2

REVIEW OF LITERATURE

The review of literature pertaining to the present study entitled **Validating the potential of *Plectranthus amboinicus* against lung cancer - *In silico*, *in vitro* and *in vivo* approaches** is discussed under the following headings.

2.1 Scenario on cancer
2.2 Lung cancer - The study of concern
2.3 Epidemiology of lung cancer
2.4 Treatment strategies for lung cancer
2.5. Modern approaches in treating lung cancer
2.6 Apoptosis
2.7 Plants as therapeutic agents for cancer treatment
2.8 *Plectranthus amboinicus* - The candidate plant
2.9 Pharmacological properties of *P. amboinicus*
2.10 Metabolite profiling of bioactive phytocompouds
2.11 Syringic acid – The compound of study
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2.14 A549 lung cancer cell lines – An overview
2.15 Mice model in cancer research

2.1 SCENARIO ON CANCER

Cancer is a puzzling and frightening disease which afflicted multicellular living beings for more than 200 million years, and there is evidence of cancer among ancestors of modern humans going back well over a million years. Unlike infectious diseases, parasites, and many environmental diseases, cancer is not primarily caused by some entity that is foreign to our bodies. Its agents of destruction are human cells that have, as it were, slipped their reins, and have been recruited and to some extent transformed into pathological organisms or the building blocks of tumors (Hausman, 2019). Cancer has emerged as an important

cause of morbidity and mortality in India. Because intense surgeries and other treatment protocols are necessary to treat this malignancy, a diagnosis of cancer puts a major financial burden on the economically poor family as well as on communities and society. Cancer is a multi-factorial disease and the contributing factors include various environmental stresses, specific genetic background and improper food habits (Padma, 2015). The National Centre for Disease Informatics and Research of the Indian Council of Medical Research at Bengaluru, India, has reported that 1.45 million cases of cancer were diagnosed in 2016 and the burden of cancer is expected to become double in the next 20 years (Rath and Gandhi, 2014; Singh *et al*., 2018). In the year 2000, Hanahan and Weinberg published the hallmarks of cancer, where they attempted to organize the dense complexities of cancer biology into six major hallmarks namely, maintaining signals for proliferation (self-sufficiency in growth signals), evading growth suppressors (insensitivity to anti-growth signals), resisting cell death (evading apoptosis), enabling replicative immortality (limitless replicative potential), inducing angiogenesis and activating invasion and metastasis (Fouad and Aanei, 2017). Four more biological processes were added to these six initial hallmarks, which include, genome instability, tumor-promoting inflammation, evading immune destruction and reprogramming of cell bioenergetics (Hanahan and Weinberg, 2011).

The symptoms and signs of cancer may include fever, pain, fatigue, skin changes, unintended weight loss or weight gain, lumps or tumors, difficulty in swallowing, changes or difficulties with bowel or bladder function, persistent cough or hoarseness, shortness of breath, chest pain and other secondary infections. The major types of cancer include carcinoma, sarcoma, melanoma, lymphoma, and leukemia. Carcinomas are the most commonly diagnosed cancers that originate in the skin, lungs, breasts, pancreas, and other organs and glands (Codima *et al*., 2021).

2.2 LUNG CANCER - THE STUDY OF CONCERN

Lung cancer is not only one of the most deadly diseases but it is also the most leading cause of death worldwide. It is ranked the second most common

cancer in men and women from mass races such as white, black, Asian, Pacific Islander, American Indian/Alaska Native, and Hispanic. It has been founded that fifteen percent of all new cancers are relative to lung cancer. Lung cancer occurs when abnormal cells rapidly grow in the lung. Due to uncontrolled reproduction of lung cancer cells, tumors are formed which blocks the airways and damage the normal lungs. As a consequence, the lungs function is depleted and soon discontinues working normally. Lung cancer takes years to develop but changes usually occur when individuals are exposed to carcinogenic agents frequently. There are two different types of lung cancer namely small cell lung cancer and non-small cell lung cancer, where both are treated very differently (Wu *et al*., 2021).

The small cell lung cancer (SCLC) also known as oat cell cancer occurs only about 1 in 10 to 3 in 20 people. It often starts in the bronchi near the center of the chest, and it tends to spread widely through the body in the early course of the disease. The cancer cells can multiply quickly, form large tumors, and spread to lymph nodes and other organs, such as the bones, brain, adrenal glands, and liver. Small cell lung cancer is caused almost always by smoking. It is very rare for someone who has never smoked to have small cell lung cancer. It grows and spreads more quickly than non-small cell lung cancer. Non-small cell lung cancer (NSCLC) is the most common type of lung cancer. This usually has a very slow growth/spread rate. There are three different forms of NSCLC which are adenocarcinomas, squamous cell carcinomas, and large cell carcinomas. Adenocarcinoma usually accounts for about 40% of all lung cancers which are found in the outer part of the lung. On the other hand, squamous cell carcinoma can be found in the center of the lung in which it accounts for about 25% to 30% of lung cancers. Large cell carcinoma can be found in any part of the lung which tends to be harder to treat due to its rapid growth and spread rate (Auclin *et al*., 2020).

The majority of lung cancer starts in the lining of the bronchi or also develops below the lining of the bronchi, the border of the lungs. The core root of lung cancer is due to smoking, either from passive smoking, or from secondhand

smoking. Since tobacco contains over 4,000 chemicals, presence of nitrosamines and polycyclic aromatic hydrocarbons proves it to be a carcinogen. Lung cancer can also develop from non smokers who have been exposed to these chemicals via inhaling tobacco smoke. This issue appears to cause an estimate of 3,000 lung cancer deaths. Another cause of lung cancer is by radon gas, a natural decay product of uranium which has been estimated to cause 12% deaths. Radon gas is invisible, and odorless in which it makes it hard to avoid from being exposed because they can travel from underground all the way to our places through the gaps in the doors, pipes, drains, etc. Another factor that can cause lung cancer can arise from families who have the history of cancer in their genes. This is very important because familial predisposition can be avoided by being knowledgeable of the causes and ways to prevent oneself from getting lung cancer. Other environmental factors such as exposure to copper, nickel, chromium, cadmium, etc can cause lung cancer (Naraki *et al.*, 2021).

In some cases, 25% of individuals with lung cancer do not show any signs or symptoms. They are usually diagnosed through the use of tools such as chest X-rays and CT Scans. Moreover, some individuals show symptoms in which they have breathing trouble, coughing, wheezing, chest pains and coughing up blood (Fig.1). Depending on the severity of the disease, it can cause from shoulder pain to paralysis, and difficulty swallowing (Krueger, 2021).

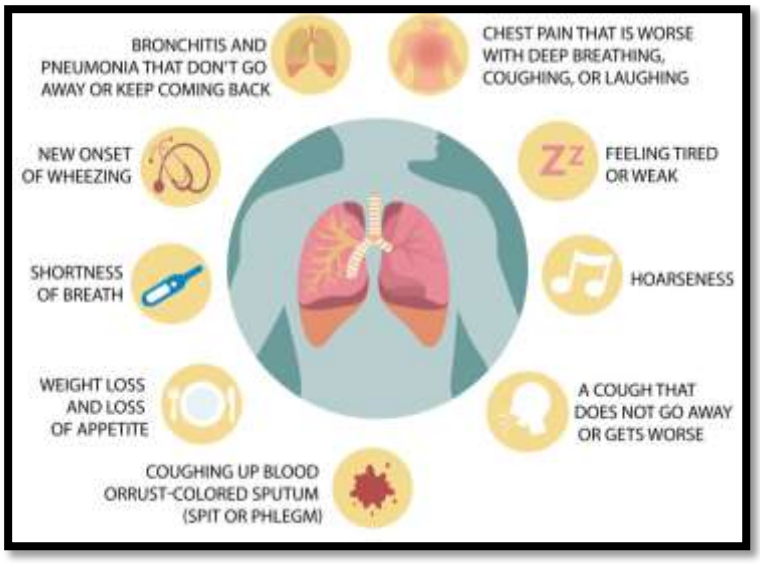

Fig. 1 Signs and symptoms of lung cancer

2.3 EPIDEMIOLOGY OF GLOBAL LUNG CANCER

Lung cancer cases and deaths are rising rapidly all over the world. In 2018, GLOBOCAN estimated 2.09 million new cases (11.6% of total cancer cases) and 1.76 million deaths (18.4% of total cancer deaths) higher than 2012 reported rates (1.8 million new cases and 1.6 million deaths), making it the most frequent cancer and cause of death in men and women. In women lung cancer is rated the third most common cancer type and the second most common cause of death (Torre *et al*., 2016 and Bray *et al.,* 2018) (Fig. 2).

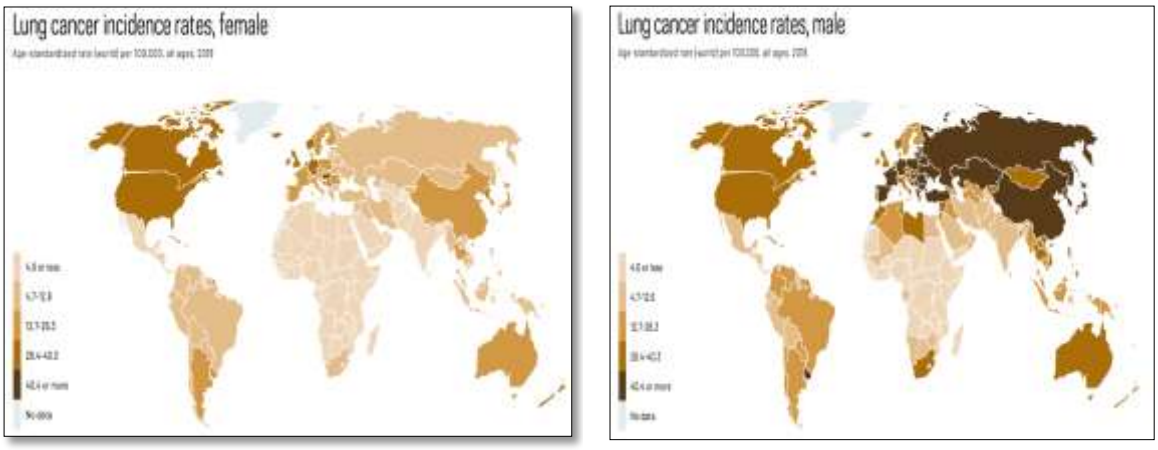

Fig. 2 Lung cancer incidence by gender

Between countries, significant variation in lung cancer incidence and demographic distribution are noted, and tobacco smoking rates and stage of economic development influence these patterns. Although cancer statistics in developing countries are less reliable, lung cancer incidence is expected to increase in developing regions with the recent increase in smoking prevalence in China, Indonesia, Eastern Europe, and the Northern and Southern parts of Africa (Aldakheel *et al*., 2021). Up to 80% of current smokers now live in low - or middle income countries, and more than one-half of lung cancer deaths occur in less developed regions. By contrast, lung cancer incidence is decreasing or expected to decrease in countries such as United States, the United Kingdom, the Nordic countries, Australia, New Zealand, Singapore, Germany, and Uruguay that took up smoking the earliest and is now successfully implementing smoking cessation and avoidance campaigns (Torre *et al*., 2015). Although the increasing lung

cancer burden globally is driven by lung cancer cases in men, most countries are also observing an increasing incidence in women. Although breast cancer is the leading cause of cancer-associated deaths in women globally, lung cancer is the leading cause of cancer death in women in several areas, including North America, Northern/Western Europe, Australia, and New Zealand. The higher mortality rates in these areas likely reflect local smoking patterns. The World Health Organization estimates that 48% of men and 10% of women globally are smokers. Although smoking prevalence is similar between men in developed and developing countries, smoking prevalence is significantly lower in women in developing countries. In areas where tobacco smoking rates in women are low, nontobacco risk factors likely play a more significant role in lung cancer development (Ng *et al*., 2014).

Lung cancer has become the most common cause of cancer death in men ages 40 and older and women ages 60 and older. The median age at lung cancer diagnosis is 70 years, and the median age at lung cancer death is 72 years. In general, lung cancer mortality increases with age until approximately ages 80 to 85, after which heart disease exceeds cancer as the most common cause of death in both genders. Although smoking patterns likely contribute to this finding, the authors noted that smoking behaviors did not entirely explain the recognized differences. The discovery of higher incidence of lung cancer in younger women demonstrates how our “traditional” view of lung cancer is changing. Overall, lung cancer incidence and mortality are highest in African American men and lowest in Hispanic women (Carioli *et al*., 2021).

Currently, the highest lung cancer incidence and mortality rates are in Kentucky, where the age-adjusted incidence per 100,000 people is 112.8 for men and 79 for women. The age-adjusted mortality per 100,000 people is 84.5 in men and 52.2 in women. In contrast, the lowest incidence and age-adjusted lung cancer death rates are in Utah, with incidences of 32.4 and 23.7 and mortality rates of 23.4 and 15.6 in men and women, respectively. Although lung cancer incidence and mortality rates are decreasing nationally, several areas of the country with higher smoking prevalence have not observed the same

improvements in lung cancer outcomes. Nationwide differences in lung cancer incidence and mortality are likely to persist until similar smoking cessation rates are achieved. Because lower income counties have much higher rates of tobacco smoking, lung cancer disparities owing to socio-economic status are analogous to those seen geographically. In poor counties (compared with affluent counties), lung cancer mortality in men is more than 40% higher, and low socio-economic status may increase the risk of death during hospitalization for a lung cancer resection (Hovanec *et al.,* 2018).

In India, lung cancer constitutes 6.9 per cent of all new cancer cases and 9.3 per cent of all cancer related deaths in both sexes, it is the commonest cancer and cause of cancer related mortality in men, with the highest reported incidences from Mizoram in both males and females. The time trends of lung cancer show a significant rise in Delhi, Chennai and Bengaluru in both sexes. The incidence and pattern of lung cancer differ as per geographic region and ethnicity and largely reflect the prevalence and pattern of smoking. The overall 5-year survival rate of lung cancer is dismal with approximately 15 per cent in developed countries and 5 per cent in developing countries (Ferlay *et al*., 2018).

2.4 TREATMENT STRATEGIES FOR LUNG CANCER

Patients with advanced NSCLC receive palliative therapies with systemic treatments and in case of local problems, radiotherapy or surgery is given. Systemic anticancer treatments are chemotherapy, targeted therapies and immune checkpoint inhibitors. The type of systemic therapy depends on tumor histology, presence or absence of driver mutations in tumors, performance status of patients and other factors. Supportive care including end-of-life care also plays a major role in patients with advanced NSCLC (Jung *et al*., 2021).

Surgery

The aim of surgery in cancer treatment is to completely remove the lung tumor and the close by lymph nodes in the chest area. The tumor should be removed with a surrounding margin or border of normal lung tissue. Several types of surgery may possibly be used for lung cancer namely pneumonectomy,

segmentectomy, a wedge, lobectomy, radiofrequency ablation and adjuvant therapy. Adjuvant therapy is the cure given after surgery to lessen the lung cancer risk returning. It involves chemotherapy, targeted and radiation therapies. The intention behind adjuvant therapy is to rid the body of any cancer cells that may still be in the body after the surgery (Casiraghi *et al*., 2021).

Radiation therapy

This is the application of high energy X-rays and other particles to destroy cancer cells. External-beam radiation therapy is given regularly by use of a machine that is outside the body. Lung cancer patients who undergo radiation therapy experience loss of appetite and fatigue. If therapy is administered in the center of the chest or neck, patients may have difficulty in swallowing or experience a sore throat (Lee *et al*., 2021).

Chemotherapy

In chemotherapy drugs are used to destroy cancer cells through preventing the cancer cells growth and division. Chemotherapy has been proven to improve the quality and length of life for the lung cancer patients in all stages. Common ways of administering chemotherapy is through placing an intravenous tube in the vein by use of needle or a capsule/ pill administered orally. The side effect of chemotherapy includes hair loss, diarrhea, appetite loss, vomiting, nausea, fatigue and risk of infection which depends on the dose used and individual (El-Hussein *et al*., 2021).

Targeted therapy

This form of treatment blocks the spread and growth of cancer cells whilst limiting harm to healthy cells. For lung cancer, the types of targeted therapy administered include anti-angiogenesis therapy (Li *et al*., 2021).

2.5 MODERN APPROACHES IN TREATING LUNG CANCER

Over the years, our understanding of disease biology has evolved, and the histological classification is now stretching to molecular classification. Newer molecular targets and driver mutations have been identified which play a major role in pathogenesis that can be addressed with therapeutic interventions. These

advancements have led to the development of more individualized treatment modalities, the so called era of "*personalized medicine*". There has been a new interest in the histological characterization of lung cancer in view of newer histology guided therapeutic modalities and genomic classification (Standfield *et al*., 2011).

At present more than 50 per cent of lung adenocarcinomas and about a third of squamous cell carcinomas are characterized based on the mutation profile. This molecular classification has led to development of targeted therapeutic strategies (Stella *et al*., 2013). Fig. 3 depicts the automated image analyser which serves as an aided scoring tool for lung cancer diagnosis.

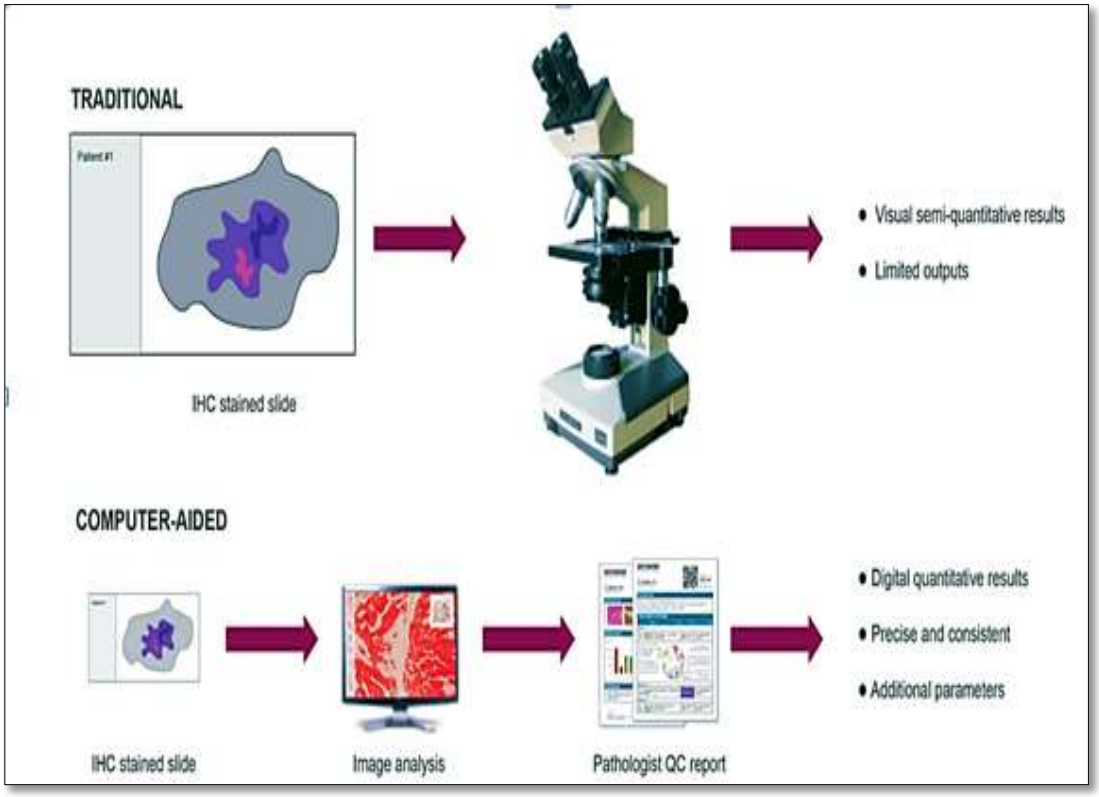

Fig.3 Automated Image Analyser – A scoring tool for lung cancer diagnosis (Widmaier *et al*., 2020)

Mutations in epidermal growth factor receptors (EGFR) best illustrate the therapeutic importance of molecular classification. EGFR mutations strongly predict the efficacy of inhibitors of EGFR with response rates higher than 70

percent reported in many studies (Mok, 2008). Genomic expression, mutational and proteomic profiling studies, as well as various mouse lung tumour models have led to the identification of additional molecular driver mutations (Motoi *et al*., 2008). Another such example of mutation driven therapy is targeting EML4-ALK (echinoderm microtubule-associated protein like 4-anaplastic lymphoma kinase) rearrangement. Biologically, EML4-ALK fusions result in protein oligomerisation and constitutive activation of the kinase (Soda *et al*., 2007). The frequency of EML4-ALK translocation ranges from 3 to 7 per cent in unselected NSCLC. Detection methods include reverse-transcriptase PCR, fluorescence *in situ* hybridization, and immunohisto-chemistry. EML4-ALK translocations are generally found in tumours with wild type EGFR and KRAS25. Tyrosine kinase inhibitor targeting ALK, crizotinib has shown a response rate of 65 per cent in previously treated patients of NSCLC that harbour ALK rearrangement and has been approved for this indication (Shaw *et al*., 2013). With these advances, the validation of targeted therapeutic compounds should ideally parallel with the development of predictive biomarkers (Li *et al*., 2013). In this context, the availability of high quality molecular testing is pivotal and should be integrated into treatment guidelines. The most exciting advances have been made in lung cancer driven by molecular changes in driver oncogenes. The first of these molecular drivers linked to specific therapy were mutations in the epidermal growth factor receptor that activated the receptor even in the absence of ligand (epidermal growth factor or transforming growth factor alpha (Dowell and Minna, 2006).

2.6 APOPTOSIS

Apoptosis or programmed cell death is a strictly controlled pathway, responsible for removal of unwanted cells, old and injured cells. The characteristics of apoptosis include morphological changes such as heterochromatin core mass, cell membrane shrink and loosing organelles position in cytoplasm. Apoptosis also gives some clues about effective anticancer therapy. Many chemotherapeutic agents were reported to event their anti-tumor effect including apoptosis of cancer cells. A wide variety of natural compounds appear to possess significant cytotoxicity as well as chemopreventive activity (Khan *et al*.,

2020). Many death and survival genes, which are regulated by extracellular factors, are involved in apoptosis. Apoptosis resistant cells and transduction pathways which inhibit apoptosis can induce non-apoptotic mechanisms of cell death and senescence, thereby preserving the antitumor effect of some anticancer agents. Several molecular markers and related signaling pathways have been revealed to play key roles in carcinogenesis by causing persistent proliferation and blocked apoptosis of epithelial cells. Most anticancer agents act via inducing apoptosis in malignant cells. The loss of sensitivity to apoptosis in malignant cells represents one of the key molecular mechanisms of resistance to therapy (Timofeeva and Gandhi, 2021).

Mitochondria mediated apoptotic pathway

The intrinsic and extrinsic cell-death pathways of apoptosis converge on the effector caspases. The intrinsic cell death pathway, also called as the mitochondrial apoptotic pathway, is activated by a wide range of signals, including radiation, cytotoxic drugs, cellular stress, and growth factor withdrawal, and involves the release of proteins (including cytochrome c) from the mitochondrial membrane space. Cytochrome c combines with an adaptor molecule, apoptosis protease activating factor 1, and also with an inactive initiator caspase, procaspase-9, within a multiprotein complex called the apoptosome. This leads to the activation of caspase-9, which then triggers a cascade of caspase (caspase-3, caspase-6, caspase-7) activation, resulting in the morphologic and biochemical changes associated with apoptosis. By contrast, the extrinsic cell-death pathway can function independently of mitochondria and is activated by cell-surface death receptors, such as Fas and Tumor necrosis factor–Related Apoptosis–Inducing Ligand receptors, directly activating the caspase cascade via an “initiator” caspase (caspase-8) within a death inducing signaling complex (Fig. 4).

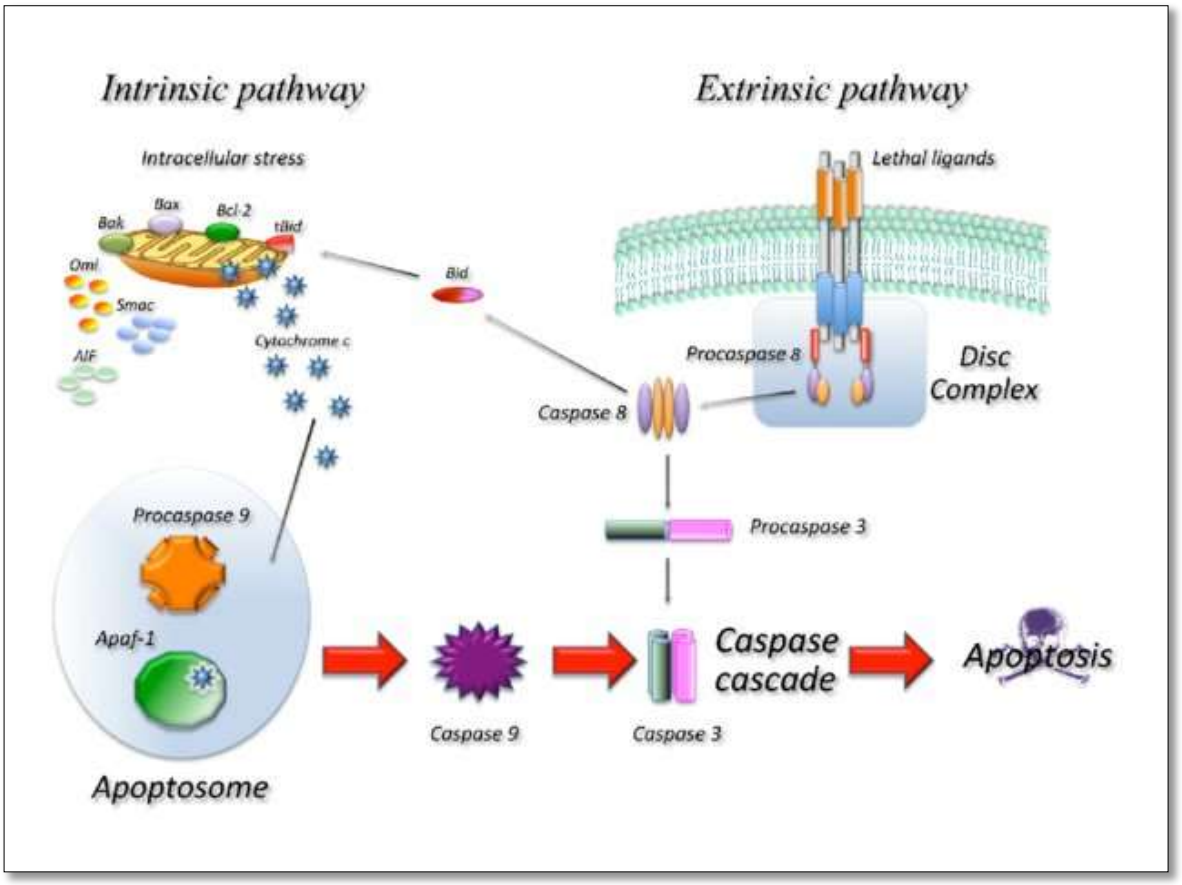

Fig. 4 Molecular pathways leading to apoptosis

Favaloro *et al.* (2012) Apoptosis is one of the major mechanisms of cell death in response to cancer therapies. Alterations in susceptibility to apoptosis not only contribute to neoplastic development but also can enhance resistance to conventional anticancer therapies, such as radiation and cytotoxic agents. Recognizing involved mechanisms in cancer development is of great importance for developing neoplastic treatment. Induction of apoptosis is one the most important marker of cytotoxic antitumor agents. It has been shown that some natural compounds including plants induce apoptotic pathways that are blocked in cancer cells (Safarzadeh *et al*., 2014 and Kang and Reynolds, 2009).

2.7 PLANTS AS THERAPEUTIC AGENTS FOR CANCER TREATMENT

A variety of plants used in native folk medicine around the world have been a good source of therapeutic agents. More than half of the commercially available drugs so far are plant derived or mimics of plant derived substances. Since many

medicinal plants are assumed to have anticancer activity, they have undergone intensive pharmacological and biological studies to prove their efficacy. For this reason, there are only a few plant-derived drugs currently available in the market for the treatment of human cancers (Sofi *et al.,* 2018).

Natural products, either in the form of total extracts or purified active compounds, have been demonstrated to play a vital role in the current management of different types of cancer, with directions being pulled toward both treatment and prevention. As proven by current cancer therapy, an increased number of anticancer drugs used in the clinic are based on natural products obtained from different plants. With respect to the main classes of natural compounds, phenolics have been intensively studied as natural compounds with chemo-preventive properties against different types of cancer due to their biological activities which include antiproliferative and proapoptotic effects (Ghitu *et al.,* 2019).

The increased interest of food and pharmaceutical industries for these phytocompounds is due to their antioxidant effect. Oxidative stress reflects an imbalance in the normal redox state of cells which can cause toxic effects through the production of free radicals. In this way, these compounds are able to prevent the damage of cell membranes and biomolecules (e.g. DNA, proteins, lipids) caused by free radicals. Oxidative stress is suspected to play a key pathogenic role in the development of different types of age-related cancer as well as neurodegenerative diseases including Parkinson's disease, Alzheimer's disease, Huntington's disease, depression or multiple sclerosis (Georgeta *et al*., 2016).

2.8 *PLECTRANTHUS AMBOINICUS* - THE CANDIDATE PLANT

In traditional system of medicine from ancient period, medicinal plants play a vital role in the treatment of various diseases. Ayurveda is one of the most practiced medical systems since it has fewer side effects when compared to other medicines. *Plectranthus amboinicus* also known as country borage which is raised in tropical, subtropical and warm temperate climate zones on the mountains in India, Nepal, Burma, Sri Lanka, Thailand and Africa. It grows well from 300 m to

1800 m of altitude on dry hill slopes exposed to the sun. It is a succulent, perennial, branched herb belonging to Lamiaceae family with four-cornered base. The leaves are very dense, succulent, hairy, grey-green, possess strongly aromatic smell and have a good mixed herbal taste. The herb easily grows in a well drained, half-shaded location and reaches approximately 50cm height. The roots are smooth, tuberous, fasiculated, 0.5-2.5 cm thick, conical, fusiform and straight, upto 20cm long. Flowers range from bluish to light lavender in colour. Racemes are fine and the front of the calyx is dented and deflexed. The plants have four seperated ovaries with different smell leaves and tuber and the leaves are also used as culinary agent in many countries (Himesh and Akilesh, 2012 and Roshan *et al*., 2010).

2.9 PHARMACOLOGICAL PROPERTIES OF *P. amboinicus*

Plectranthus amboinicus is one such medicinal plant which is used to treat many diseases like cold, cough, fever, sore throat, asthma, flu, eczema, and cardiovascular disorders etc.The plant possesses various therapeutical potentials such as antimicrobial, anti-inflammatory, antidiabetic, anxiolytic, antineoplastic, analgesic, antimalarial, antibiofilm efficacy, diuretic, wound healing and antiplatelet aggregation activities. Presence of various phytoconstituents or secondary metabolites such as flavonoids, glycosides, phenols, tannins, alkaloids and terpenoids impart strong flavor and medicinal property to the plant. The plant possesses nutritional properties which is attributed by its phytochemicals and are highly valued in the pharmaceutical industry. It does not possess any side effects when compared to other medical system and can be used as a flavoring agent or incorporated as ingredients in the preparation of traditional food. The essential oils present in leaves and stem are used in the cosmetics and skin care (Kumar *et al*., 2020). Altogether the plants possess valuable therapeutic effects and can act as an alternative to cure various ailments or diseases. Few pharmacological properties of the candidate plant is discussed below:

Duraiswamy *et al.* (2021) conducted a study on the *in vitro* and *in vivo* inflammatory activities of *Plectranthus amboinicus*. The *in vivo* study was carried out on the formation of induced paw edema test. Group of rats were pretreated

with formalin which causes inflammation in the hind paw of rats. The act of inflammation was confirmed by the paw licking behaviour of the rats. The mice which were treated with the aqueous and ethyl acetate extracts of *P. amboinicus* showed recovery rate within 30 minutes. The reduction of inflammation rate was more in ethyl acetate (35%) when compared to aqueous extract (25%). The reduction rate of ethyl acetate extract is attributed to the carvacrol present in it. The *in vitro* study was carried out on the inflammation produced using the IC-21 macrophage cells and the inflammation were indicated by the production of nitric acid in the IC-21 macrophages. From the results it was concluded that the aqueous and ethyl acetate extracts of *Plectranthus amboinicus* inhibited nearly 37% and 52% of the nitric acid production.

Suresh *et al.* (2020) reported that ethanolic extract of *Plectranthus amboinicus* demonstrated anticancer activity against A549 human lung cancer cell line by inducing apoptosis. The study primly reported the antioxidant and anticarcinogenic effects of *P. amboinicus* phytofabricated gold nanoparticles towards lung adenocarcinoma cells under *in vitro* conditions. The herbal concentrate, size and dosage of gold nanoparticles were the key variables that determined the anticarcinogenic efficacy.

Laila *et al*. (2020) revealed the methanolic extract of cytotoxicity of *Coleus amboinicus* against colon cancer cell lines. The extract showed potent anticolon cancer activity against WiDr cells as compared with the regular 5-flurouracil drug. The methanol extract induced cell death at low concentrations mainly by apoptosis, as multiple genes such as BAX, p53 are upregulated and the antiapoptotic gene such as Bcl-2 have been downregulated. A mode of cell death for the methanol extract corresponded to low concentration apoptosis with intrinsic pathway, as it upregulated Caspase 9 but downregulated Caspase 8. The findings obtained from the study of LC-MS suggested the presence of several useful bioactive compounds which would contribute to *Coleus amboinicus* anticancer properties.

Ramalingam *et al.* (2020) reported the antihyperglycemic activity of the rosmarinic acid isolated from *Plectranthus amboinicus.* Type 2 diabetes was induced in rats by feeding high fat diet and streptozotocin as control. Rosmarinic acid was isolated from the methanolic extract of *Plectranthus amboinicus* and injected into the diabetics induced rats. The rats showed a decrease in blood glucose level and increase in plasma insulin levels and also a decreased HOMO-IR index in diabetic rats.

Paramasivam *et al.* (2020) identified the biological activity of the *Plectranthus amboinicus* and its phytochemical constituents. The larvicidal activity was assessed by treatment against larva of *Aedes aegypti.* The extract of *P. amboinicus* showed larvicidal activity against fourth instar and the reactivity was more in ethyl acetate extract followed by methanol, hexane and aqueous extract. Toxicity assay was done using non targeting organisms like zebra fish embryo and brime shrimp.

Kushali *et al.* (2019) reviewed the inhibitory activity of *Plectranthus amboinicus* against the α-amylase and α-glycosidase. These enzymes play a vital role in digesting the carbohydrates and converting them into glucose. The methanolic extract of fraction 2 isolate of *P. amboinicus* was found to inhibit the activity of enzymes at varied concentration of doses. This activity of fraction 2 isolate of *P. amboinicus* can help to reduce the levels of post prandial sugars.

Hasibuan and Sumaiyah (2019) synthesised nanoparticle from the ethanolic extract of *P. amboinicus* leaves and the findings of the study revealed significant anti-proliferative and pro-apoptosis effects on T47D breast cancer cell lines.

Matias *et al*. (2019) assessed the presence of antitumor lead compounds in *Plecranthus madagascariensis*. Various solvent extracts were prepared and major components for cytotoxicity were identified and evaluated in human cancer cell lines, including breast cancer (MDA-MB-231, MCF-7), colon cancer (HCT116) and lung cancer (NCI-H460, NCI-H460/R) and also in healthy lung cells (MCR-5). Royleanones exhibited high selectivity for lung cancer cells and also

demonstrated similar growth inhibition against cancer cell line (NCI-H460) and its multidrug-resistant version (NCI-H460/R), which over expresses the multidrug-resistant protein 1 (MDR1 or P-glycoprotein), indicating the compounds 6β,7α-dihydroxyroyleanone and 7α-acetoxy-6β-hydroxyroyleanone are not substrates for efflux pump.

Ismayil and Nimila (2019) studied the antimicrobial capability of *Plectranthus amboinicus* leaves and discovered the inhibition zone of hydroalcohol extracts on bacterial strains namely *Klebsiella pneumoniae* and *Shigella flexneri*. The results showed that *Klebsiella pneumonia* and *Shigella flexneri* was more sensitive to alcohol and aqueous extracts of *P. amboinicus* respectively.

The antimicrobial activity of essential oil extracted from *Plectranthus amboinicus* using methanol and chloroform was tested against various bacterial and fungal strains by Sivaranjani *et al*. (2019). Methanol extract displayed high antimicrobial activity when compared with chloroform extract and hence found to be effective against the bacterial and fungal isolates tested.

Swamy *et al.* (2017) tested the antibacterial and antifungal activity of acetone, methanol and hexane extracts of *Plectranthus amboinicus*. The antimicrobial activity was exhibited by all the three extracts but the methanolic extract showed maximum activity when compared to acetone and hexane extracts. Among the tested microbes, *Bacillus subtilis* and *Candida albicans* were found to be susceptible to all forms of extracts.

Swamy *et al.* (2017) evaluated the antioxidant properties of hexane, methanolic and acetone extracts. The antioxidant properties were estimated by DPPH free radical scavenging and ferric reducing antioxidant potential assays. The DPPH activity was high in methanolic extract than acetone and hexane extracts. The ferric reducing antioxidant potential assay revealed that the highest ferric ion reducing potential was exhibited by methanolic extract followed by acetone and hexane extract.

Manimekalai *et al*. (2016) reported that methanol and ethyl acetate extract of *Plectranthus amboinicus* showed a dose-dependent inhibition against the biofilm forming *Streptococcus pyogenes* isolated from pharyngitis patients. Among the solvent extracts, the methanol extract showed significant activity against the test pathogens at minimal concentration. The crude extract is rich in biologically active phytochemicals which might have interacted directly or indirectly in the process of biofilm formation by *Streptococcus pyogenes*. In addition, *P. amboinicus* controls the biofilm formation of the upper respiratory tract pathogen *Streptococcus pyogenes*.

Rai *et al*. (2016) documented antitumor activity of hexane extracts of *P. amboinicus*. The findings demonstrated a major inhibition of Sarcoma-180 tumor development in mice treated with the hexane extract of *Plectranthus amboinicus*. A dosage of 350 mg/kg of *P. amboinicus* hexane extracts significantly decreased S-180 tumor growth with 66% inhibition, while doses of 100, 150 and 250 mg/kg decreased the inhibition to 44%, 45% and 47% respectively. Prior to and after the treatment there were no major changes in the body weight.

Gupta and Negi (2015) performed the antimicrobial activity of *Plectranthus amboinicus* leaf extracts in model food system like cabbage and papaya against natural microorganisms in chicken meat. The acetone and ethyl acetate leaf extracts reduced the bacterial count in food systems and reduced the leakage of cell constituents to an extent of 40 to 80% and 60 to 95% respectively.

Silitonga *et al*. (2015) reported significant improvement in the immunoglobulin (IgG, IgM) and lysozyme levels in rats treated with ethanolic leaf extract of *Plectranthus amboinicus.* Janakiraman and Somasundaram (2014) evaluated the anti-inflammatory effect of hydroalcoholic extract *Plectranthus amboinicus* (HAEPA) leaves against *in vitro* protein denaturation. In order to test the anti-inflammatory properties of the extract the plant extracts were incubated with egg albumin at various concentrations. Acetaminophen was used as normal medication of choice, and the study narrated the HAEPA's concentration-dependent protein denaturation inhibition. Hence, HAEPA has significant anti-inflammatory effect against denaturation of *in vitro* proteins and this effect may be

due to polyphenolic content, and may be more synergistic than a single compound.

Chen *et al.* (2014) demonstrated the extraction of phenolic component from hexane extract of *Plectranthus amboinicus*. The anti-inflammatory activity was related to modulation of antioxidative enzymes in the liver with a decreased malondialdehyde level. Also, they observed the production of TNF-α and cyclooxygenase 2 in the tissue of edema paw induced in mice. *In vitro* studies revealed the production of pro inflammatory mediators in RAW 264.7 cell line. Chiu *et al.* (2012) studied the inhibitory effect of *P. amboinicus* over pro-inflammatory mediators and they found that the plant is having both anti - inflammatory as well as analgesic activities. They stated that it is used in folklore medicine for relieving pain and inflammation.

Asres *et al*. (2013) reported that *Plectranthus amboinicus* leaf extract possess potent antimicrobial (both antibacterial and antifungal) activities. In particular, the leaf juice is used to treat wounds and has anti-influenza properties. The existence of chemical structure, antimicrobial and modular activities of essential oils from *Plectranthus amboinicus, Plectranthus ornatus* and *Plectranthus barbatus* was investigated by Galvão Rodrigues *et al*. (2013). Using microdilution process, they carried out antimicrobial studies against different bacterial strains and the test showed that the bacterial strains were immune to essential oils.

Bhatt and Negi (2012) demonstrated the highest polyphenolic content in the solvent extract of *Plectranthus amboinicus* leaves, with substantial total antioxidant and DPPH free radical-scavenging properties. Manjamalai and Berlin (2013) reported that the essential oil from *Plectranthus amboinicus* has important antioxidant property against stress-created lung cancer in both *in vitro* and *in vivo* models which could be due to the existence of phytochemical compounds such as carvocrol and thymol.

Antianxiety study was done by Tiwari *et al.* (2012) on albino rats by elevated plus maze (EPM) and light dark method to induce anxiety. The ethanolic

and aqueous extract of *Plectranthus amboinicus* increased the arm entries in open arms and decreased the time spent in the maze. In addition to that, higher doses of *Plectranthus amboinicus* did not have sedative effect instead it had stimulation effect on the rats and decreased the anxiety. Similar study was done by Archana (2013) on estimating the anxiolytic activity of alcoholic extract of *Plectranthus amboinicus*. The results proved that the increase in time in the light area and the head dip movement and head dip duration without locomotion.

Manjamalai *et al*. (2012) analyzed the pharmacological activities of *Plectranthus amboinicus* and reported its antitumor and cytotoxic activities. The administration of leaf extract to the laboratory rats showed regulatory effects on the formation of calcium oxalate stones.

Shenoy *et al.* (2012) studied the hepatoprotective activity of *Plectranthus amboinicus*. Hepatotoxicity in rats was induced in rats by paracetamol treated with ethanolic extract of *Plectranthus amboinicus*. The heptatoprotective activity was tested by analyzing the liver histopathology and serum constituent analysis. The results showed that the ethanolic extracts of *P. amboinicus* made the tissue enzyme levels to normal and reduced the changes induced by paracetamol.

Chiu *et al.* (2012) studied the analgesic properties of *Plectranthus amboinicus* by acetic acid induced writhing test, where the rats were injected with formalin in the paw to induce pain. The rats after acetic acid injection were subjected to aqueous extract of *Plectranthus amboinicus* which reduced the acetic acid induced writhing response in rats. The extract also reduced the pain induced by formalin which was confirmed by the decreased licking of paw by rats.

Hafeeza *et al*. (2011) evaluated oregano *(Oreganum vulgare spp.),* ajowan *(Trachyspermum ammi*) and Indian borage *(Plectranthus amboinicus)* extracts for radical scavenging activity (RSA), antioxidant activity (AA), total polyphenol (TP) and flavonoid content (TFC) analyses. Oregano demonstrated maximum radical scavenging activity for aqueous and ethanolic extracts at a concentration of 50ppm respectively, followed by ajowan and Indian borage. Oregano and ajowan extracts displayed greater antioxidant activity than Indian borage. Aqueous

oregano extracts had the highest TP (Gallic acid equivalents) and TFC (Catechin equivalents) contents of 27.7% and 50.6% as compared to ajowan (6.7%, 24.4%) and Indian borage (4.2%, 5.5%). Synergistic experiments have shown that adding oregano extract significantly increased the RSA of ajowan and Indian borage extracts at a concentration of 50ppm.

Anti-inflammatory activity was recorded by Manjamalai *et al.* (2011) using the methanolic extract of *Plectranthus amboinicus* in oedema of rats which were induced by egg albumin, formalin, xylene, carageenan and formaldehyde. The results showed that the extract of *Plectranthus amboinicus* inhibits the prostaglandin formation in carageenan induced paw edema. The inflammation in egg albumin and formaldehyde induced paw edema was inhibited by extracts of *P. amboinicus* by blocking the release of histamine and 5-HT. The ear edema induced by xylene was inhibited by the high dose of extracts and it inhibited the phospholipase A_2. They concluded that the mode of action was somewhat similar to that of paracetamol.

Vishwanathaswamy *et al.* (2011) reviewed the antidiabetic activity of *Plectranthus amboinicus* and conducted a study by administering the ethanolic extract to the alloxan induced diabetic rats and the results revealed significant reduction in the glucose level.

Patel *et al.* (2010) conducted a research on the diuretic property of ethanolic and aqueous extract of *Plectranthus amboinicus* in male albino rats and used furosemide as a standard. The results proved that the ethanolic extract of *Plectranthus amboinicus* was nearly similar to that of the diuretic condition produced by furosemide. The sodium, potassium and chlorine ion concentration was also increased. The diuretic property of ethanolic extract was more when compared to aqueous extract of *Plectranthus amboinicus* and it is attributed to the phytochemicals present in them.

Roshan *et al.* (2010) demonstrated the antinociceptive and antipyretic effects of *Plectranthus amboinicus*. Toxicity was induced by tail immersion and acetic acid induced writhing method. Antipyretic activity was induced using

injection of brewer's yeast. The results showed that the ethanolic and aqueous extract reduced the acetic acid induced writhing in mice and also showed protection against tail immersion in rats. Both ethanolic and aqueous extract exhibited antipyretic effect and reduced the fever induced by brewer's yeast.

Chang *et al.* (2010) described the use of *Plectranthus amboinicus* on rheumatoid arthritis and it was induced on the Lewis rats by injection of collagen. The collagen injected rats developed arthritis after 20 days of injection. The rats which were treated with high doses of *Plectranthus amboinicus* inhibited the weight loss of rats and also had low arthritic index. The swelling in footpad of mice was also reduced to 70% due to the treatment with higher dose of *Plectranthus amboinicus.* This also reduced the level of serum C - reactive protein which is the marker of systemic inflammation. High doses of *P. amboinicus* inhibited the inflammatory cytokines like TNF-α, IL-6 and IL-1β.

Palani *et al*. (2010) stated that ethanolic extract of *Plectranthus amboinicus* has anti-clastogenic potency against anticancer drugs. Leaves are antilithic, antispasmodic, carminative and stimulant which are useful in treating urinary diseases and vaginal discharge. The leaf juice is carminative when mixed with sugar, given to children in colic. It is beneficial for asthma, calculus, chronic cough, dyspepsia, fever, gonorrhea and piles. It is externally used in conjunctivitis and bruised leaves are locally applied for headache. The expressed juice is used in epilepsy and other convulsive disorders and the plant extract is used in the treatment of gastrointestinal troubles.

Gurgel *et al.* (2009) performed anti-inflammatory and antitumor activities of the hydroalcoholic extracts from the leaves of *P. amboinicus*. A significant reduction of the paw edema was observed at doses of 150, 250 and 350 mg/kg of the hydroalcoholic extracts of *P. amboinicus*. The highest percentage of reduction of the paw edema was observed in the groups treated with 250mg/kg (41%) and 350mg/kg (33%) of the hydroalcoholic extracts of *P. amboinicus*. Interestingly, the lowest percentage of inhibition of paw edema was observed in the groups that were treated with 10mg/kg of the indomethacin, a non-steroidal anti-inflammatory drug.

2.10 METABOLITE PROFILING OF BIOACTIVE PHYTOCOMPOUDS

In nature, plants encompass a wide range of therapeutically valued bioactive compounds. These natural compounds are widely used in both traditional and modern therapies for improving human health with relatively less or no side effects. Globally, various medicinal plants have been well explored to discover novel drug molecules to combat the threat of ever-increasing human diseases. *Plectranthus amboinicus* possess about 30 nonvolatile and 76 volatile compounds which attributes to various pharmacological properties (Arumugam *et al*., 2016). In general, various solvents are used for extracting secondary metabolites from different parts of the plants. Moreover, the extraction of phytocompounds and yield mainly depends on the type of solvents and the method of extraction (Hayouni *et al*., 2007).

Sulaiman *et al.* (2018) evaluated the leaves of *P. amboinicus* through liquid chromatography tandem mass spectroscopic method and identified the presence of major polyphenolics. The photometric estimation reveals the presence of phenolic content such as flavonoids and tannins in the extracts and ethanol extract revealed the highest polyphenolic content. The LC-MS analysis led to the structural identification of 14 compounds by evaluating the m/z values compared with their pattern of mass fragmentation on the dissociation caused by collision.

Swamy *et al*. (2017) confirmed the occurrence of a total 46 phytocompounds in different solvent extracts through GC-MS analysis. Some of the major compounds included carvacrol (37.7%), tetracontane (16.6%), squalene (15.6%), tetrapentacontane (13.7%), and phytol (12.9%). Extraction of solvents influenced the recovery of phytocompounds and the highest pharmacological activities in the methanol extract could be correlated to the presence of additional bioactive compounds.

Manimekalai *et al*. (2016) identified a total of 32 compounds from the GC-MS analysis of the leaf extract. The major compounds were methylsulfonylmethane, nonadecanoic acid, phenol,2-methoxy(2-propenyl), undecyltrichloroacetate, and cholest-22-ene-21-ol, 3,5-dehydro-6-methoxy-,

pivalate. In addition, compounds such as hexadecanoic acid, stigmasterol, and propanaoic acid were also present.

The phytochemical studies revealed the presence of various flavonoids like quercetin, apigenin, luteolin, salvigenin, genkwanin and volatile oil in the leaves of *P. amboinicus*. Because of their wider pharmacological activities *Plectranthus* has to be identified as a traditional medicine. Herbal medicines have received greater attention as an alternative to clinical therapy and the demand for these remedies has currently increased (Deena *et al.,* 2002).The pharmacological properties such as urolithiasis (Patel *et al.,* 2010), fungitoxic (Murthy and Ramalashkmi, 2009), antibacterial (Vijayakumar 2008), antimalarial (Periyanayagam *et al.,* 2008) and anti-inflammatory(Jia *et al.,* 2010) have been reported.

Sabrina *et al*. (2014) identified 19 monoterpenoid compounds in *Plectranthus spp.*, amongst them, the major constituents are 3-carene, alpha-terpinene, o-cymene, gamma-terpinene, camphor and carvacrol was abundantly present. The major compounds are 3-carene, carvacrol and camphor accounts for 58.03% of the total oil compositions and have been proved to produce sensitizing potential and allergic effect.

Gupta *et al*. (2013) evaluated the pharmacological and phenolic content of *Plectranthus amboinicus* leaves from ethyl acetate and acetone extracts. The crude leaf extracts indicated the presence of phenolics such as caffeic acid, coumaric acid, rutin, quercetin and gallic acid. The acetone extract demonstrated higher antioxidant and antimutagenicity activity than the ethyl acetate extract.

El-hawary *et al*. (2012) reported the presence of antioxidant, diuretic, anti-inflammatory, cytotoxic and antimicrobial activities in the leaves of *Plectranthus amboinicus*. The total polyphenolics and tannins were found to be high in the stems and roots respectively. The stem and root possess caffeic acid, rosmarinic acid, coumaric acid, chrysoeriol, luteolin, quercitin and eriodyctiol.

Patel *et al.* (2010) reported the pharmocognostic descriptions of the leaves of *P. amboinicus*. Preliminary phytochemical analysis and thin layer chromatography indicated the existence of alkaloids, carbohydrates, glycosides,

proteins, amino acids, flavonoids, quinones, tannins, phenolic compounds and terpenoids. The results were applicable in setting pharmacognostic standards for plant monograph recognition, purity, consistency and preparation.

While Murthy *et al*. (2009) reported carvacrol as a major (70%) component in *Plectranthus spp*. In contrast, Grace *et al*. (2011) and Selvakumar (2012) reported much lower carvacrol composition (13-14%). *Coleus amboinicus* has the range of carvacrol component between 58 – 65% (Murthy *et al.,* 2009). The difference of the compositions could be due to geographical region, plant variety, age of plant, extraction and drying techniques (Murthy *et al*., 2009). Therefore, the composition of chemical components varies as the studied plant was of different geographic regions viz., Malaysia, India and Brazil.

The presence of bioactive compounds like carvacrol and camphor could contribute to the antimicrobial activity as plant extract and essential oils has long term being recognized to possess medicinal properties (Tajkarimi, 2010). The high inhibitory effect of *P. amboinicus* is likely due to its high content of carvacrol and camphor, which efficient antimicrobial known (Shunying *et al.,* 2005). Additionally, the component in lower composition also could contribute to antimicrobial activity such as alpha-terpinene, gamma-terpinene and o-cymene.

2.11 SYRINGIC ACID – THE COMPOUND OF STUDY

Srinivasulu *et al*. (2018) highlighted the natural sources of syringic acid and the use of phytochemicals to control human diseases which has been considerable public and scientific interest in recent days (Fig. 5). Syringic acid (SA), a phenolic compound often found in fruits and vegetables is synthesized via shikimic acid pathway in plants. It shows a wide range of therapeutic applications in the prevention of diabetes, cardiovascular diseases, cancer, cerebral ischemia, liver and brain as well as it possess antioxidant, antimicrobial, anti-inflammatory, antiendotoxic, neuro and hepatoprotective activities. It has an effective free radical scavenging activity and alleviates the oxidative stress markers.

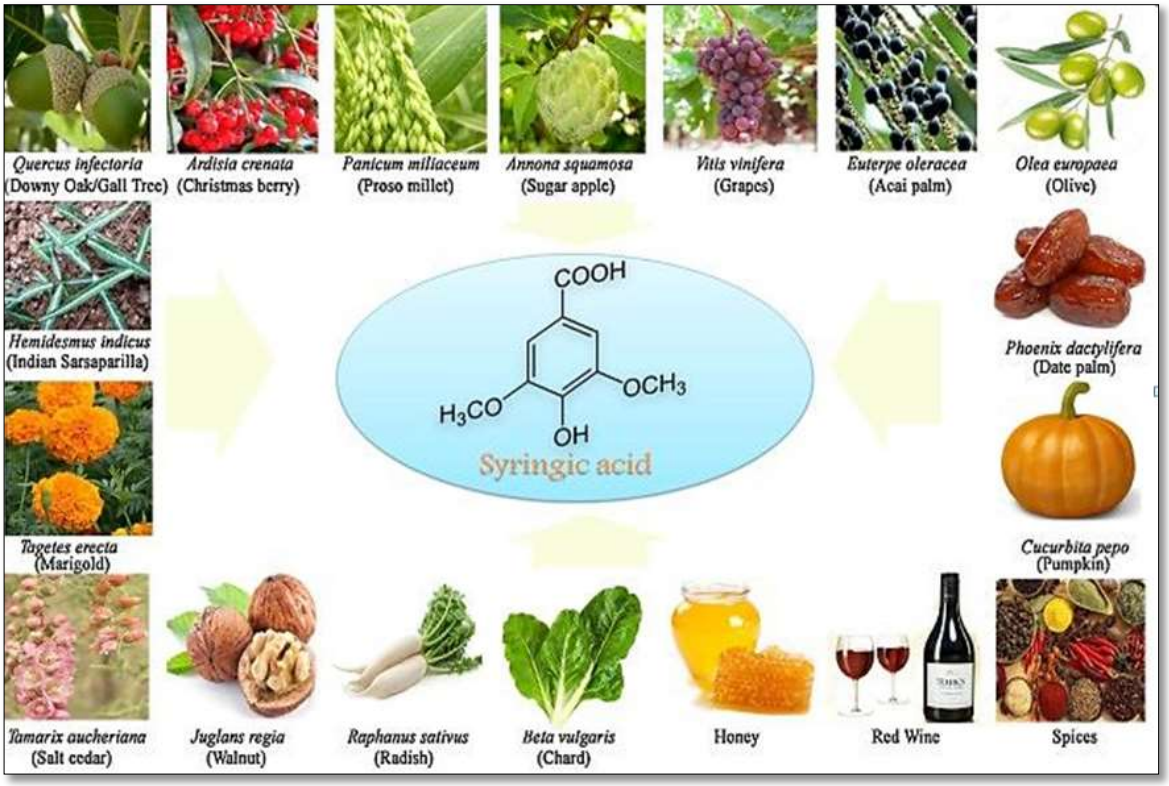

Fig. 5 Natural sources of syringic acid (Srinivasulu *et al*., 2018)

The therapeutic property of SA is attributed by the presence of methoxy groups onto the aromatic ring at positions 3 and 5. The strong antioxidant activity of SA may confer its beneficial effects for human health. It has the potential to modulate enzyme activity, protein dynamics and diverse transcription factors involved in diabetes, inflammation, cancer and angiogenesis. *In vivo* experimental data and histopathological studies on SA activity has delineated its possible therapeutic mechanisms. Besides usage in biomedical field, SA has greater industrial applications in bioremediation, photocatalytic ozonation, and laccase based catalysis (Srinivasulu *et al*., 2018).

SA is one of the abundant phenolic compound present in olives, dates, spices, pumpkin, grapes (Pezzuto, 2008), acacia palm (Pacheco-Palencia *et al*., 2008), honey, red wine and other plants. Lignin the component of plant cell wall is made up of abundant aromatic compounds in nature. Along with other phenolic compounds, syringic acid is also contributing to the structural integrity of the lignin (Kiran *et al*., 2014).

SA is found in few fungal species of pharmacological importance. The phenolic compounds, particularly syringic acid isolated from *Inonotus obliquus* (mushroom) might suppress the growth in HCT116 cells and also exhibited inhibitory effects on DNA topoisomerases and DNA polymerases (Stanikunaite *et al*., 2009). The ethanolic extract of *Elaphomyces granulatus*, a truffle-like fungus consists of syringaldehyde and syringic acid which was evaluated for cyclooxygenase-2 enzyme inhibitory and antioxidant activities using mouse macrophages as experimental model (Karamac *et al*., 2005).

2.12 THERAPEUTIC APPLICATIONS OF SYRINGIC ACID

Non small lung cancer is the most commonly diagnosed type of lung cancer, accounting for approximately 85% of all cases (Barzi and Pennell, 2010). More than 70% of them are in stages III and IV when diagnosed making curative surgery difficult. Hence there is an urgent necessity to create new strategies by understanding the molecular mechanism of anticancerous agents and to further provide the basis for more effective treatments (Andreescu *et al*., 2005). Validation of nutraceutical and pharmaceutical targets is essential for the prediction of physiological and side effects of a compound. Fig. 6 depicts the biomedical applications of syringic acid.

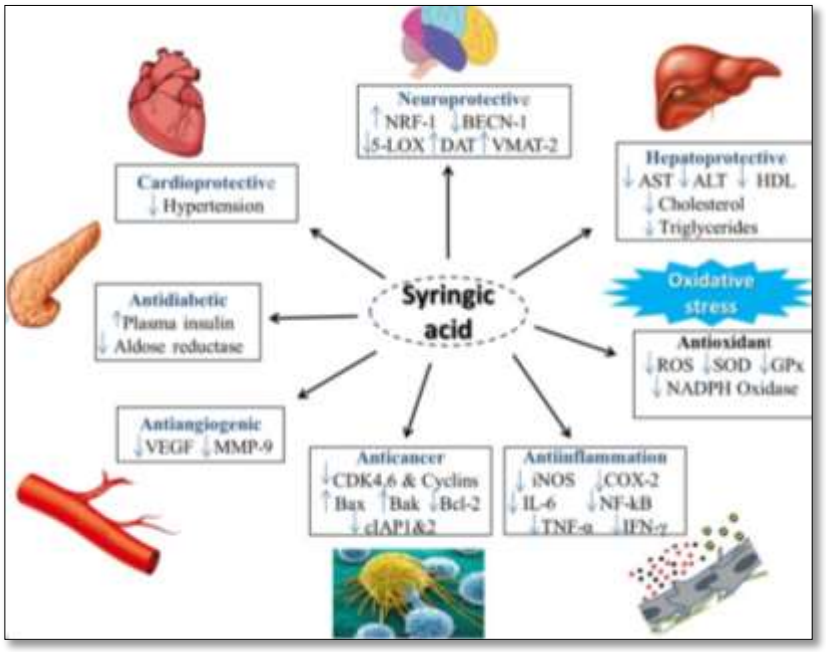

Fig. 6 Biomedical applications of syringic acid (Srinivasulu *et al*., 2018)

Treatment with chemotherapeutic agents is largely dependent on their ability to trigger cell apoptosis in tumor cells (Vansteenkiste, 2007). Thus an alternative therapy by identifying effective anticancer agents using natural compound has substantial hope in anticancer therapy. Flavonoids and phenolic acids are the most important groups of secondary metabolites and bioactive compounds in plants. Emerging studies show an increasing demand for phenolic compounds to encounter the activity of cancerous transformation of cells (Kim *et al*., 2003).

Syringic acid has long been used as traditional medicine and is known to have antioxidant, hepatoprotective, neuroprotective and anticancer effects. Studies regarding the anticancer effect of SA against squamous carcinoma cell (SCC)-25, human oral SCC (OSCC) has not been studied. SA inhibited the proliferation and induced cytotoxicity in SCC-25 cells in a concentration-dependent manner. SA treatment caused apoptosis-related morphological changes as evidenced by the dual staining and the modulation of apoptotic marker gene expressions. SA treatments modulated Bcl-2/Bax homeostasis and increased the expressions of cytochrome c and caspases 3 and 9 respectively. Thus SA specifically induced cell death and inhibited the proliferation in OSCC cells through intrinsic/mitochondrial apoptosis pathway, suggesting it as an effective agent for the treatment of human OSCC (Abijeth and Ezhilarasan, 2020).

Gheena and Ezhilarasan (2019) studied the impact of syringic acid for its hepatoprotective, anti-inflammatory, immunomodulatory, free radical scavenging, and antioxidant activities. The study was aimed to evaluate the cytotoxic effect of SA against human hepatoma (HepG2) cell line and reported that treatment with SA caused significant downregulation of Bcl-2 gene expression. Thus SA had a cytotoxic effect on human HepG2 cell line, and might be a promising agent in anticancer research.

Srivastava *et al* (2018) reported that SA showed free radical scavenging activity with 2, 2-diphenyl-1- picrylhydrazyl and beta-carotene. While, another study added that SA alleviated the oxidative stress markers and prevents acute pancreatitis in L-arginine induced rat (Cikman *et al*., 2015).

Studies by Abaza *et al*. (2013) suggested that syringic acid is a naturally occurring phenolic compound found in many plants and food compounds. Syringic acid from *Tamarix aucheriana* possessed antimitogenic and chemo-sensitizing activities in human colorectal cancer cells. SA exerted its chemotherapeutic and chemo-sensitizing effects through an array of mechanisms including cell-cycle arrest, apoptosis induction, inhibition of cell proliferation, cell migration, angiogenesis, NFκB DNA-binding and proteasome activities.

Experimental findings by Shi *et al*. (2016) have suggested that SA was able to control the growth of *Cronobacter sakazakii*, an opportunistic pathogen in food products. Coherently, another experimental study suggested that SA and its synthetic analogs have shown antibacterial activity against *Salmonella typhi* and *Staphylococcus aureus* (Cheemanapalli *et al*., 2016).

Karthik *et al*. (2014) revealed that syringic acid induced apoptosis in A549 cells in a dose dependent manner. The study reported that there were significant apoptotic cells observed at 30µM compared to that of control. Significantly there was a 5 fold increase of apoptotic ratio observed in 30µM of SA treated cells. Propidium iodide staining was done to stain the nuclei of treated and control cells for additional confirmation of apoptosis induction by SA. The A549 cells treated with SA shows condensed nuclei which were fragmented to form apoptotic bodies confirmed its anticancer activity. Angiogenesis, the formation of new blood vessels is essential for the conservancy of normal cell functions. The dysfunction and/or increase in angiogenesis may lead to uncontrolled cell proliferation of cells resulting in cancer. Inhibiting angiogenic factors using natural compound has substantial hope in cancer research. Syringic acid, a naturally occurring phenolic acid was tested for its antiangiogenic property using the zebrafish embryo as *in vivo* model. Morphological observations of SA treated embryos were analyzed to evaluate the toxicity of the compound. The findings altogether suggest that syringic acid may have antiangiogenic activity by down regulating VEGF mediated pathway thereby having potential therapeutic benefit and promises to be a weapon against cancer (Karthik *et al*., 2014).

Oorabi *et al*. (2013) showed that proteosome inhibition leads to growth arrest in the G_1 phase of the cell cycle and/or induction of apoptosis. However, it was found that some of these inhibitors do not induce apoptosis in several human normal cell lines. This selective activity makes proteasome inhibition a promising target for new generation of anticancer drugs. Clinical validation of the proteasome, as a therapeutic target in oncology, has been provided by the dipeptide boronic acid derivative, bortezomib. Syringic acid, a known phenolic acid, isolated from the methanol extract of *Tamarix aucheriana* was shown to possess proteasome inhibitory activity.

The phenolic skeleton of SA possesses antimicrobial activity against several microorganisms. It inhibited the growth of methicillin-resistant *Staphylococcus aureus*, isolated from wound exudates. Among all 4-hydroxybenzoic acid derivatives, SA can only show anti-mutagenicity against *Salmonella typhimurium* tester strain TA-100 (Manuja *et al*., 2013). An *in vitro* study of SA was found to be effective against *Ganoderma boninense* (a pathogen causes basal stem rot and disease in oil palm trees) at lowest concentration of 0.5 mg/ml (Chong *et al*., 2011).

The mycelia of the edible mushroom *Lentinula edodes* were found to secrete phenolic compounds, such as syringic acid and vanillic acid. These compounds have radical scavenging activity, and were examined for their oxidative stress and hepatoprotective effect on CCl_4-induced chronic liver injury in mice. Both these compounds inhibited the activation of cultured hepatic stellate cells, which play a central role in liver fibrogenesis, and maintained hepatocyte viability. These data suggested that the administration of syringic acid and vanillic acid could suppress hepatic fibrosis in chronic liver injury (Itoh *et al*., 2010).

Kampa *et al*., (2004) concentrated on the antiproliferative action of caffeic acid, syringic acid, sinapic acid, protocatechuic acid, ferulic acid and 3,4-dihydroxy-phenylacetic acid which were concentrated on T47D human breast cancer cells for testing their antioxidant activity. A number of possible mechanisms were involved such as interaction with membrane and intracellular receptors and nitric oxide production were observed.

Belkheiri *et al*. (2010) suggested that syringic hydrazones (synthetic analogs of SA) possess a dual role of radical and carbonyl scavenger activity and prevents oxidative and carbonyl stress in the pathophysiology of atherosclerosis.

Syringic acid inhibited the UVB-induced phosphorylation of mitogen-activated protein kinases and Akt signaling pathways as well as epidermal growth factor receptor (EGFR). Syringic acid treatment further inhibited intracellular reactive oxygen species, protein-tyrosine phosphatase-κ activity, a regulator of EGFR activation and N-acetyl-l-cysteine along with SA inhibited UVB-induced nicotinamide adenine dinucleotide phosphate oxidase activity. Thus identification of molecular targets and its response to the drugs will pave a right way in cancer therapy (Ha *et al*., 2018).

2.13 *IN SILICO* STUDIES ON CANCER TARGETS

Every year, approximately 6.7 million deaths take place around the world due to various types of cancer (ACS, 2020). A wide array of cytotoxic agents and radiotherapy were used in the cancer treatment and they have limitations in their usage like side effects, and efficacy (Kim *et al*., 2003 and Stopeck and Thompson, 2012). Therefore, the development of better effective therapeutics for the cancer treatment from natural products remains continues because of its minimal side effects (Da Rocha *et al*., 2001, Vermani and Garg, 2002 and Gurib-Fakim, 2006).

In silico drug design using virtual screening, absorption, distribution, metabolism and excretion (ADME) / Tox data analysis, automated docking and molecular dynamics simulations for the determination of lead compounds for further *in vitro* analysis is a cost effective strategy. Accurate prognostic stratification of non-small cell lung cancer has become an important clinical reference when designing therapeutic strategies for cancer patients. With this clinical application in mind, Lai *et al*., (2020) developed deep neural network combining heterogeneous data sources of gene expression and clinical data to accurately predict the overall survival of NSCLC patients. Based on microarray data from a cohort set (614 patients), seven well-known NSCLC biomarkers were used to group patients.

Ahamed *et al*. (2020) investigated the *in silico* activity of the compounds 9-octadecenoicacid and 3-dodecanol through docking with 3OGN protein via AutodockVina program. The compound 9-octadecenoicacid showed good binding affinity (3.3 kcal/mol) than 3-dodecanol (3.0 kcal/mol) in 2RS2 protein respectively. Hydrogen bonding is one of the significant factors in the stability of protein ligand bonding, and the favorable bond distance amongst the H-donor and the H-acceptor atoms is less than 3.5 Å. Thus docking studies are key component to inspect the interaction and binding mode between the compounds 9-octadecenoicacid and 3-dodecanol.

Gurung *et al*. (2020) aimed the elucidation of plausible mechanisms of anticancer activity of the *Adenium obesum* by evaluating the binding interaction of its nine major selected compounds with macromolecular receptors implicated in the initiation and progression of cancer using various *in silico* approaches. Molecular docking studies revealed structural insights into possible binding modes of major active compounds of *A. obesum,* and identified the best docked compound for each target. The compound 1 (16-anhydro-3-acetylgitoxigenin) was found to be best docked (showed a high binding affinity, good number of hydrogen bonds and hydrophobic interactions with their respective molecular targets which play a key role in the pathogenesis of cancer) to four targets CDK-2, topoisomerase-II, VEGFR-2 and topoisomerase-I, whereas compound 2 (12b-Hydro xypregna-4,6,16-triene-3,20-dione), compound 7 (Lup-20(29)- ene-3,28-diol) and compound 8 (Quercetin 3,30 -dimethyl ether) were found to be best docked to CDK-6, Bcl-2 and Telomere: G-quadruplex respectively with favorable drug-like properties. Thus, these compounds can be promising leads for the design of specific target inhibitors which would help with management of the disease.

Zhao *et al*. (2016) investigated the strategy to discover novel lead compounds from an in-house database of Traditional Chinese Medicinal (TCM) compounds against epithelial growth factor receptor (EGFR) protein for targeting non-small cell lung cancer. After virtual screening of an initial dataset of 2,242 TCM compounds, leads were identified based on binding energy and ADME / Tox

data and subjected to automated docking followed by molecular dynamics simulation. Triptolide, a top compound identified by this vigorous *in silico* screening, was then tested *in vitro* on the H2347 cell line carrying wild-type EGFR, revealing an anti-proliferative potency similar to that of known drugs against NSCLC.

A significant study by Heble *et al*. (2016) revealed the anticancer property of bioactive molecules through *in silico* docking analysis of phytoconstituents from *Crocus sativus, Curcuma longa, Cassia occidentalis* and *Moringa oleifera. In silico* docking analysis was performed using Molegro Virtual Docker. The parameter used for the docking analysis are MolDock score, Rerank score and H-bond interactions. The target for anticancer activity was thymidylate synthase (PBD ID: 1HVY). The phytoconstituents of four medicinal plants and the standard drugs Ralitrexed, 5-Fluorouracil and Vinblastine were obtained from the PubChem database and drug bank in .mol format for comparison respectively. The comparative anticancer activity of the phytoconstituents of four medicinal plants are analysed by docking score and binding energy. It was analysed from the parameters of docking that the phytoconstituents from *Crocus sativum* showed better anticancer activity compared to that of the standard drugs.

Hasibuan *et al*. (2016) performed the *in vitro* study using 2,5-diphenyl tetrazolium bromide against T47D, MCF-7, HeLa and WiDr cell lines. *In silico* docking was carried using PLANTS program and visualized by Yasara program. The model of three dimension enzyme structures used in this research were epidermal growth factor receptor (EGFR), phosphatidylinositol-3-kinase (PI3K), estrogen receptor-alpha (ER-α), ER-beta (ER-β), and human EGFR 2 (HER-2). The tests showed that the inhibitory concentration of β-sitosterol was 50% and β-sitosterol and ZSTK474 inhibited EGFR and PI3K with good docking score when compared with tamoxifen.

Karthik *et al*. (2014) investigated the molecular interaction behind the mechanism of inhibition between SA and Bcl-2 which was done using Autodock 4v2 and the interaction was viewed using Pymol. SA shows good binding energy

(- 6.55 kcal/mol) and formed three hydrogen bond with the active site of Bcl-2 and thereby effectively inhibits the antiapoptotic protein.

Phosrithong *et al*. (2010) investigated the potential molecular targets for anticancerous compounds and docking was performed using the enzymes (cyclin-dependent protein kinase 2 (CDK-2), CDK-6, DNA topoisomerases I and II) and receptor proteins (B-cell lymphoma 2 (Bcl-2), vascular endothelial growth factor receptor 2 (VEGFR-2), and the telomere: G-quadruplexes) involved with cell cycle, cell growth, and DNA replication. The docking results revealed that lupeol exhibited better binding interaction to CDK-2 and Bcl-2 than the known CDK-2 and Bcl-2 inhibitors. Epigallocatechingallate (EGCG) was found to bind to CDK-6 with tighter interaction than several reported CDK-6 inhibitors. Flavopiridol, a synthetic flavonoid, was best bound to DNA topoisomerase I. Green tea catechin was best docked with topoisomerase II and VEGFR-2 and quercetin showed very good binding interaction with telomere: G-quadruplex.

Furey *et al*. (2000) stated that machine learning algorithms are powerful tools that apply input features (biomarkers) to capture the complicated interdependencies between the features to accurately predict clinical outcomes. In addition, predicting cancer prognosis can be improved by appropriately modelling the interactions between biomarkers compared with the single biomarker approach. A deep neural network is composed of non-linear modules represent multiple levels of abstraction (Bengio, 2009). Goodfellow *et al*. (2016) emphasized the significance that deep learning algorithms can extract high-level abstractions from different types of data sources and provide superior performance compared with traditional machine learning methods.

2.14 A549 LUNG CANCER CELL LINES – AN OVERVIEW

Research into various diseases, such as cancer, often relies on identifying drugs that influence cell growth and metabolism, or induce cell death (Wang *et al*., 2004). Stem-cell-based therapies in the context of regenerative medicine and tissue engineering rely on understanding how cells differentiate and interact with other cells, tissues, and materials. Primary stem, progenitor, and lineage-specific

cells are the gold standards for studying cell growth and behavior *in vitro*. However, the use of primary cells can be hampered by an unreliable supply, the difficulty of performing isolation and culture procedures *in vitro*, and loss of phenotype with increasing time in culture. For example, primary pulmonary alveolar type II (ATII) epithelial cells lose their distinctive phenotype over a period of 1–2 weeks when cultured *in vitro*, as they undergo spontaneous differentiation resulting in expression of features characteristic of alveolar type I (ATI) cells (Fuchs *et al*., 2003).

To overcome these limitations, cell lines are often used as models for primary cells. These cells are typically derived from cancerous tissue or by immortalization of primary cells through retroviral transfection or transduction. Cell lines are generally easier to culture than primary cells, have a high proliferation rate, long lifespan and maintain their phenotype in culture. However, the main disadvantage of cell lines is that the phenotype they express may not be consistent with the true phenotype of their primary counterparts (O'Hare *et al*., 2001).

The human A549 adenocarcinoma cell line has been used in lung cell biology as a model for ATII cells. A549 cells, as found in the lung tissue of their origin, are squamous and responsible for the diffusion of substances such as water and electrolytes, across alveoli. The A549 cell line was derived from a type II pneumocyte lung tumor which expresses the characteristic features of ATII cells, including synthesis of phospholipids, cytoplasmic lamellar bodies (Lbs), and apical microvilli (Giard *et al*., 1973). These highly specialized cells produce surfactant, a multifunctional lubricant that reduces surface tension and prevents alveolar collapse during ventilation. Since then, A549 cells have been used for *in vitro* studies of surfactant production and regulation of surfactant systems (Salmona *et al*., 1992).

If A549 cells are cultured *in vitro*, they grow as a monolayer, adherent or attaching to the culture flask. The cells are able to synthesize lecithin and contain high levels of unsaturated fatty acids, which are important to maintain membrane

phospholipids. A549 cells are widely used as a type II pulmonary epithelial cell model for drug metabolism and as a transfection host. When grown for sufficient long time in cell culture, A549 cells may begin to differentiate, as signaled by the presence of multilamellar bodies. A549 cells have served as models of alveolar Type II pulmonary epithelium, finding utility in research examining the metabolic processing of lung tissue and possible mechanisms of drug delivery to the tissue. In context of lung cancer drug development, the cells have served as testing grounds for novel drugs such as paclitaxel, docetaxel and bevacizumab both *in vitro* and *in vivo* through cell culture and xenografting, respectively. Single-cell tracking of A549 has enabled to elaborate pedigree-tree profiles and demonstrate correlations in behavior among sister cells. Such observations of correlations can be used as proxy measurements to identify cellular stress and inheritance as a response to drug treatment (Thomas *et al.,* 1998). A549 has also been employed in viral research and associated protein expression changes as a consequence of viral infection. Although A549 is a cancer cell line, it has also been studied for its response to tuberculosis, specifically the production of chemokines as it is induced by the invading bacteria (Lin *et al.,* 1998).

2.15. MICE MODEL IN CANCER RESEARCH

With the effective control of severe infectious diseases and the extension of human life expectancy, cancer has become one of the major diseases that seriously endanger human health. The development and research of new diagnostic methods and innovative treatment tools are essential to reduce the global incidence of cancer. The animal experiment is an important bridge between cell experiment and clinical experiment. Under certain conditions, the occurrence and development of animal diseases are similar to that of human beings, and animals have similar anatomy, physiology and heredity to human beings. Therefore, animal models are often used to study human diseases. In cancer research, the use of animal models can help us understand the genetic basis of cancer and the role of specific genes and gene mutations in the occurrence and development of cancer, which also facilitates the development and testing of antineoplastic drugs. With the continuous development of precision and

personalized medicine, researchers are looking for standardized and personalized tumor models that are more similar to human tumors (Xu *et al.,* 2019).

Animal models are valuable tools for studying the biology and genetics of human cancers as well as for preclinical investigation of anti-cancer therapeutics and cancer prevention. The mouse has been the traditional animal model for basic and preclinical studies of cancer, which plays important and complimentary roles as models of cancer research. The mouse genome is highly homologous to the human genome, which can simulate a series of biological characteristics such as the occurrence, development and metastasis of human cancer cells in vivo, and has the advantages of convenient feeding, low price, strong reproductive ability, easy maintenance and easy gene modification. It provides a good tool for cancer research and a valuable platform for drug discovery and verification (Bock *et al*., 2014). Genetically engineered mouse models of cancer have been generated by a variety of interventions such as chemical or physical mutagenesis, viral infection, insertion of transgenes, homologous recombination, and the recently developed gene edition. Studies from the genetically engineered models and xenograft models have led to discovery of the molecular basis of tumor initiation, growth, and metastasis, as well as being utilized for anti-cancer drug discovery and testing (Lunardi *et al.,* 2014 and Lehman and Stairs, 2015). Thus, translating animal models of cancer in combination with molecular tumor profiling and developing clinically useful targeted therapeutics are expected to produce a positive impact towards the goal of precision treatment that is personalized for patients with malignant diseases.

The detailed description of the methodology adapted for the present study is presented in the following chapter.