Chapter - 6

Summary

Cancer is still considered one of the deadliest diseases globally, with lung cancer being a leading cause of cancer-related deaths. Lung cancer is more prevalent in males than females, and it remains a significant public health concern worldwide, with high mortality rates. Lung cancer can be classified into two types: non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC), with NSCLC being the most common form of lung cancer. The primary cause of lung cancer is exposure to tobacco smoke, air pollution, and genetic mutations. Current treatments for lung cancer, such as surgery, chemotherapy, and radiation therapy, often have severe side effects, and the high relapse rate and mortality rate remain significant concerns. For NSCLC, several drugs are available; however, they frequently cause severe side effects and toxicity. So, the use of safe and natural therapeutic agents helps reduce the risk of toxic side effects associated with conventional treatments. Natural medicines has been used from centuries to treat various diseases and has emerged as a great alternative to conventional treatments, mostly due to its minimal side effects. The active phytocomponents present in them play a vital role in their therapeutic benefits. Herbs and botanicals are natural therapeutic agents which have been tested as an alternative agent against cancer. Numerous studies have investigated the effects of phytocomponents in herbs on cancer cell lines, and many have shown promising results.

In this study, we have explored the anticancer effect of phytocomponents in bark of *Bauhinia variegata* on lung cancer cell lines (A549 and H460) and outlining potential therapeutic molecular mechanism involved in this process.

In order to isolate the phytocomponents present in bark of *Bauhinia variegata*, bark was collected, washed, dried and extracted with various solvents based on their polarity i.e., from non-polar to polar solvents were utilised. After extraction, the preliminary phytochemical screening of bark extracts of *Bauhinia variegata* revealed the presence of various phytocomponents such as fat and oil, carbohydrates, steroids, coumarins, tannins, alkaloids, saponins, cardiac glycosides, flavonoids and phenols. Next, crude extracts were screened for antioxidant and anticancer activity by DPPH assay and MTT assay respectively. The methanolic and water extracts of *Bauhinia variegata* showed maximum antioxidant activity

with the strongest DPPH radical scavenging activity among all the extracts. Among all the potential bark extracts, Petroleum ether bark extract (PEBE) and Chloroform bark extract (CBE) of *Bauhinia variegata* were found to be most potent on A549 and H460 lung cancer cell lines respectively. The anticancer potential of two screened bark extracts, PEBE and CBE, was evaluated through various assays such as scratch assay, DNA fragmentation, ROS measurement, Caspase activity and different fluorescence microscopy techniques. These assays suggested that PEBE and CBE possess strong anticancer properties. The extracts were found to induce apoptosis in respective lung cancer cells, as evidence by caspase activity and DNA fragmentation. PEBE and CBE were also observed to inhibit cancer cells migration, a crucial aspect of cancer metastasis. They also generate high levels of ROS, which can cause oxidative stress leading to cell death in cancer cells. Thus, all these results provided strong evidence for the potential of PEBE and CBE as promising anticancer agents. PEBE and CBE were further characterized by TG/DSC analysis, FTIR and GC-MS analytical techniques. The GC/MS chromatogram of the PEBE and CBE of *Bauhinia variegata* showed the presence of Oleic acid, palmitic acid, Heptadecanoic acid, Erucic acid, Trans-2-Undecen-1-ol, Stearic acid hydrazide, Tert-Hexadecane thiol, Heptadecane, 2,6,10,15-tetramethyl, Phthalic acid, nonyl tridec-2-yn-1-yl ester, 12- Methyl- E, E-2,13-octadecadien-1-ol in PEBE and Phenol,2,4-bis (1,1 – dimethyl ethyl), Hexadecanoic acid, ethyl ester, Oleic acid, 7-Methyl-Z-tetradecen-1-ol acetate, 4-(1,1-Dimethylallyl)-9-methoxy-7H-furo (3,2-g) [1] benzopyran-7-one, -Hydroxy-3(3,5-dimethyoxyphenyl)-benzo(b)furan in CBE.The collection of thermal and FTIR analysis data for a plant-based raw material is crucial for establishing standards in the production of a therapeutic products. This information helps to ensure the quality, efficacy and safety of plant-based products. The study found that thermal disposal of *Bauhinia variegata* PEBE and CBE leads to the release of harmless by products, which can increase the usage of this effective anti-cancer drug in pharmaceutical applications. This safe disposal method could potentially be used as a commercial claim.

In order to isolate the bioactive phytocomponents from active extracts, i.e., PEBE and CBE of *Bauhinia variegata*, the PEBE and CBE extracts were fractionated using column chromatography and different partially purified fractions were collected. The partially purified fractions obtained from silica gel column chromatography of PEBE and CBE of *Bauhinia variegata* were tested for the detection of various phytocompounds using TLC and sub-fractionated based on their similar TLC profiles. Based upon characterization by GCMS, followed by identification by NIST library, percentage of compounds present in PEBE as well

as the literature study (reported as effective anticancer phytomolecules in previous studies), Oleic acid, Palmitic acid and Heptadecanoic acid, were identified to have cytotoxic effect and were checked on A549 cells in dose and time dependent manner. Oleic acid showed to be most effective on A549 cells at 48hr treatment. Simultaneously, the partially purified fractions obtained from CBE of *Bauhinia variegata* were sub-fractionated and the cytotoxic effect of different fractions was checked on H460 cells in dose and time dependent manner. Purified fractions F3 (PFF3) and F4 (PFF4) of CBE of *Bauhinia variegata* exhibited a cytotoxic potential against H460 cells at 24 h treatment in a dose and time dependent manner. Characterization of PFF3 and PFF4 of CBE was done by FTIR, GCMS, NMR and HPLC. Different analytical methods revealed the presence of mixture of compounds in the PFF3 & PFF4 of CBE of *Bauhinia variegata*. PFF3 fraction showed highest proportion of different fatty acids and Coumarin derivates while PFF4 showed the highest proportion of ϒ- Sitosterol and different triterpenoids which have been shown to possess anti-cancer properties in various *in vitro* and *in vivo* studies. Further, combination effect of PFF3 and PFF4 of CBE with Paclitaxel (PTX) was checked, to lower the dosage of Paclitaxel (PTX) which is currently being used for the treatment. Combining PTX and PFF3 had a synergistic impact on H460 cells *in vitro*, and an increase in the level of induced apoptosis may be the cause of the potentiation of the cytotoxicity against the H460 cells.

Further the effect of these compounds as anti-cancer compounds was studied using scratch assay. Studies were also done on effect of various cell death parameters using fluorescence microscopy and protein expression of molecules important for apoptosis (western blot analysis). Paclitaxel and Gemcitabine were used as standard drugs in this study. Oleic acid treatment at a defined concentration resulted in a gradual reduction in the number and rate of migrated cells in A549 cells, while H460 cells exhibited slower migration and wound healing capacity with defined concentrations of PFF3 and PFF4 as well as with PFF3 + Paclitaxel (PXT) treatment in comparison to control cells, depending on the dose and time. Moreover, Oleic acid treatment increased the production of reactive oxygen species (ROS) in A549 cells in a concentration-dependent manner, while PFF3 and PFF4 promoted ROS generation in H460 cells. The combination of PFF3 and PXT synergistically induced more ROS build up and apoptosis in H460 cells than the single dose treatments. By using AO/EtBr staining, it was observed that treating H460 cells with different concentrations of PFF3, PFF4, and the combination of PFF3+PTX induced chromatin condensation and the development of apoptotic bodies. Further, the fluorescence densities found increased in response to increasing

concentrations of PFF3 and PFF4 on H460 cells measured by Annexin V and PI staining by fluorescence microscopy. The combination of PFF3 & PXT resulted in more cells showing Annexin V and PI staining, indicating late apoptosis or necrosis. Cells treated with different concentrations of PFF4 showed more PI positive and less Annexin V stained cells, indicating they are in the late apoptotic and necrotic stage. These findings suggest that the compounds induced apoptosis in H460 cells. To understand the reduction in cell viability in response to Oleic acid (OA) treatment, A549 cells were treated with varying concentrations of Oleic acid for 48 hours, which resulted in the formation of comet like tails indicating a significant increase in DNA damage. In contrast, H460 cells treated with higher concentrations of PFF3 and PFF4, as well as the combination of PFF3 & PXT showed an increase in DNA damage, leading to chromosomal DNA fragmentation and subsequent apoptotic cell death. Additionally, increasing concentrations of Oleic acid in A549 cells and PFF3 and PFF4, as well as the combination of PFF3 & PXT in H460 cells caused a decrease in mitochondrial membrane potential ($\Delta\Psi m$). The fluorescence microscope was used to detect autophagy in A549 cells by labelling with MDC, a marker for autophagy. On treating the cells with different concentrations of Oleic acid for 48 hours, it was observed that higher concentrations of Oleic acid induced the accumulation of MDC-labelled particles in A549 cells. To investigate how Oleic acid causes cell death in A549 cells, a western blot analysis was conducted to assess the levels of proteins related to apoptosis. The main mechanism observed in the study was apoptosis induction in A549 cells caused by Oleic acid. This was evidenced by the increased expression of cleaved PARP and cleaved caspase 3. Furthermore, the decrease in p-Akt expression may have contributed to the induction of apoptosis. In H460 cells, PARP and caspase-9 expression was observed. This study concludes that Oleic acid (an important component of PEBE) induce apoptosis in A549 cells. This is the first report on the effect of oleic acid on A549 cells. PFF3, PFF4 as well as the combination of PFF3+ PTX induced apoptosis in H460 cells and these too can be promising therapeutic molecules as per this study.

To summarize, the major conclusions of the present study:

1. The antioxidant activity of different extracts was observed and resulted that methanolic and water extracts showed maximum antioxidant activity with the strongest DPPH radical scavenging activity among all the extracts.

2. Petroleum ether bark extract (PEBE) and Chloroform bark extract (CBE) were found to have the most potent anticancer effect on A549 and H460 cells respectively.

3. PEBE and CBE induced apoptosis of A549 and H460 cell lines by activating caspase-3 signaling cascade. Study proposes the involvement of mitochondria mediated cell death pathway.

4. Separation of components by column chromatography and characterization of PEBE by GC/MS analysis showed Oleic acid to be one of the important components and further studies with Oleic acid proved it to be most potent on A549 cells.

5. Purified fractions F3 (PFF3) and F4 (PFF4) of CBE exhibited cytotoxic potential against H460 cells.

6. Palmitic acid, Linoleic acid and Alloisoimperatorin accounting for maximum percentage of the total area, were the main compounds identified in PFF3. ϒ- Sitosterol, Friedelan-3-one & Lupeol of PFF4 were the main compounds identified in PFF4 and might be responsible for the cytotoxic effect on H460 cell. The synergistic effects of Paclitaxel (PTX) and PFF3 were also examined in this study to reduce the dosage of PTX currently being utilised and both showed synergistic effect at defined concentration and time-point on H460 cells.

7. For both cell lines, the anticancer activity was seen to be due to an increase in ROS with alteration in mitochondrial membrane potential.

8. The changes in the expression of Akt, p-akt, Caspase 3 proteins, PARP which stimulated apoptosis in A549 cells and expression of PARP and caspase-9 proteins in H460 cells were observed. Despite attempts the precise molecular pathways for each individual compound and distinct target molecules could not be identified.

In conclusion, this study has been instrumental in identifying bioactive components with promising anti-cancer effects in the extracts from bark of Bauhinia variegata. The compounds and fractions isolated from effective bark extracts demonstrate a potential for the development of new herbal anti-cancer drugs.