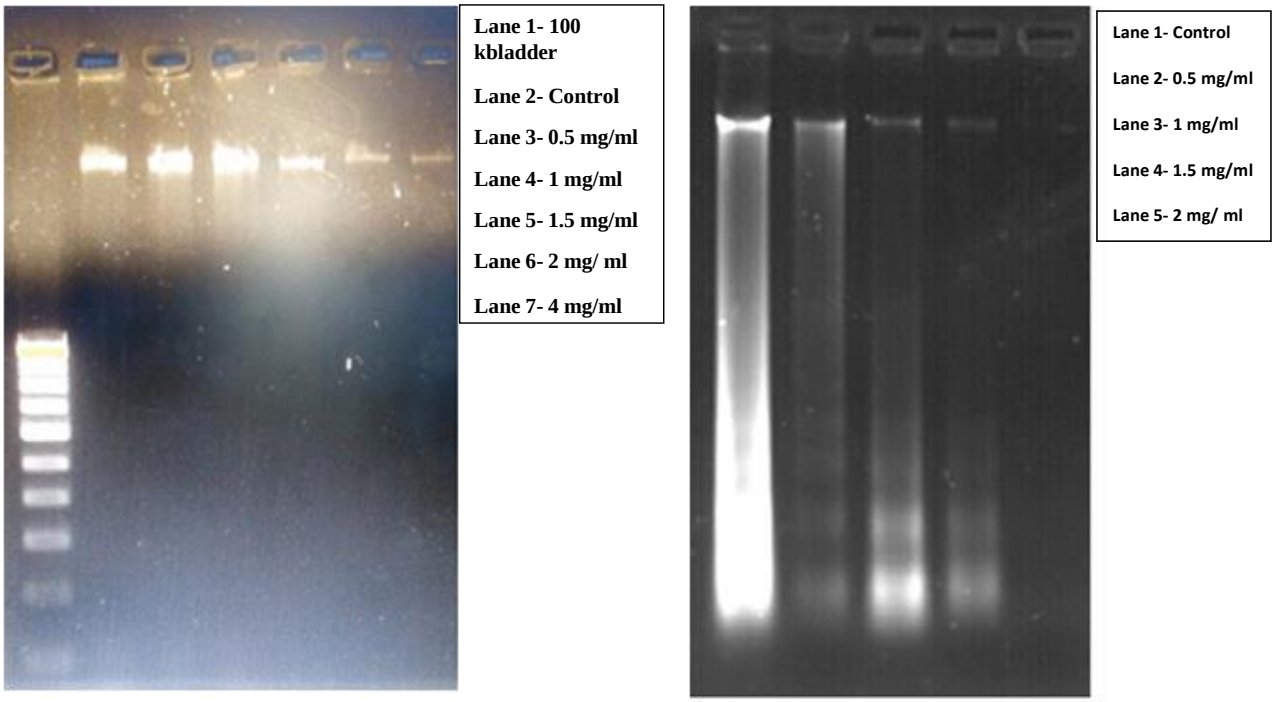
(ii)

Figure 3.12 Representative images for the results of *in vitro* scratch test to assess effect of CBE on cell migration; n = 3. CBE inhibits migration of H460cells (i) Wound healing assay to determine the effect of CBE on H460 cell migration. Quantitative analysis of wound size (µm) within 30 h. is measured here in (ii) Error bars indicate the standard error of the mean of three independent experiments: ***P<0.001.

3.6.5 DNA Fragmentation Studies

3.6.5.1 PEBE Induces Cellular DNA Fragmentation in A549 Cells

In order to explain the mechanism of cell apoptosis mediated by PEBE, we performed a DNA fragmentation assay, since DNA fragmentation is the characteristic property or apoptosis. Treatment of cells with different concentrations of PEBE for 48 h, led to a decrease in band intensity of DNA with increasing concentration of PEBE in 1 % agarose gel electrophoresis as shown in Figure 3.13a. A typical DNA ladder pattern of internucleosomal fragmentation was observed with after 48 h of treatment as shown in Figure 3.13b. The late stages of apoptosis are characterized by damage (fragmentation) of DNA[27]. These data suggest that PEBE extract is an effective inducer of Apoptosis.



DNA bands intensity decreasing with increasing concentrations ofPEBE on A549 cells. 1% gel

A

DNA fragmentation with increasing concentrations of PEBE on A549 cells. 1.8% gel

b

Figure 3.13 Representative images of DNA laddering in A549 cells (a) Cells treated with different concentrations of PEBE for 48 h results in decrease in DNA bands with increasing concentration of PEBE in 1% agarose gel electrophoresis, (b) Cells treated with increasing concentrations of PEBE for 48 h and results in typical laddering pattern in 1.8% agarose gel electrophoresis.

3.6.5.2 CBE Induces Cellular DNA Fragmentation in H460 Cells

Fragmentation of genomic DNA (light fragments) was observed in H460 cell line treated with 2 and 4 mg/ ml of CBE for 24 h as shown in Figure 3.14. A typical ladder pattern of internucleosomal fragmentation was observed in H460 cell line after 24 h at higher concentrations of CBE. Low-molecular-weight DNA from these cells was resolved in 1.8 % agarose gels. These data suggest that CBE is a potent inducer of apoptosis. Further studies are needed to establish the role of the interaction of CBE with DNA in cancer cells.

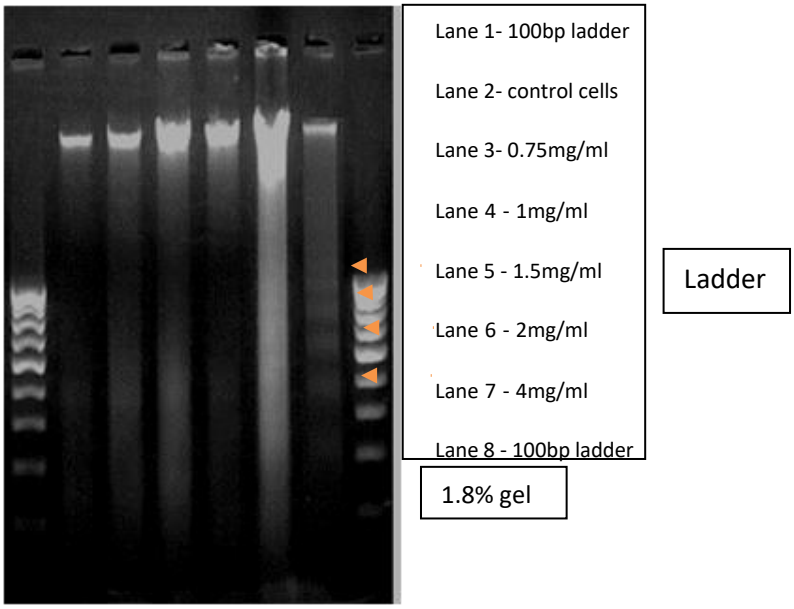


Figure 3.14 Representative images of DNA laddering in H460 cells. Cells treated with increasing concentrations of CBE for 24 h and results in typical laddering pattern in 1.8% agarose gel electrophoresis.

3.6.6 Quantification of ROS

3.6.6.1 Effects of PEBE on Intracellular ROS Levels of A549 Cells- DCHF-DA Assay

ROS levels were examined at indicated time points (0 to 10 h) after PEBE treatment on A549 cells and it was seen that the ROS levels reached a maximum at about 6 h (29.5 %, as compared

to 8 % at 0 h), but subsequently decreased as shown in Figure 3.15. On treatment with PEBE, intracellular ROS elevates at initial hours and decrease subsequently which suggest they could be activating downstream signaling pathway resulting in apoptosis. These results indicate that ROS production is an early phase event in apoptosis induced by PEBE.

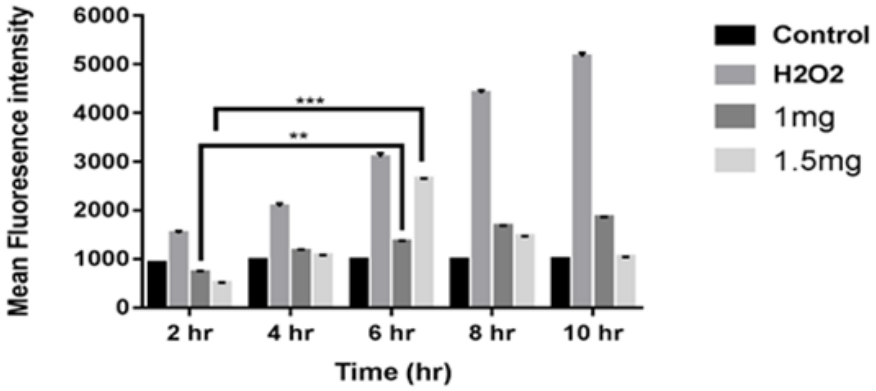


Figure 3.15 Time - dependent changes in ROS levels of A549 cells at different concentrations of PEBE. Data expressed as mean ± SEM, **p < 0.01, ***p < 0.001 as compared to control.

3.6.6.2 Effects of CBE on Intracellular ROS Levels of H460 Cells - DCHF-DA Assay

Recent studies have shown that ROS levels in a cell may have a significant role to play in the outcome of therapeutic agents28. After exposure of H460 cells to CBE, ROS levels first increase then there is a little decrease at 6h and then stability increase within the cells as shown in Figure 3.16. Data showed that CBE increased ROS generation from 2 to 10h treatment.

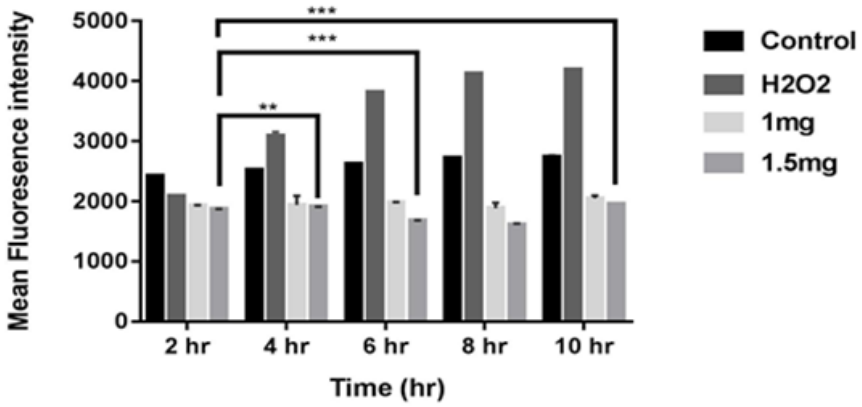


Figure 3.16 Time - dependent changes in ROS levels of H460 cells at different concentrations of CBE. Data expressed as mean ± SEM, **p < 0.01, ***p < 0.001 as compared to control.

3.6.7 Alterations in Nuclear Morphology - DAPI Staining

DAPI staining was done after treatment of A549 cells with PEBE (1 mg/ml) showed chromatin condensation, nuclear fragmentation ("horse-shoe" like appearance of nucleus) and cell shrinkage with an increase in apoptotic bodies in cells treated with 1.5 mg/ml PEBE for 48 h as shown in Figure 3.17 a, b, c. The control cells had round homogenous nuclei. The morphological changes associated with apoptosis such as margination of nucleus, chromatin condensation and nuclear fragmentation marked by arrows in Figure 3.17 d, e, f in H460 cells after 24h treatment with increasing concentrations (IC50) of extracts is very distinct.

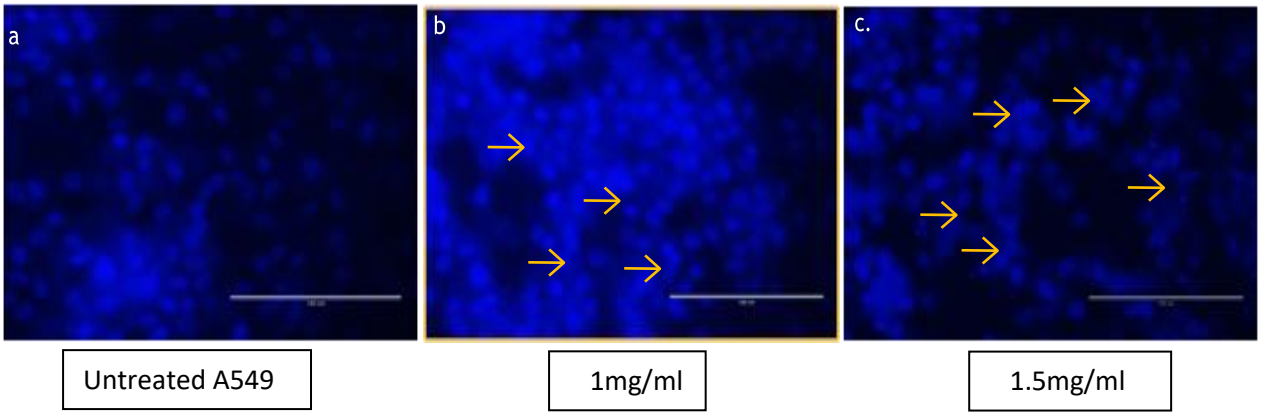


Figure 3.17 The effect of PEBE on apoptotic potential in A549 cells was evaluated using DAPI staining. a) control group; b) in the presence of 1 mg/ml; c) 1.5mg/ml of PEBE of *Bauhinia variegata* for 48 h under fluorescence microscope, Scale bar- 100 um, Mag- 40x.

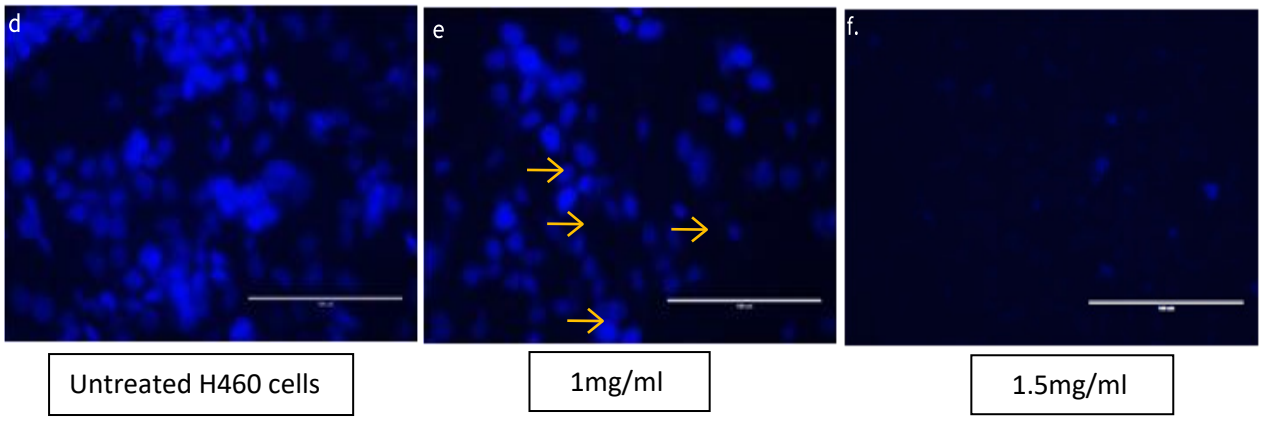


Figure 3.17 Effect of CBE on apoptotic potential in H460 cells was evaluated using DAPI staining d) control H460 cells e) 1 mg/ml f) 1.5 mg/ml CBE of *Bauhinia variegata* for 24 h under fluorescence microscope, Scale bar-100 um, Mag- 40x. Arrow indicates chromatin

condensation, nuclear fragmentation, horse- shoe shape nuclei and cell shrinkage in treated cells as compared to control cells.

3.6.8 AO/EtBr Staining

3.6.8.1 AO/EtBr Staining in A549 cells

Live cells with normal morphology were abundant in the A549 control group whereas early apoptotic cells were observed on treatment with 1 mg/ml PEBE concentration. Both early and late apoptotic cells were observed in A549 cell line treated with 1, 1.5 and 2 mg/ ml concentrations. The 2 mg/ml treatment showed the greatest number of apoptotic bodies, and the cells were mostly in the late apoptotic stage as shown by arrows in Figure 3.18 a, b, c, d. Live cells appeared green, while early apoptotic appeared bright green or yellow and late apoptotic appeared red with condensed and fragmented nuclei.

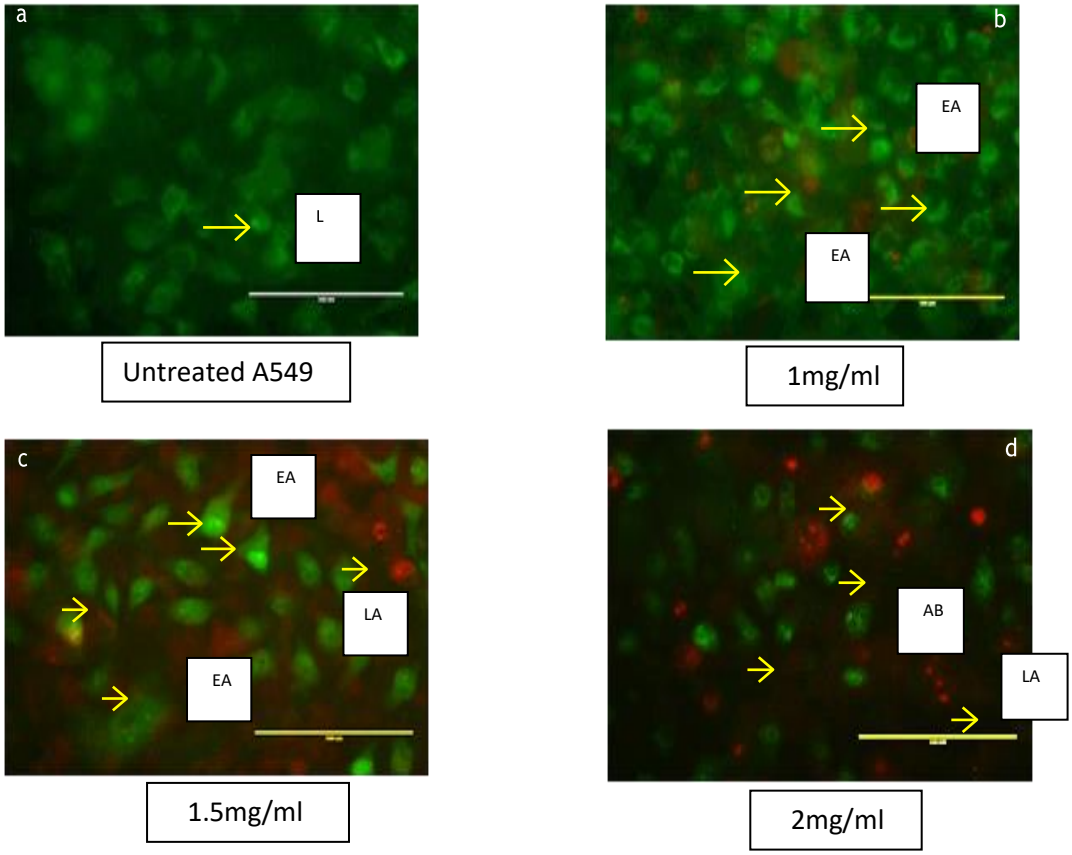


Figure 3.18 A549 cells were stained by AO/EB and observed under fluorescence microscope: **a)** A549 control group; **b)** in the presence of 1mg/ml; **c)** 1.5mg/ml; **d)** 2mg/ml of PEBE for 48h. Control wells were treated with equivalent amount of medium alone. Green live cells showed normal morphology with uniform nuclei; yellow early apoptotic cells showed nuclear margination and

chromatin condensation. Late orange/red apoptotic cells showed fragmented chromatin and apoptotic bodies.

3.6.8.2 AO/EtBr Staining in H460 cells

For H460 cell line, live cells with normal morphology were abundant in H460 control group. H460 cell line treated with 1 mg/ml CBE showed early apoptotic cells while H460 cell line treated with 1.5 mg/ml CBE showed late apoptotic bodies as shown in Figure 3.18 e, f, g.

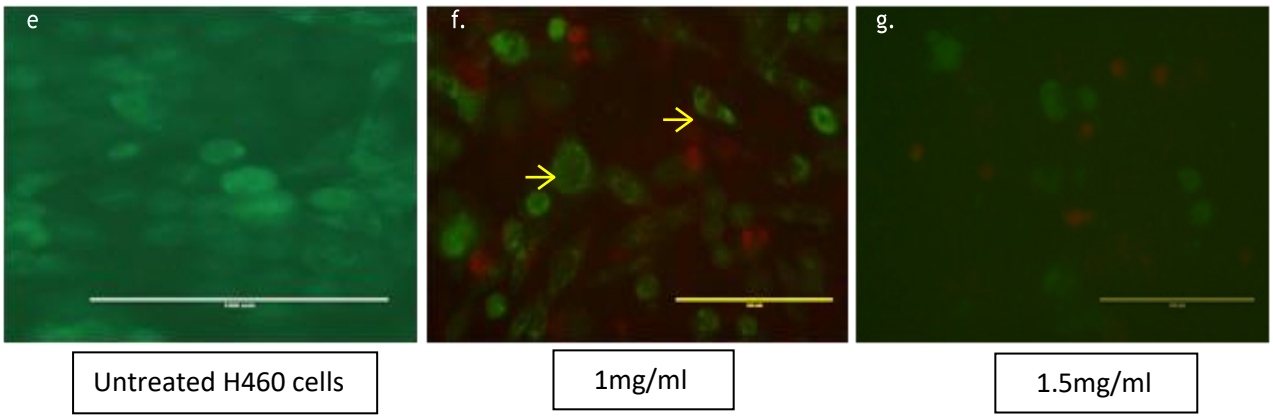


Figure 3.18 H460 cells were also stained by AO/EB and observed under fluorescence microscope: e) control group; f) in the presence of 1mg/ml; g) 1.5mg/ml of CBE for 24h. Green live cells showed normal morphology with uniform nuclei; yellow early apoptotic cells showed nuclear margination and chromatin condensation. Late orange/red apoptotic cells showed fragmented chromatin and apoptotic bodies.

3.6.9 Caspase-3 Activity in Cell Lines after PEBE and CBE Treatment

Caspase-3, a marker of apoptosis and has shown to be adequate for potent activation of apoptosis29,30. Caspase 3 activity significantly increased in PEBE treated A549 cells and CBE treated H460 cells at the IC50 value after 24 h to 48 h treatment. After 48 h of incubation of A549 cell line with PEBE there was a 3-fold increase in caspase-3 levels as compared to A549 control cells as shown in Figure 3.19 (a).

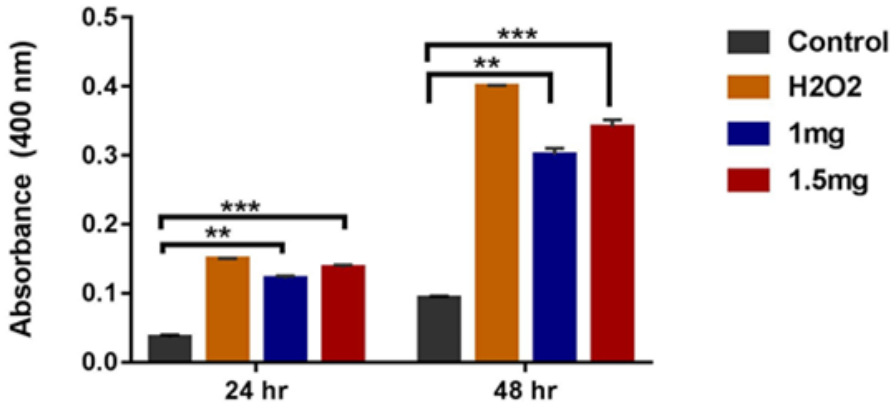


Figure 3.19 a) A549 cells were seeded in 24 well plates, then treated with PEBE in concentration and time - dependent manner. Caspase-3 activity were measured spectrophotometrically by detection of chromophore pNA at 405nm. Data expressed as mean ± SEM, n=3 **p < 0.01, ***p < 0.001 as compared to control.

Caspase-3 activity significantly increased at IC50 value of CBE from 12 h to 24 h treatment in **H460 cell line** as shown in Figure 3.19 (b). From these data, we can say that Caspase-3 may also function before or at the stage when commitment to loss of cell viability is made.

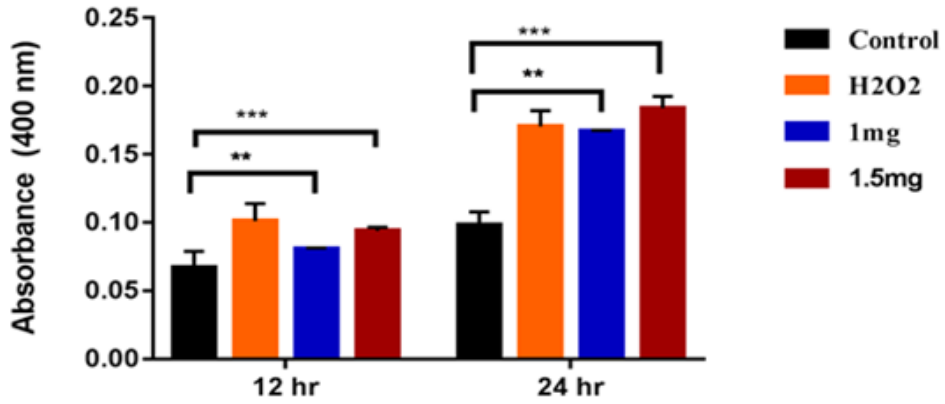


Figure 3.19 b) H460 cells were seeded in 24 well plates, then treated with CBE in concentration and time - dependent manner. Caspase-3 activity were measured spectrophotometrically by detection of chromophore pNA at 405nm. Data expressed as mean ± SEM, n=3 **p < 0.01, ***p < 0.001 as compared to control.

3.6.10 Alteration in Mitochondrial Membrane Potential by TMRM Staining

Cell health can be assessed by proper functioning of mitochondria which can be monitored by observing changes in mitochondrial membrane potential (MMP). The role of intrinsic apoptosis pathway was further validated by the changes in mitochondrial membrane potential in A549 cells treated with PEBE (1 mg, 1.5 mg) for 48 h and H460 cells treated with CBE at (1 mg,1.5 mg) for 24 h. PEBE and CBE significantly decreased the mitochondrial membrane potential (ΔΨm) in A549 as shown in Figure 3.20 a, b, c and H460 cells as shown in Figure 3.20 d, e, f with increasing concentrations.

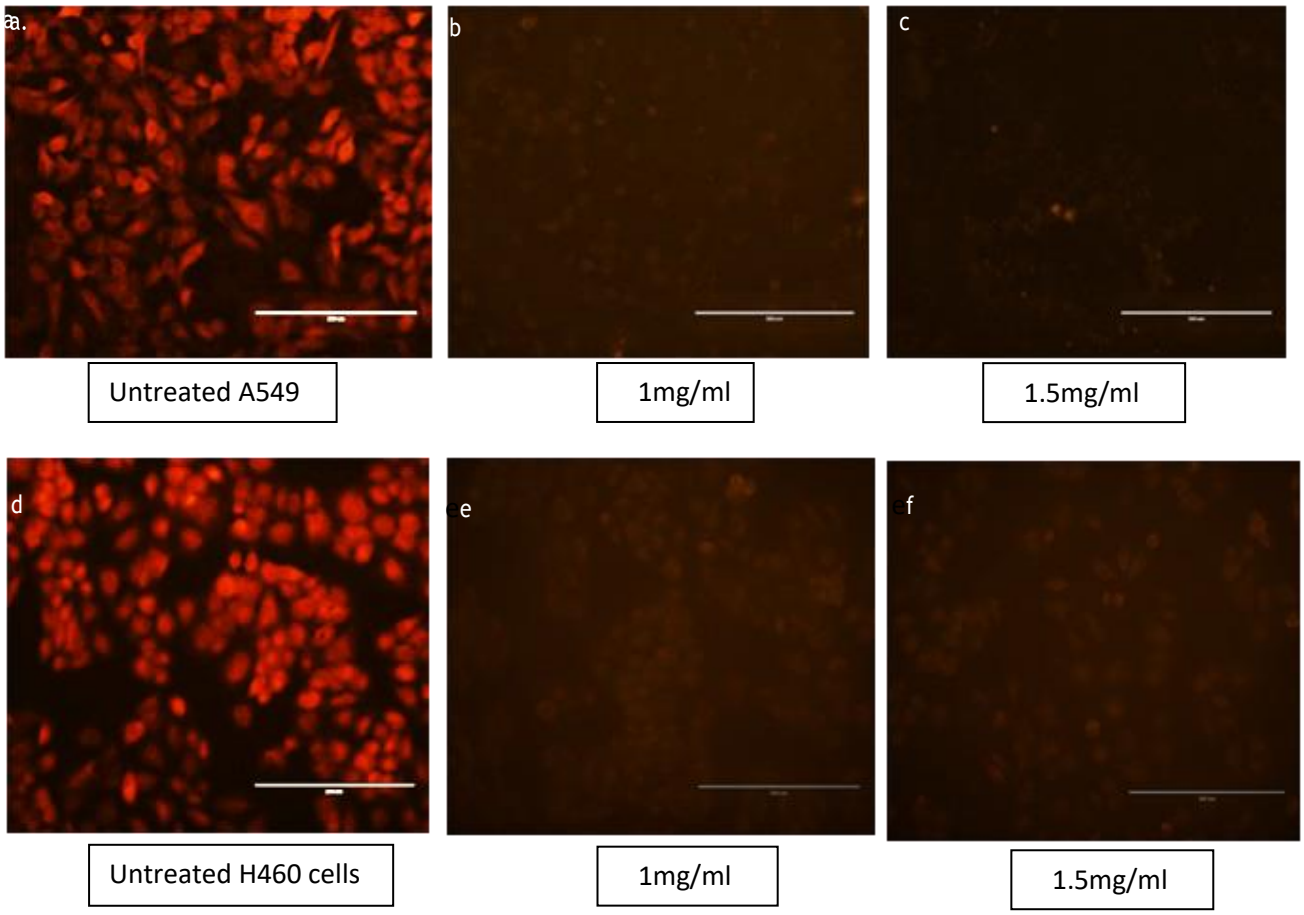


Figure 3.20 Cells were stained with TMRM and imaged by standard fluorescence techniques. PEBE (1mg/ml, 1.5mg/ml) for 48 h did not show significant change in TMRM fluorescence intensity in A549 cells (a - c), while CBE (1mg/ml, 1.5mg/ml) for 24 h significantly decreased TMRM florescence intensity in H460 cells (d - f).

3.6.11 Conclusion

Our data validates with proof of mechanism the efficacy of *Bauhinia variegata* PEBE against A549 cells proliferation and that of CBE against H460 cells thus targeting two types of lung cancer cell lines widely[36] accepted as most common forms of lung cancer. The extracts of *Bauhinia variegata* bark extracts were differently inhibiting A549 cells proliferation but petroleum ether extract was found to be the foremost potent inhibitor of A549 cells proliferation. MTT assay showed that the effect was optimal at 48 h treatment. Colonogenic study revealed that PEBE showed significant anti-tumorigenic activity. Treatment with PEBE showed a decrease in DNA bands and fragmentation of genomic DNA was observed at 48 h treatment. ROS production is an early phase in apoptosis induced by PEBE treatment. Caspase-3, an important effector of apoptosis got activated by PEBE treatment on A549 cells after 48

h. The decrease in MMP of A549 cells treated with various concentrations of PEBE was observed using TMRM dye as compared to control cells proving apoptosis.

On the other hand, H460 cells proliferation was maximally affected by but chloroform bark extract with IC50 of 1 mg/ml at 24 h. Colonogenic ability of CBE shows significant difference in mean tumor diameter. CBE acted *via* the apoptotic pathway & showed fragmentation of genomic DNA in dose and time dependent manner. Increase in ROS was responsible for CBE-induced apoptosis in H460 cell line. Unravelling the molecular mechanism showed that CBE causes the activation of caspase-3 in H460 cells at24 h treatment. Further, there is a decrease in MMP ofH460 cells treated with various concentrations of CBE as compared to untreated cells, which was observed using TMRM dye.

Thus, it can be said that PEBE and CBE induced apoptosis of A549 and H460 cell lines is through the activation of caspase-3 signaling and involves mitochondrial cell death mediated pathway, leading to observed anti cancerous effect as shown in Figure 3.22. A pictorial representation of the summary of this work is shown in Figure 3.21.

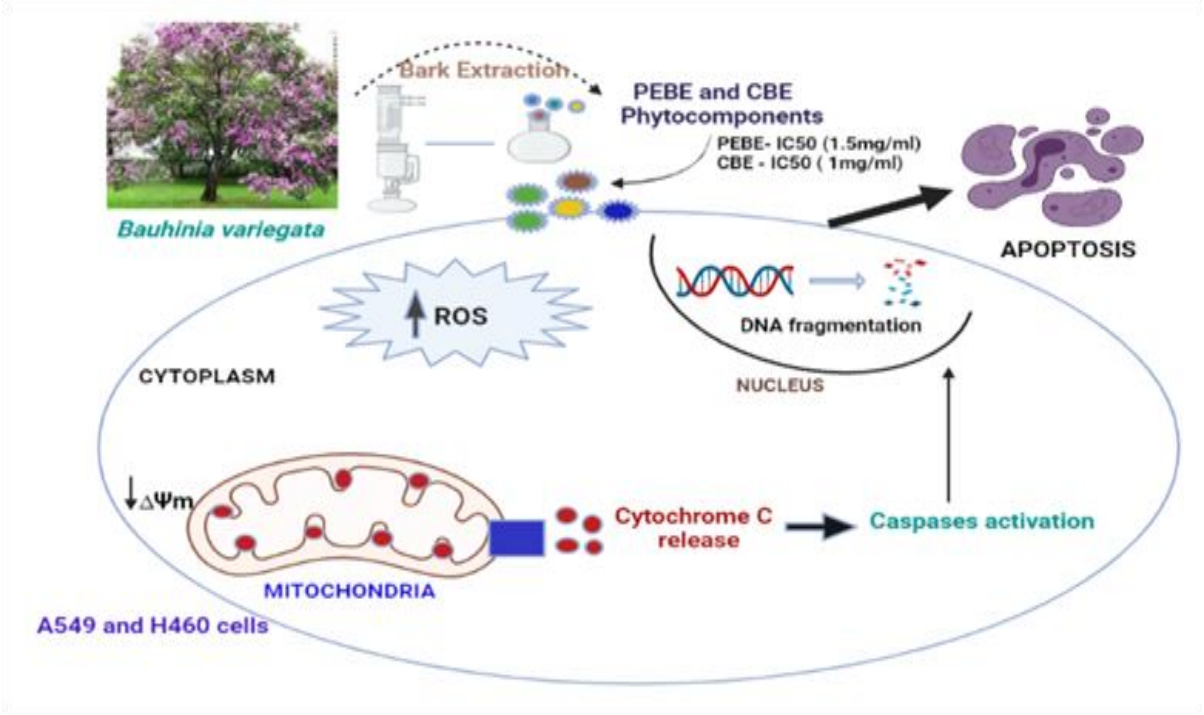


Figure 3.21 Schematic representation of the mechanism of anticancer action of PEBE and CBE of *Bauhinia variegata*.

3.7 Chemical characterization of *Bauhinia variegata* L. bark extract by TG-DSC, FT-IR and GC-MS analytical techniques.

3.7.1 Introduction

Thermal analysis has become a significant tool for characterization of dry extracts and plant products. TG alongside other techniques helps in better understanding of the organic molecules especially if the compounds are intended for pharmaceutical use[37]. FTIR analysis will help reveal the chemical composition in PEBE and CBE. The standardization and characterization of dried extracts of therapeutic plants have been demonstrated by numerous authors as uses of various thermal analysis techniques in pharmaceutical technology[38,39].

The method of drug treatment aims at finding a product which attends market necessities as well as provides efficacy, safety and quality. This thermal analysis is going tobe useful in studying the behavior of drugs degradation during its dumping process after its expiration since it may also release some toxic bye products which may be harmful for the society. The objective of this section is to characterize the dried extracts of *Bauhinia variegata* by TG/DSC analysis and FTIR coupled with GC-MS (TG- DSC-FTIR-GC-MS).

3.7.2. Thermal analysis parameters (TGA/ DSC)

The analysis of the change within the mass of a sample on heating is understood as thermogravimetric analysis (TG). For TGA procedure, about 4 mg of PEBE and CBE samples were analyzed within the temperature interval of 30–600°C with a continuing heating rate of 10 °C /min using Al_2O3 crucible under a nitrogen flow of 50 ml/min during a (NETZSCH STA 449F3Jupiter. 2005). All mass loss percentages were determined using Proteus analysis software. The DSC curves of PEBE and CBE were obtained during a NETZSCH DSC 200F3 Maia, using PAN aluminum crucibles with about 3-4 mg of samples under nitrogen atmosphere at the flow of 50 mL/min. The pan was sealed and transferred to the heating chamber to equilibrate for 2hrs. As a reference, a pan that was empty was used. Rising temperature experiments were conducted at the temperature range from 25 to 300 °C and heating rate of 10° C /min. Software called Proteus was used to analyze the data.

3.7.3 Fourier-transform infrared spectroscopy (FTIR)

Dried PEBE and CBE, 10 mg was encapsulated in 100 mg of KBr pellet so as to organize translucent sample discs. The powdered sample was loaded in FTIR spectroscope (BRUKER, FTIR spectrophotometer, Alpha II) with a scan range from 500 to 4000 cm[40].

3.7.4 Gas chromatography–mass spectrometry Analysis for identification of compounds (GC-MS)

GC-MS analysis was administered on a Perkin Elmer Turbo-mass coupled with GC-Auto-XL, MS at 70eV using helium as carrier gas[40].

3.8 Results and Discussion

3.8.1 Thermal analysis parameters of extracts:

In this thermograph, it can be observed that with increase in temperature, the substances undergo gradual decomposition,then the change within the mass of the substances occurs with the breaking of chemical bonds at the elevated temperatures. This analytical technique is often used for characterization of plant extracts. The TG curves obtained for *Bauhinia variegata* extracts under N2 atmosphere (pyrolysis) are presented in Figure 3.22 a and 3.22b. The extract thermal comportment was divided into three sequential steps, with the primary one associated with loss of loosely bound water due to sample dehydration. In graph's, until 200°C, PEBE and CBE showed thermal stability with minor variations in mass. Around 150 - 200 ◦C, a change in mass% was observed as PEBE – 1.73% and CBE – 4.24% and possibly related to non-oxidative degradation of extracts. Exothermic peaks arerepresentative during the thermal degradation of plant extracts.

Finally, from 350 to 600 ◦C, the PEBE was thoroughly degraded, leaving 17.96% residual mass. For CBE, from 320 to 600 ◦C, the extract got fully degraded leaving 21.34% residual mass. This is often mostly due to non-oxidative atmosphere used for thermal analysis. As observed in TGA results of extracts, thermal events like mass losses were within the range of 30–100 ◦C (Phase I), 100–360 ◦C (Phase II) and 360–600 ◦C (Phase III).

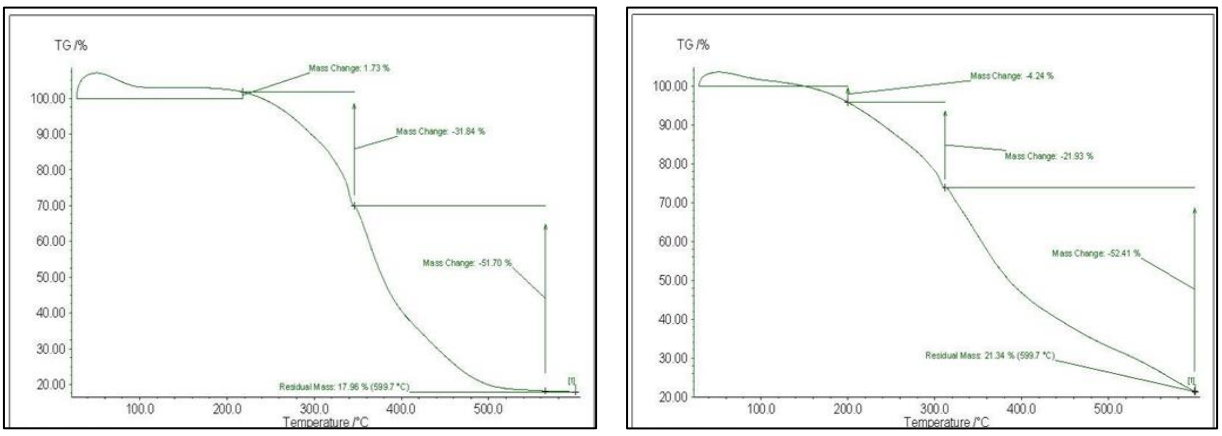


Figure 3.22 a) TG curves for dried PEBE and b) CBE of *Bauhinia variegata*

DSC (differential scanning calorimetry) is a technique to measure the changes in materials as a function of temperature and time. In Figure 3.23 a and 3.23 b, the schematic DSC curve shows typical thermal effect. The curve shows three transition phases, glass transitions labelled (1), the peak due to crystallization labelled as (2) and eventually the decomposition labelled as (3). Peak area corresponds to the enthalpy involved during the process. For PEBE, the melting temperatures, within the differential scanning calorimetry (DSC) curve, occurred between 55 and 100°C (peak at 79.4°C) indicating a mix of the extract. For CBE, the melting temperature arose between 50 - 70°C (peak at 51.2°C). The decomposition processes begin above 140°C for PEBE and for CBE and it starts above 110°C.

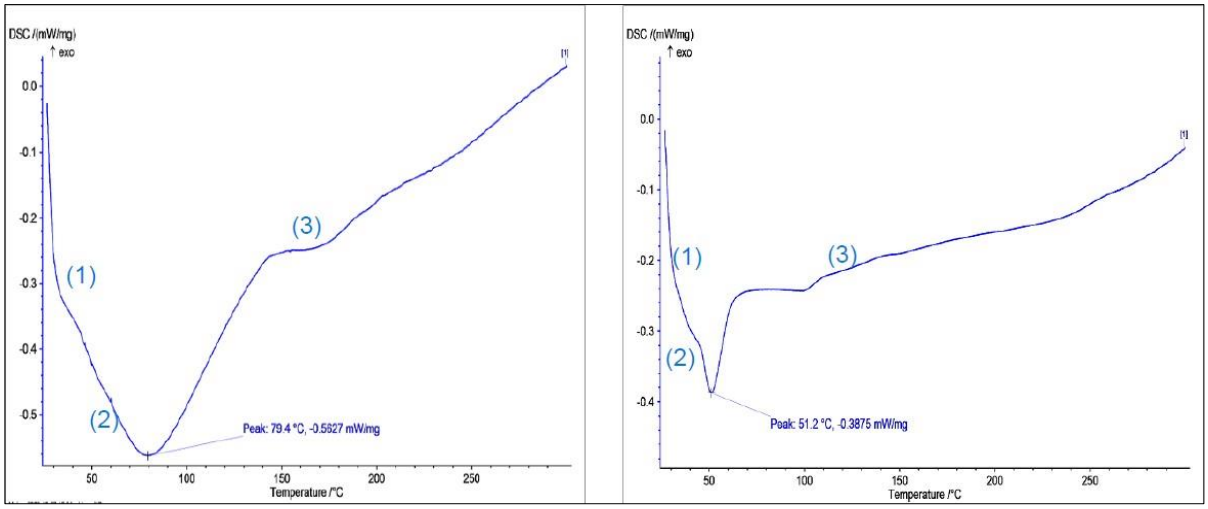


Figure 3.23 a) DSC curves for dried PEBE and b) CBE of *Bauhinia variegata*

3.8.2 Fourier-transform infrared spectroscopy (FTIR)

The data on the peak values and the possible functional groups (obtained by FTIR analysis) present in the bark extracts (prepared in PE, CHL) of *Bauhinia variegata* are presented in Figure 3.24 a, 3.24b and Table 3.4.

Petroleum ether (PE) extract: P.E extract of *Bauhinia variegata* exhibited a characteristic band at 1460 cm^{-1} indicating the presence of C-H group, 1734 cm^{-1} pair of carbonyls (C=O) group, 2926.34 cm^{-1} for C-H stretching and 3437.92 for -OH group.

Chloroform (CHL) extract: The characteristic absorption band were exhibited at 3019.75 cm^{-1} (for OH group), 2927.24 cm^{-1} (for C-H stretching), 2854.93 cm^{-1} (for OH group), 1215.55 and 1261.33 cm^{-1}.

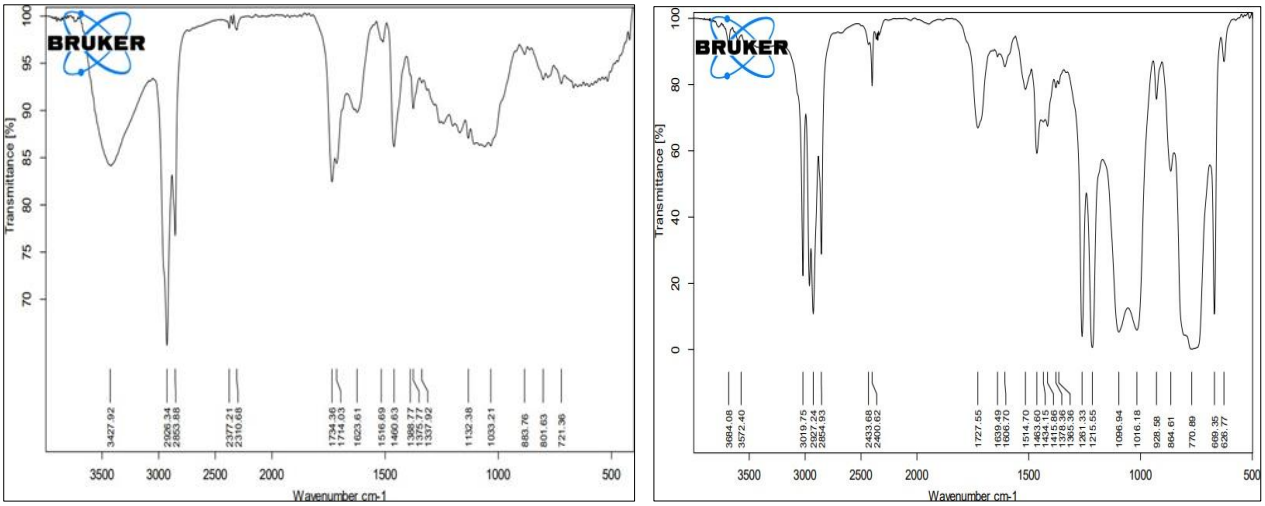


Figure 3.24 a) FTIR graph of PEBE of *Bauhinia variegata* and b) CBE of *Bauhinia variegata.*

Table 3.4 FTIR spectral peak values and type of groups obtained for the bark extract (in petroleum ether and chloroform bark extract solvents) of *Bauhinia variegata*

Extracts (Petroleum Ether)	Peak values	Type	Intensity	Extracts Chloroform	Peak values	Type	Intensity
Petroleum Ether	1388.77	C-H bending	m	**Chloroform**	669.35	C-Br stretch	S
	1460.63	C-H bend	m		770.89	C-H bending	S
	1516.69	C-N=O	S		928.58	C-H stretch	m
	1623.61	C=C group	V		1016.18	C-OH group	S
	1714.03	C=O carbonylgroup	S		1096.94	C-OH group	S
	1734.36	C=O carbonylgroup	S		1215.55	C=O carbonyl group	S
	2853.88	C-H stretching	S		1261.33	C=O carbonyl group	S

	2926.34	C-H stretching	S		1463.60	C-H stretching	m
Petroleum Ether	3427.92	-OH group	V		1727.55	C=O carbonyl group	S
					2433.68	-NH group	m
					2854.93	-OH group	V
					2927.24	C-H stretching	S
				Chloroform	3019.75	-OH group	V

3.8.3 Gas Chromatography/Mass Spectrometry Analysis

The GC/MS chromatogram showed the presence of various compounds in PEBE and CBE of *Bauhinia variegata* as shown in table 3.5 and 3.6. Identification of the phytochemicals is based on the peak area; retention time and molecular formula (table 3.5. and table 3.6).

Table 3.5. Phytocomponents identified in the petroleum ether bark extract (PEBE) of *Bauhinia variegata* by GC/MS. CAS: Chemical abstract service

S. No.	RT (min.)	Name of compound	Molecular formula	Molecular weight	CAS
1.	9.05 min.	Benzene,1,3- bis (1,1-Dimethyl ethyl)	$C_{14}H_{22}$	190	1014-60-4
2.	9.43 min.	Trans-2- Undecen-1-ol	$C_{11}H_{22}O$	170	75039-84-8
3.	9.87 min.	Trans-2-undecen-1-ol	$C_{11}H_{22}O$	170	75039-84-8
4.	11.52 min.	Oxalic acid, allyl hexadecyl ester	$C_{21}H_{38}O_4$	354	-
5.	12.92 min.	1-Iodo-2-methylundecane	$C_{12}H_{25}I$	296	73105-67-6
6.	13.56 min.	Phenol,2,4 -bis (1,1-dimethylethyl)	$C_{14}H_{22}O$	206	96-76-4

7.	16.05 min.	Heptadecane,2,6,10,15 -tetramethyl	$C_{21}H_{44}$	296	54833-48-6
8.	19.92 min.	Stearic acidhydrazide	$C_{18}H_{38}N_2O$	298	4130-54-5
9.	20.61 min.	Phthalic acid, nonyl tridec-2-yn-1-yl ester	$C_{30}H_{46}O_4$	470	-
10.	23.46 min.	n-hexadecenoicacid	$C_{16}H_{32}O_2$	256	57-10-3
11.	25.04 min.	Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1
12.	25.96 min.	Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1
13.	26.97 min.	2-Methyl-Z-4-tetradecene	$C_{15}H_{30}$	210	-
14.	27.38 min.	Oleic acid	$C_{18}H_{34}O_2$	282	112-80-1
15.	27.64 min.	Erucic acid	$C_{22}H_{42}O_2$	338	112-86-7
16.	27.96 min.	Tert- Hexadecanethiol	$C_{16}H_{34}S$	258	25360-09-2
17.	29.88 min.	Heptadecanoicacid	$C_{17}H_{34}O_2$	270	506-12-7
18.	30.76 min.	12- Methyl- E, E-2,13-octadecadien-1-ol	$C_{19}H_{36}O$	280	-

Table 3.6 Phytocomponents identified in the chloroform bark extract (CBE) of *Bauhinia variegata* by GC/MS.

S.N.	**Retentio ntime**	**Name of compound**	**Molecular formula**	**Molecular Weight**	**CAS**
1	11.04 min.	Phenol,2,4-bis (1,1dimethylethyl)	C14H22O	206	96-76-4
2	18.82 min.	Hexadecanoic acid, ethyl ester	C18H36O2	284	628-97-7

3	21.96 min.	Oleic acid	C18H34O2	282	112-80-1
4.	23.97 min.	Oleic acid	C18H34O2	282	112-80-1
5	25.54 min.	7-Methyl-Z tetradecen-1-ol acetate	C17H32O2	268	-
6	27.89 min.	Oleic acid	C18H34O2	282	112-80-1
7	29.65 min.	4-(1,1-Dimethylallyl)-9-methoxy-7H-furo(3,2-g) [1] benzopyran-7-one	C17H16O4	284	34155-80-1
8	30.35 min.	6-Hydroxy-3-(3,5di methyoxypheny) -benzo (b)furan	C16H14O4	270	241472

3.9 Conclusion

Data on thermal and FT-IR analysis on a plant derived raw material is important to establish parameters on the development of therapeutic products which will help in insuring its quality, safety and efficacy. This study also showed that thermal disposal of *Bauhinia variegata* PEBE and CBE results in the release of harmless bye products which definitely increase the application of this efficient anti-cancerous drug in pharmaceutical applications where this safe disposal may be used as a commercial claim.

3.10 References

1. Jyoti, S. SS, Kamal K, et al. Diversity, distribution, indigenous uses and conservation prioritization of the economically important floristic diversity in Nadaun Block of Hamirpur District, Himachal Pradesh. *Int J Biodivers Conserv*. 2014;6(7):522-540. doi:10.5897/ijbc2014.0710

2. Sengupta R, Desai D. Physicochemical and preliminary phytochemical studies on Kachnar bark (*Bauhinia variegata*). 2015;7(7):67-71.

3. Gilman EF, Watson DG, Klein RW, Koeser AK, Hilbert DR, Mclean DC. *Bauhinia variegata* : Orchid Tree 1. Published online 2018:2-4.

4. Science A, No V. An ethnobotanical study of madhya pradesh 1: plants used against various disorders among tribal women t r sahu. 1982;(3):178-181.

5. Mahajan SG, Mehta AA. Phcog Rev . : Plant Review Rakta Kanchan (*Bauhinia variegata*): Chemistry , Traditional and Medicinal uses- a review. 2007;1(2):314-319.

6. Nariyal V, Sharma P. Kanchnar (*Bauhinia variegata*) As a Medicinal Herb: a Systematic Review. *Int J Adv Res*. 2017;5(9):587-591. doi:10.21474/ijar01/5364

7. Khare P, Kishore K, Sharma DK. Review Article Historical aspects , Medicinal uses , Phytochemistry and Pharmacological review of *Bauhinia variegata*. 2018;4(5):546-562.

8. **Tanvi K, Akash D, Pushpa R. Phytochemical evaluation and Chemical characterization of *Bauhinia variegata* L . bark extract by TGA / DSC , FT-IR and GC-MS analytical techniques : Pharmaceutical Aspects. 2022;26(7):120-126.**

9. Mishra A, Sharma AK, Kumar S, Saxena AK, Pandey AK. *Bauhinia variegata* leaf extracts exhibit considerable antibacterial, antioxidant, and anticancer activities. *Biomed Res Int*. 2013;2013. doi:10.1155/2013/915436

10. Ks K, Neivashini M, Xc O, Nabila P, Khan NH. Madridge Qualitative Study on the Phytochemical Constituents of the Flower Buds of *Bauhinia variegata*. 2000;2(1):47-51. doi:10.18689/mjpr-1000108

11. Bodke YD. Isolation of Phytoconstituents from the Stem Bark of *Bauhinia variegata* Linn. 2014;2(9):150-156.

12. Uddin G, Sattar S, Rauf A. Preliminary Phytochemical , *In vitro* Pharmacological Study

of *Bauhinia alba* and *Bauhinia variegata* flowers. 2012;1(4):75-79. doi:10.5829/idosi.mejmpr.2011.1.4.1117

13. Shahana S, Pratima A, Nikalje G. Brief Review on Bauhinia variegata: Phytochemistry, Antidiabetic and Antioxidant potential. *Am J PharmTech Res*. 2017;7(1). https://www.researchgate.net/profile/Anna-

14. Hollman PCH. Evidence for health benefits of plant phenols : local or systemic effects ? 2001;852(March). doi:10.1002/jsfa.900

15. Kumar S, Baniwal P, Kaur J, Kumar H. Kachnar (*Bauhinia variegata*) 18. 2020;(December). doi:10.1007/978-981-15-7285-2

16. Antidiabetic activity of stem bark of *Bauhinia variegata* in hyperglycemic rats. 2012;3(1):64-66. doi:10.4103/0976-500X.92522

17. Monga G, Koppula S, Devi K, Srivastava S, Kumar S. Exploring Efficacy of *Bauhinia variegata* as Medicinal Herb in Combating Different Clinical Conditions : A Systematic Review. Published online 2022:1-13. doi:10.23880/beba-16000175

18. Bhandari J, Muhammad BT, Thapa P, Shrestha BG. Study of phytochemical, anti-microbial, anti-oxidant, and anti-cancer properties of Allium wallichii. *BMC Complement Altern Med*. 2017;17(1):1-9. doi:10.1186/s12906-017-1622-6

19. Parikh PM, Ranade AA, Govind B, et al. Lung cancer in India: Current status and promising strategies. *South Asian J Cancer*. 2016;05(03):093-095. doi:10.4103/2278-330x.187563

20. Debevec L, Debeljak A. Multidisciplinary management of lung cancer [1]. *J Thorac Oncol*. 2007;2(6):577. doi:10.1097/JTO.0b013e318060f16d

21. Lu T, Yang X, Huang Y, et al. Trends in the incidence, treatment, and survival of patients with lung cancer in the last four decades. *Cancer Manag Res*. 2019;11:943-953. doi:10.2147/CMAR.S187317

22. Huang CY, Ju DT, Chang CF, Muralidhar Reddy P, Velmurugan BK. A review on the effects of current chemotherapy drugs and natural agents in treating non-small cell lung cancer. *Biomed*. 2017;7(4):12-23. doi:10.1051/bmdcn/2017070423

23. Jin S, Park H-J, Oh YN, et al. Anti-cancer Activity of Osmanthus matsumuranus Extract

by Inducing G2/M Arrest and Apoptosis in Human Hepatocellular Carcinoma Hep G2 Cells. *J Cancer Prev*. 2015;20(4):241-249. doi:10.15430/jcp.2015.20.4.241

24. Richardson JSM, Sethi G, Lee GS, Malek SNA. Chalepin: Isolated from Ruta angustifolia L. Pers induces mitochondrial mediated apoptosis in lung carcinoma cells. *BMC Complement Altern Med*. 2016;16(1):1-27. doi:10.1186/s12906-016-1368-6

25. Galadari S, Rahman A, Pallichankandy S, Thayyullathil F. Reactive oxygen species and cancer paradox: To promote or to suppress? *Free Radic Biol Med*. 2017;104(January):144-164. doi:10.1016/j.freeradbiomed.2017.01.004

26. Aggarwal V, Tuli HS, Varol A, et al. Role of reactive oxygen species in cancer progression: Molecular mechanisms and recent advancements. *Biomolecules*. 2019;9(11). doi:10.3390/biom9110735

27. Hensley P, Mishra M, Kyprianou N. Targeting caspases in cancer therapeutics. *Biol Chem*. 2013;394(7):831-843. doi:10.1515/hsz-2013-0128

28. Olsson M, Zhivotovsky B. Caspases and cancer. *Cell Death Differ*. 2011;18(9):1441-1449. doi:10.1038/cdd.2011.30

29. Jelínek M, Balušíková K, Schmiedlová M, et al. The role of individual caspases in cell death induction by taxanes in breast cancer cells. *Cancer Cell Int*. 2015;15(1):1-16. doi:10.1186/s12935-015-0155-7

30. Zhao T, Pan H, Feng Y, Li H, Zhao Y. Petroleum ether extract of Chenopodium album L. Prevents cell growth and induces apoptosis of human lung cancer cells. *Exp Ther Med*. 2016;12(5):3301-3307. doi:10.3892/etm.2016.3765

31. Franken NAP, Rodermond HM, Stap J, Haveman J, van Bree C. Colonogenic assay of cells in vitro. *Nat Protoc*. 2006;1(5):2315-2319. doi:10.1038/nprot.2006.339

32. Liang CC, Park AY, Guan JL. In vitro scratch assay: A convenient and inexpensive method for analysis of cell migration in vitro. *Nat Protoc*. 2007;2(2):329-333. doi:10.1038/nprot.2007.30

33. Hsia TC, Yu CC, Hsu SC, et al. Cantharidin induces apoptosis of H460 human lung cancer cells through mitochondria-dependent pathways. *Int J Oncol*. 2014;45(1):245-254. doi:10.3892/ijo.2014.2428

34. Lin W, Ye H. Anticancer activity of ursolic acid on human ovarian cancer cells via ROS and MMP mediated apoptosis , cell cycle arrest and downregulation of PI3K / AKT pathway. 2020;25(2):750-756.

35. Alizadeh J, Zeki AA, Mirzaei N, et al. Mevalonate Cascade Inhibition by Simvastatin Induces the Intrinsic Apoptosis Pathway via Depletion of Isoprenoids in Tumor Cells. *Sci Rep*. 2017;7(February):1-14. doi:10.1038/srep44841

36. **Khanna T, Dave A, Purani S, Vedamurthy J, Jivani D, Robin P. *Bauhinia variegata* Bark Extract: Assessment of its Anti-proliferative and Apoptotic Activities on A549 and H460 Lung Cancer Cell Lines. *J Nat Remedies*. 2022;22(2):175-195. doi:10.18311/jnr/2022/28740**

37. Ford JL, Mann TE. Fast-Scan DSC and its role in pharmaceutical physical form characterisation and selection. *Adv Drug Deliv Rev*. 2012;64(5):422-430. doi:10.1016/j.addr.2011.12.001

38. Jamir K, Badithi N, Venumadhav K, Seshagirirao K. Characterization and comparative studies of galactomannans from Bauhinia vahlii, Delonix elata, and Peltophorum pterocarpum. *Int J Biol Macromol*. 2019;134:498-506. doi:10.1016/j.ijbiomac.2019.05.080

39. Sousa R, Figueirinha A, Batista MT, Pina ME. Formulation Effects in the Antioxidant Activity of Extract from the Leaves of Cymbopogon citratus (DC) Stapf. *Molecules*. 2021;26(15):4518. doi:10.3390/molecules26154518

40. Pakkirisamy M, Kalakandan SK, Ravichandran K. Phytochemical screening, GC-MS, FT-IR analysis of methanolic extract of Curcuma caesia roxb (black turmeric). *Pharmacogn J*. 2014;9(6):952-956. doi:10.5530/pj.2017.6.149