Synopsis of the Ph.D. thesis on

Effect of phytocomponents from *Bauhinia variegata* L. on Lung Cancer cell lines.

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Introduction:

Cancer is an uncontrolled proliferation of cells. Exponential increase in cancer incidences is a global burden. The incident rate of lung cancer is increasing rapidly with increasing mortality. 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[1,2]. Tobacco consumption is one of the key risk factors for lung cancer. The other risk factors are genetic susceptibility, diet, alcohol consumption, environmental exposure, and air pollutants. There are two main subtypes of lung cancer: Non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). Non-small cell lung cancer (NSCLC) contributes for about 85 % of the lung cancer cases while 15 % cases are SCLC[2]. The most typical NSCLC is adenocarcinoma which constitute about 40%, followed by large cell carcinoma which is around 15 %[3]. Hence, in this study A549 (adenocarcinoma) and H460 (large cell carcinoma) cell lines are used. Moreover, major cause for therapeutic failure in NSCLC is drug resistance which leads to tumour recurrence and disease progression. The 5-year relative survival rate of lung cancer has increased with time, but less than 21%[3]. Cancer treatments include chemotherapy, radiation therapy and surgery. Though multiple synthetic chemotherapeutic agents have been developed and some are in clinical trials, their therapeutic scale is restricted by their toxicity. The poor survival rate along with low efficacy and side effects of chemotherapy (20-30 %) are major causes of concern in lung cancer[4,5]. The side effects related to present drugs motivates scientists to search for anticancer compounds from natural sources such as plant phytochemicals.

Natural products have been used since ancient times for the treatment of different types of diseases. Phytocomponents have lower toxicity providing an attractive alternative in cancer therapy[6,7]. Plant of our interest is *Bauhinia variegata,* a species in the legume family, Fabaceae. It is commonly known as Mountain Ebony, which is a medium-sized deciduous tree found throughout India. *Bauhinia variegata* L. has been mentioned in traditional texts to have multiple pharmacological activities[8] with preliminary proof of *in-vitro* cytotoxic activity of leaf and bark extracts on different types of cancers. The fact that *Bauhinia variegata* is regarded as medicinal in traditional medicine is not in disagreement with recent findings. For example, it is seen to have anti-ulcer activity[9]. Leaf extracts of the plant have shown molluscicidal activity against the snail *Lymnaea acuminata* which is the vector for fasciolosis[10]. Leaf extracts have also shown Antinociceptive and Anti-inflammatory qualities[11]. Anti- inflammatory activity is in fact, shown by bark extracts as well[12]. Hepatoprotective activity in intoxicated mice is also shown by bark extracts[13]. Antidiabetic activity is shown in hyperglycemic rats using bark extract. Apart from these medicinal uses, the plant also negatively affects the growth of several microorganisms. Anti-bacterial activity was seen in Leaf methanol extract bark acetone and bark methanol extract[14]. Even flowers from the plant have shown antibacterial as well as antioxidant activity[15]. The anticancer potential of the plant is also being recognized in recent years. Bark ethanol extract of *Bauhinia variegata* has shown antitumor activity against Dalton's ascetic lymphoma in Swiss albino mice[16], chemo preventive and cytotoxic effect in liver tumour and cell lines[17], antitumor activity against Ehrlich Ascites Carcinoma Induced Mice[16]. Methanol extracts have shown anticarcinogenic and antimutagenic potential in melanoma in Swiss Albino mice[18].

Government of India has given a lot of emphasis on bringing its traditional ayurvedic knowledge to greater acceptability through validation using biochemical mechanisms involved[19].

Cancer cells has increased ROS levels as compared to their normal counterparts and are detoxified by complex antioxidative mechanisms[20]. Progression of cancer has been shown to follow changes in ROS[19-20]. A fall out in Oxidative stress happens due to imbalance between the systems which generates and scavenges ROS.

Apoptosis is the cell's natural mechanism for programmed cell death. The apoptotic pathway is activated by both intracellular and extracellular signals. There are two different pathways that lead to apoptosis: the intrinsic and extrinsic pathways that correlate with the signal type[22]. They are also referred to as the mitochondrial and death receptor pathways, respectively[22]. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. Upon receiving specific signals instructing the cells to undergo apoptosis several distinctive changes occur in the cell. A family of proteins known as caspases are typically activated in the early stages of apoptosis[23]. These proteins breakdown or cleave key cellular components that are required for normal cellular function including structural proteins in the cytoskeleton and nuclear proteins such as DNA repair enzymes. The caspases can also activate other degradative enzymes such as DNase, which begin to cleave the DNA in the nucleus. Typically, the cell begins to shrink following the cleavage of lamins and actin filaments in the cytoskeleton. The breakdown of chromatin in the nucleus often leads to nuclear condensation and in many cases the nuclei of apoptotic cells take on a "horse-shoe" like appearance. Cells continue to shrink, packaging themselves into a form that allows for their removal by macrophages. These phagocytic cells are responsible for clearing the apoptotic cells from tissues in a clean and tidy fashion that avoids many of the problems associated with necrotic cell death[22].

Cancer cells can circumvent apoptosis making proteins involved in the apoptotic cascades which can be as ideal targets for cancer therapy[24]. Re-establishing apoptotic programming in malignant cells selectively kills tumour cells and caspases as primary inducers of apoptosis, provide an ideal platform to develop effective therapeutic strategies for cancer[25,26]. Here, we report the biochemical basis for the effective anticancer potential of *Bauhinia variegata* bark extracts as well as its active phytocomponents on lung cancer cell lines.

Key Question:

Can the extract and isolated phytocomponents from *Bauhinia variegata* be able to decrease the proliferative ability, adhesion and migratory ability of the lung cancer cells? Do these effects occur via the apoptotic pathway?

Objectives of the study:

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

1A (1) Extraction of phytochemicals from *Bauhinia variegata* bark extracts by Soxhlet extraction method.

(2) Determination of anti-oxidant potential activity of *Bauhinia variegata* bark extracts.

1B. To study the effects of active extract of *Bauhinia variegata* on cytotoxic, cell migratory and genotoxic parameters of **A549 and H460 cells.**

1.B.1. Cell proliferation Assay – MTT assay

1.B.2. Soft agar Assay

1.B.3. Cell Migration Assay

1.B.4. Apoptosis-mediated DNA fragmentation.

1.B.5. To study the effect of active extract on apoptosis (*In vitro)*.

- To check if the extract induces cell death via apoptosis by Fluorescence microscopy

1.B.6. To study the effect of active extract on intracellular ROS levels.

1.B.7. To check the effect of active extract on Caspase-3 levels.

1C. Chemical characterization of *Bauhinia variegata* L. bark active extracts by TGA/DSC, FT-IR and GC-MS analytical techniques.

2: Isolation and identification of effective phytocomponents from active extracts of *Bauhinia variegata* L. by analytical methods and to examine its effects on cytotoxic parameters in lung cancer cell lines.

3. To study the effect of active fractions/ isolated phytocomponents of *Bauhinia variegata* L. on cell migration & cell death parameters in lung cancer cell lines.

3.1. Scratch assay in A549 and H460 cells treated with effective Phytocomponents.

3.2. ROS generation by DCFDA in A549 & H460 cells.

3. 3. Fluorescence microscopy:

3.3.1. Comet assay for cell death in A549 & H460 cells.

3.4. Elucidating the mechanism of action of the active compound linked with biological activities in A549 and H460 cell lines by expression of pathway related proteins by Western blot analysis.

Plan of Work

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

1A (1) Extraction of phytochemicals from *Bauhinia variegata* bark extracts by Soxhlet extraction method.

- *Bauhinia variegata* bark was collected from Waghai botanical garden, Dang, Gujarat during December- January each year and was validated by the Department of Botany, The Maharaja Sayajirao University of Baroda, India.
- The bark was washed, surface sterilized with 0.1 % mercuric chloride, rinsed - shade dried, powdered and packed into a thimble for extraction by Soxhlet method with eluotropic series for 8-12 hr. The dry sample was dissolved in DMSO to form a 100 mg/ml stock & filtered by a 0.22 µm syringe filter for further use.
- Qualitative analysis of *Bauhinia variegata* bark extracts were done using standard procedures.

1B. To study the effects of active extract of *Bauhinia variegata* on cytotoxic, cell migratory and genotoxic parameters of **A549 and H460 cells.**

- Human lung cancer cell lines A549 and H460 were obtained from (NCCS, Pune, India). Cell lines were grown as per the standard protocol in controlled environment and supplements[27].
- The lung cancer cell lines were treated with *Bauhinia variegata* bark extracts with different concentrations of bark extracts for 24 h, 48 h and 72 h time points and viability of cells was measured by MTT assay; metastatic ability was determined through Scratch assay and effect on DNA integrity was shown by gel electrophoresis.
- Petroleum ether bark extract (PEBE) and Chloroform bark extract (CBE) was most cytotoxic on A549 and H460 lung cancer cell lines respectively.

1C. Chemical characterization of *Bauhinia variegata* L. bark active extracts by TGA/DSC, FT-IR and GC-MS analytical techniques.

- TGA/DSC, FTIR and GC-MS study of PEBE and CBE was done.

2: Isolation & identification of effective phytocomponents from active extracts of *Bauhinia variegata* L. by analytical methods and to examine its effects on cytotoxic parameters in lung cancer cell lines.

- Standardisation of solvent system for PEBE and CBE was done followed by Column Chromatography and TLC analysis of isolated fractions.
- A549 cell line was treated with standards of anticancer compounds studied by GC-MS. Effect of isolated fractions/ phytocomponents was checked on H460 cell line.

3. To study the effect of active fractions/ isolated phytocomponents of *Bauhinia variegata* L. on cell migration & cell death parameters in lung cancer cell lines.

- Cell migration ability of A549 and H460 cells was observed in presence of respective cytotoxic compounds at definite time-period.
- Cell death parameters were observed in both cell lines in in presence of respective cytotoxic compounds at definite time-period.
- Mechanism of cell death in A549 cell line was observed by Western blotting.

Results:

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

1A (1) Extraction of phytochemicals from *Bauhinia variegata* bark extracts by Soxhlet extraction method.

Bauhinia variegata bark was ground after washing and drying, the powder so obtained was weighed and subjected to solvent extraction by Soxhlet apparatus. Various fractions of crude extracts were collected.

(2) Determination of anti-oxidant potential activity of *Bauhinia variegata* bark extracts.

Bark extracts of *Bauhinia variegata* were prepared by different solvents using Soxhlet apparatus and tested for their antioxidant potential by DPPH assay. 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.

1B. To study the effects of active extract of *Bauhinia variegata* on cytotoxic, cell migratory and genotoxic parameters of **A549 and H460 cells.**

1.B.1. Cell proliferation Assay – MTT assay

- Petroleum ether and n-Hexane extracts showed the foremost cytotoxic effect on A549 cell line as compared to other extracts at 48 h treatment.
- Petroleum ether bark extract (PEBE) has been selected for the further study due to lesser yield of n-hexane extract with IC50 of 1.6 mg/ml.
- The cytotoxicity of CBE on H460 cells was time dependent demonstrating a potent and definite growth inhibitory effect with IC50 of 1.0 mg/ml for 24 h.

1.B.2. Soft agar Assay

- PEBE significantly decreased A549 colony growth in a concentration-dependent manner.
- CBE significantly decreased colony growth of H460 cells in a concentration-dependent manner.

1.B.3. Cell Migration Assay

- In A549 cells, as compared with the control group, gradual reduction was noticed within the number and rate of migrated cells with PEBE treatment.
- 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 & showed slower migration and wound healing capacity.

1.B.4. Apoptosis-mediated DNA fragmentation

- 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. A typical DNA ladder pattern of internucleosomal fragmentation was observed with after 48 h of treatment.
- A typical ladder pattern of internucleosomal fragmentation was observed in H460 cell line after 24 h at higher concentrations of CBE.

1.B.5. To study the effect of active extract on apoptosis (*In vitro)*

- To check if the extract induces cell death via apoptosis, Fluorescence microscopic studies were done by using DAPI & AO/EtBr staining

- DAPI staining was done after treatment of A549 cells with PEBE showed chromatin condensation, nuclear fragmentation ("horse-shoe" like appearance of nucleus) and cell shrinkage with an increase in apoptotic bodies at defined concentrations.
- Live cells with normal morphology were abundant in the A549 control group whereas early apoptotic cells were observed on treatment with different concentrations of PEBE. Both early and late apoptotic cells were observed in cells treated with defined concentrations of PEBE on A549 cells.
- For H460 cell line, live cells with normal morphology were abundant in H460 control group. H460 cell line treated with CBE showed early apoptotic cells & late apoptotic bodies at defined concentrations.

1.B.6. To study the effect of active extract on intracellular ROS levels

- On treatment with PEBE, intracellular ROS elevates at initial hours and decrease subsequently which suggest they could be activating downstream signalling pathway resulting in apoptosis. These results indicate that ROS production is an early phase event in apoptosis.
- In H460 cells, ROS levels first increase then there is a little decrease at 6h and then stability increase within the cells.

1.B.7. To check the effect of active extract on Caspase-3 levels

- Caspase 3 activity significantly increased in PEBE treated A549 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.
- Caspase-3 activity significantly increased at IC50 value of CBE from 12 h to 24 h treatment in H460 cell line.

Summary – Objective 1 (a& b):

Thus, it can be concluded that phytocomponents from *Bauhinia variegata* hindered the normal growth of A549 and H460 cancer cell lines by inducing apoptosis, inhibiting colony formation, decreasing cell migration, increasing intracellular ROS levels & activating Caspases.

1C. Chemical characterization of *Bauhinia variegata* L. bark active extracts by TGA/DSC, FT-IR and GC-MS analytical techniques.

Petroleum ether (PE) extract - PE extract of *Bauhinia variegata* exhibited a characteristic band at 1460 cm -1indicating 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.

- The GC-MS chromatogram of the PEBE of *Bauhinia variegata* showed the presence of 18 compounds.
- There are eight phytochemicals present in CBE.
- TGA/DSC analytical technique is often used for characterization of plant extracts.

Summary – Objective 1(C):

- PE extract of *Bauhinia variegata* exhibited a characteristic band at 1460 cm -1indicating 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.
- 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.
- The GC-MS chromatogram of the PEBE of *Bauhinia variegata* showed the presence of 18 compounds and CBE showed the presence of 08 compounds.
- The TG analysis of PEBE and CBE showed mass loss within three steps, in different temperature ranges. The DSC curves for the dried extracts of Bauhinia variegata showed that thermal processes occur between 50 – 100°C for PEBE and 50-70°C for CBE.

2: Isolation & identification of effective phytocomponents from active extracts of *Bauhinia variegata* L. by analytical methods and to examine its effects on cytotoxic parameters in lung cancer cell lines.

2.1. To isolate and characterize phytocomponents from PEBE extract by Column Chromatography.

- Standardisation of solvent system for PEBE was done – PE:BZ: EA (8:1:1)
- The Column Chromatography of the extract with standardised solvent system lead to extraction of 91 fractions , followed by Thin Layer Chromatography to find individual Redardation factor.

2.2. To isolate and characterize phytocomponents from Chloroform bark extract (CBE) by Column Chromatography.

- Standardisation of solvent system for CBE was done - Chloroform: Ethyl Acetate: methanol– 7:2:1.

- The Column Chromatography of the extract with standardised solvent system lead to collection of 70 fractions from CBE which were pooled down into 9 sub-fractions depending upon solvent system and same band patterns and Rf value was also calculated.

2.1.1. To examine the effect of active fractions/ isolated phytocomponents of *Bauhinia variegata* L. on cytotoxic parameters in lung cancer cell lines.

- **Effect of isolated phytocomponents from PEBE on A549 cells.**

- The percentage of all compounds in the extract were calculated from GS-MS profile.

- Heptadecanoic acid (28%), Oleic acid (10.47%), and n-hexadecanoic acid (Palmitic acid) (1%) were choosen for further experiments based on their percentages present in the extract and as well as literature studies on them. Due to difficulty in separating these compounds and the small quantities present in the fraction, further experiments were carried with standards of these compounds on A549 cells.

- Oleic acid conjugated to BSA showed cytotoxic effect on A549 cells for a time period of 48 hours, while palmitic acid did show any significant cytotoxic effect on A549 cells even after treatment for 72 hours.
- There was effective and significant cytotoxic effect observed after treating A549 cells with Heptadecanoic acid after 72hrs with defined concentrations.
- So, oleic acid is chosen as stable cytotoxic phytocomponent for further experiments on A549 cells.

2.2.1. Cytotoxic effect of fractions of CBE on H460 cell line in time and dose dependent manner.

- Effect of F1 to F9 fractions obtained from CBE was checked on H460 cell line by MTT assay.
- F3 & F4 showed most constant cytotoxic effect at different concentrations for 24hrs, 48hrs & 72hrs on H460 cells. F3 & F4 fractions are chosen for further experiments.
- As there was not much difference in the IC50 value of fractions F3 & F4 at 24hrs, 48hrs & 72hrs treatment, therefore 24hrs treatment with IC50 of 120µg/ml for F3 & IC50 of 130µg/ml for F4 was used for further experiments.

2.2.1.1. To characterize the effective isolated phytocomponent/s from CBE using Spectroscopic Techniques (FTIR) and GCMS techniques.

- The presence of carbonyl groups and few others functional groups was confirmed by Fourier-transform infrared spectroscopy (FTIR).

- By evaluating functional groups found in FTIR data & by looking into previous GC-MS results of CBE, we can conclude that maybe mixture of compounds can be present in the fractions F3 & F4.

- GC-MS results showed the presence of mixture of compounds in F3 & F4 fractions.

Active fractions F3 & F4 is not a single compound but a mixture of compounds, so the pharmacological property shown by the active fractions F3 & F4 might be due to single compound or the synergistic effect of all the compounds in composite.

3. To study the effect of active fractions/ isolated phytocomponents of *Bauhinia variegata* L. on cell migration & cell death parameters in lung cancer cell lines.

3.1. Scratch assay in A549 and H460 cells treated with effective Phytocomponents

- A549 cells showed slower migration and wound healing at Oleic acid different concentration as compared with control (not treated).
- There was prominent cell growth retardation observed after treating A549 cells with Oleic acid for 30 and 36hrs.
- H460 cells showed slower migration and wound healing in treatment with F3 fractions at different concentrations & in F4 fractions also as compared to control. Therefore, fractions F3 & F4 impairs cell migration at these concentrations.

3.2. ROS generation by DCFDA in A549 & H460 cells

- ROS generation significantly increased after oleic acid treatment in A549 cells at defined concentrations.
- F3 & F4 fractions increased ROS generation in H460 cells at 24 h treatment at defined concentrations.

3. 3. Fluorescence microscopy:

3.3.1. Comet assay for cell death in A549 & H460 cells

- DNA damage was measured in A549 cells treated with various doses of Oleic acid.
- Tail Length were used to evaluate the level of OA-induced DNA damage in A549 cells measured by the Comet assay under alkaline conditions (pH > 13). DNA damage was detected at all concentrations of OA and increased in a concentration-dependent manner at 48 h.
- F3 & F4 induced DNA damage of NCI-H460 cells *in vitro*. The comet assay was selected for determining DNA damage. F3 & F4 fractions provoked DNA damage in NCI-H460 cells in a dose-dependent manner. The higher concentration of both fractions led to a longer DNA migration smear (comet tail) at 24h.

3.4. Elucidating the mechanism of action of the active compound linked with biological activities in A549 and H460 cell lines by expression of pathway related proteins by Western blot analysis.

- OA induced apoptosis and significantly decreased the expression of p-Akt in A549 cells, which means that OA may block the Akt signaling pathway.

- OA induced apoptosis and significantly decreased the expression of p-Akt in TSCC cells, which means that OA may block the Akt signaling pathway.

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- **Elucidating the mechanism of action of the active compound in H460 cell line by Western blot analysis – WORK IN PROGRESS.**

Conclusion:

In summary, the results of this study show that Oleic acid has valuable anticancer effects on A549 cells *in vitro* through induction of cell death via apoptosis. Based on these findings, we conclude that OA may potentially serve as a therapeutic agent for A549 cells.

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1. **Tanvi Khanna**, Akash Dave, Sejal Purani, Jagath Vedamurthy, Dhaval Jivani and Pushpa Robin*. *Bauhinia variegata* Bark Extract: Assessment of its Anti-proliferative and Apoptotic Activities on A549 and H460 Lung Cancer Cell Lines. *J. Nat. Remedies;* April 2022; 22(2): 175 – 195. doi: 10.18311/jnr/2022/28740.

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Achievements: