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Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma: a Network Pharmacology study

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CANDIDATE'S DECLARATION

I Anjali Sinha, Roll Number: 2K21/MSCBIO/62 student of M.Sc. Biotechnology, hereby declare that the project dissertation titled - "**Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma : a Network Pharmacology Study"** which is submitted by me to the Department of Biotechnology**,** Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is original and not copied from any source with proper citation. This work has not previously formed the basis for the award of any degree, Diploma Associateship, fellowship or other similar title or recognition.

The details of the conference paper are given below:

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CERTIFICATE

I hereby certify that the Project Dissertation titled "**Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma : a Network Pharmacology Study"** which is submitted by Anjali Sinha, Roll No.: 2K21/MSCBIO/62, Department of Biotechnology**,** Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record of the project work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part or full for any Degree or Diploma to the University or elsewhere.

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Anjali

ABSTRACT

With a high incidence and death rate, squamous cell lung cancer (LUSC) is a serious issue for the world's health. Drug toxicity and medication resistance continue to be major obstacles in the treatment of LUSC. Finding new therapeutic drugs is therefore urgently needed for the management of LUSC. Natural alkaloids, which comes from the *Tabernaemontana alternifolia* plant of Apocynaceae family, has shown promising anticancer benefits against many cancer forms, including LUSC. Molecular processes of *T. alternifolia's* phtochemicals in LUSC haven't been fully understood, yet. In this work, we looked at their potential as a treatment for LUSC by focusing on its molecular targets. To identify the molecular targets and pathways of them for the treatment of LUSC, we undertook network pharmacology analysis. Our in-silico docking studies using AutoDock vina showed that 9-methoxycamptothecin, camptothecin and heyneanine can reduce LUSC cell growth and trigger apoptosis. Importantly, they suppressed genes related to proliferation, angiogenesis, DNA repair, and cell cycle control. They also prevented the expression of important oncogenic factors such as MMPs, KDR and MET, etc. These findings imply that several molecular pathways can be targeted by *T. alternifolia's* phtochemicals to treat LUSC. The molecular mechanisms underpinning their anticancer actions in LUSC are significantly clarified by this work. New therapeutic approaches for the treatment of LUSC may be developed as a result of these findings. To validate the safety and effectiveness of *T. alternifolia's* phtochemicals as a possible treatment drug for LUSC, more preclinical and clinical trials are required.

CONTENTS

CHAPTER 1

1.1 INTRODUCTION

As one of the most common forms of cancer and the major cause of cancer-related deaths worldwide, lung cancer is one of the most prevalent forms. It is estimated that 1.8 million people worldwide die from lung cancer every year. There are over 2 million lung cancer diagnoses worldwide each year.https://doi.org/10.5114/wo.2021.103829

Worldwide, squamous cell carcinoma (LUSC) is the second most likely histological subtype of lung cancer and the leading cause of death and morbidity. https://doi.org/10.1016/j.molmed.2019.04.012

LUSC treatment and detection have improved tremendously in recent years, but the prognosis remains dismal, with a low survival rate and frequent recurrences. **Siegel RL, Miller KD, Jemal A. 2019. Cancer statistics, 2019. Cancer J Clin. 69:7–34** 10.3322/caac.21551

Previous research has uncovered numerous changed genes and genomic pathways as well as the complicated genetic landscape of LUSC. However, few targetable driver mutations have so far been identified, in striking contrast to lung adenocarcinoma, and targeted treatments for LUSC continue to be ineffective. Treatment for LUSC has been transformed by immunotherapy, which is now recognised as the new standard of care. 10.1038/s41388-021-01723-7

Radiation treatment, chemotherapy, and surgery are further therapies for lung LUSC. However, the effectiveness of these treatments is constrained, and they frequently have detrimental side effects. This emphasises the necessity for cutting-edge and efficient treatment modalities to increase lung cancer patients' chances of survival and quality of life.

Traditional medicine has used natural remedies made from plants to treat a variety of illnesses, including cancer. Modern medicinal compounds that have been demonstrated to have anti-cancer properties have been derived from a variety of plants. A plant species found in Asia and Africa is T. alternifolia, also known as tree jasmine. In conventional medicine, it has been used to treat a wide range of illnesses, such as cancer, inflammation, and fever. https://doi.org/10.3390/plants10020313

Recent research has shown that T. alternifolia extracts have anti-cancer potential against a variety of cancer types, including lung cancer. It has been shown that these extracts can decrease cell proliferation and trigger apoptosis in a variety of cancer cell lines. https://doi.org/10.3390/plants10020313

The intricate interactions between medications and their targets in biological systems are the focus of network pharmacology, which blends network biology, systems biology, and pharmacology.https://doi.org/10.1016%2FB978-0-12-801814-9.00005-2

Network pharmacology has become a potent technique for identifying new therapeutic targets and the mechanisms that regulate the actions of natural compounds in recent years. The molecular mechanisms underlying the therapeutic advantages of natural compounds can be investigated using network pharmacology, which can also be utilised to find prospective therapeutic targets and drug development routes. Recently, network pharmacology has gained popularity as a tool in cancer research for identifying novel therapeutic targets and creating fresh anti-cancer medications. 10.1016/j.tips.2021.11.004

1.2 OBJECTIVES

The objectives of this project are

1.- To treat lung squamous cell carcinoma (LUSC), determine the molecular targets and pathways that Tabernaemontana alternifolia's phytochemicals interact.

2.- Examine the impact of active phytonutrients from Tabernaemontana alternifolia on the development and apoptosis of LUSC.

3.- Analyse the effects of the phytochemicals in Tabernaemontana alternifolia on gene and pathway expression for proliferation, angiogenesis, DNA repair, and cell cycle control of LUSC.

CHAPTER 2. REVIEW OF LITERATURE

Lung squamous cell carcinoma (LUSC) develops from the squamous cells that line the lungs' airways. It is one of the two primary subtypes of NSCLC, along with lung adenocarcinoma, which is the other subtype. About 25 to 30 percent of all instances of lung cancer are LUSC.**https://doi.org/10.1038/s41467-021-22801-0.** The bronchi and bigger airways of the lungs are lined with squamous cells, which are flat, scale-like cells. These cells develop into LUSC when they go through a cancerous transformation. Smoking exposes lung tissue to carcinogens that can result in genetic changes and abnormalities in the squamous cells, which can lead to the development of LUSC.**Montserrat Sanchez-Cespedes, Steven A. Ahrendt, Steven Piantadosi, Rafael Rosell, Maria Monzo, Li Wu, William H. Westra, Steven C. Yang, Jin Jen, David Sidransky; Chromosomal Alterations in Lung Adenocarcinoma from Smokers and Nonsmokers[1]. *Cancer Res* 2 February 2001; 61 (4): 1309–1313.** The most common LUSC symptom is a centrally positioned lung tumour that frequently obstructs the airways. Symptoms such as coughing, chest pain, wheezing, and recurrent respiratory infections may be present. LUSC has the potential to metastasis, or spread, to other parts of the body, such as the lymph nodes, distant organs, and bones, like other types of lung cancer.

2.1 Prevalence

Due to its high incidence and fatality rates, lung squamous cell carcinoma (LUSC) is a serious health concern for people all over the world. It makes up between 25 to 30 percent of all instances of non-small cell lung cancer (NSCLC), making it one of the most prevalent kinds of lung cancer. About 85% of lung cancer cases are NSCLC, making LUSC a significant contributor to the overall burden of lung cancer.**https://doi.org/10.1016/j.semcancer.2020.07.009** Geographically, LUSC prevalence varies, with higher rates seen in some places. **https://doi.org/10.1038/ng.3891** It is more common in developing nations, particularly in areas with high smoking rates and tobacco consumption. However, LUSC continues to be a worldwide problem that has an impact on people everywhere.

Age, gender, and smoking all have an impact on the incidence rates of LUSC. Most occurrences of LUSC are caused by current or previous smokers, making smoking the biggest risk factor. Other risk factors that affect the development of LUSC include exposure to secondhand smoke, environmental toxins, and workplace dangers including asbestos and radon. LUSC has a significant impact on world health, primarily because of its high death rates. The most common cause of cancer-related fatalities in both men and women is lung cancer, particularly LUSC. LUSC typically has a poor prognosis

than other forms of lung cancer, because it is frequently discovered at an advanced stage when curative treatment options are few.

The death rates linked to LUSC emphasise the pressing need for better methods of detection, prevention, and treatment. Despite improvements in medical science and therapeutic approaches, the overall survival rate for LUSC remains low. The high fatality rates are a result of the aggressive nature of the disease as well as the fact that there are few viable treatments available for cases in their advanced stages.

A thorough strategy that combines public health initiatives to lessen tobacco use, support early identification and screening programmes, and improve treatment choices is needed to address the global burden of LUSC. To improve patients outcome of LUSC, further research is required to better understand the underlying mechanisms of LUSC development, find novel therapeutic targets, and create cutting-edge treatment modalities.

2.2 Significance

The relationship of lung squamous cell carcinoma (LUSC) with a number of variables that contribute to its aggressive behaviour and treatment difficulties is what gives LUSC its clinical significance. These elements underline the necessity of giving LUSC specific consideration in clinical practise and research.

1. The majority of instances of LUSC are seen in smokers who are currently smoking or former smokers. LUSC is significantly related with smoking. The genetic mutations and anomalies that the toxins in tobacco smoke can cause in the squamous cells lining the airways can result in LUSC. The association between smoking and LUSC highlights the significance of tobacco control policies and programmes for quitting smoking in lowering the prevalence of this illness.

2. The LUSC are aggressive in nature and have a tendency to metastasize early. It frequently manifests as a central tumour that blocks the airways, causing symptoms including coughing, chest pain, and breathing problems. Its dismal prognosis is a result of LUSC's early dissemination to local lymph nodes and distant organs. Because LUSC is aggressive, early identification and treatment are essential for enhancing patient outcomes.

3. Compared to other kinds of lung cancer, LUSC presents unique challenges. Another non-small cell lung cancer subtype, lung adenocarcinoma, responds to targeted therapy more successfully than LUSC does. Since LUSC is frequently discovered in an advanced stage, curative surgical approaches are less effective.

LUSC is frequently treated with chemotherapy and radiation therapy, however the effectiveness of these therapies may change according on the disease's stage. Further complicating therapeutic techniques is the aggressive behaviour of LUSC, which raises the risk of disease recurrence and therapy resistance.

4. LUSC has a lower prevalence of targetable genetic abnormalities than lung adenocarcinoma, which commonly harbours actionable mutations. As a result, people with LUSC have less access to certain targeted medicines. Research is now being done on discovering new therapeutic targets and creating individualised therapy plans that are specific to LUSC.

2.3 Pathways involved

The deregulation of important signalling pathways is a major factor in enhancing tumour development, survival, and metastasis in lung squamous cell carcinoma (LUSC). The PI3K/Akt/mTOR pathway, the MAPK/ERK route, and the Notch signalling pathway are among the many pathways that are frequently changed in LUSC, leading to its pathophysiology. For the purpose of locating possible therapeutic targets and creating cutting-edge therapeutic approaches, it is crucial to comprehend these dysregulated pathways.

1. PI3K/Akt/mTOR Pathway Cell growth, proliferation, survival, and metabolism are all governed by this pathway. This pathway is frequently dysregulated in LUSC, which increases cell survival and proliferation. The PI3K/Akt/mTOR pathway can be activated by genetic changes, such as activating mutations in PIK3CA (encoding the catalytic subunit of PI3K) or loss of tumour suppressor PTEN (a negative regulator of the process). This pathway's activation encourages cell cycle progression, prevents apoptosis, and boosts angiogenesis, which aids in the development of tumours and their resistance to treatment.

2. MAPK/ERK Pathway: The MAPK/ERK pathway controls cell proliferation, survival, and differentiation as well as the transmission of extracellular signals to the nucleus. Dysregulation of this pathway is frequently seen in LUSC. The MAPK/ERK pathway can be activated by mutations or amplification of receptor tyrosine kinases (RTKs), including EGFR and FGFR. Additionally, abnormal activation of this pathway may result from abnormalities in downstream signalling molecules like KRAS. In LUSC, MAPK/ERK signalling activation encourages cell proliferation, invasion, and metastasis.

3. Notch Signaling Pathway: The Notch signaling pathway plays a crucial role in cell fate determination, differentiation, and proliferation. Dysregulation of the Notch pathway is

frequently observed in LUSC. Abnormal activation of Notch signaling, through mutations or overexpression of Notch receptors (Notch1-4) or downstream effectors, promotes cell survival, proliferation, and epithelial-to-mesenchymal transition (EMT) in LUSC. Notch signaling is also implicated in maintaining cancer stem cells and contributing to therapeutic resistance in LUSC.

These dysregulated signaling pathways in LUSC are interconnected and often cross-talk with each other, forming a complex network of molecular interactions. The activation of these pathways promotes tumor progression, metastasis, and resistance to therapy. Targeting these pathways has emerged as a promising therapeutic approach in LUSC. Several targeted agents and inhibitors against components of these pathways are being investigated in preclinical and clinical studies.

2.4 Current treatment

Lung squamous cell carcinoma (LUSC) is typically treated using a multimodal approach that combines different treatment modalities based on the stage of the disease and individual patient characteristics. The current treatment modalities for LUSC include surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. Here is an overview of each modality:

1. Surgery: Surgery plays a central role in the treatment of early-stage LUSC. The main surgical approach is called lobectomy, which involves the removal of the affected lobe of the lung. In some cases, pneumonectomy (removal of the entire lung) or segmentectomy (removal of a smaller portion of the lung) may be performed. Lymph node dissection or sampling is also carried out to assess the spread of cancer. Surgical resection aims to remove the tumor and any nearby lymph nodes to achieve complete tumor removal.

2. Radiation Therapy: Radiation therapy uses high-energy X-rays or other forms of radiation to kill cancer cells and shrink tumors. It can be used in combination with surgery or as the primary treatment for locally advanced LUSC that cannot be surgically removed. Radiation therapy may be delivered externally (external beam radiation) or internally through the placement of radioactive sources (brachytherapy). It is also employed for palliative purposes to alleviate symptoms and improve quality of life in advanced-stage LUSC.

3. Chemotherapy: Chemotherapy is a systemic treatment that uses drugs to kill cancer cells throughout the body. It is typically administered either before surgery (neoadjuvant chemotherapy) to shrink tumors and facilitate surgical resection, or after surgery (adjuvant chemotherapy) to destroy any remaining cancer cells. In advanced or metastatic LUSC, chemotherapy may be used as the primary treatment to control the disease. Combination chemotherapy regimens, such as platinum-

based drugs (cisplatin or carboplatin) in combination with other agents (e.g., paclitaxel, gemcitabine), are commonly employed.

4. Targeted Therapy: Targeted therapy involves using drugs that specifically target molecular alterations or genetic mutations driving the growth and survival of cancer cells. In LUSC, targeted therapies are typically utilized for tumors with specific mutations, such as EGFR mutations or FGFR alterations. However, targeted therapies are more commonly used in lung adenocarcinoma than in LUSC, as LUSC has a lower frequency of targetable genetic alterations.

5. Immunotherapy: Immunotherapy has revolutionized the treatment of advanced-stage lung cancer, including LUSC. Immune checkpoint inhibitors, such as drugs targeting PD-1 (programmed cell death protein 1) or PD-L1 (programmed death-ligand 1), help the immune system recognize and attack cancer cells. Immunotherapy has shown significant efficacy in a subset of LUSC patients, particularly those with high PD-L1 expression.

The choice of treatment for LUSC is based on a number of variables, such as the disease's stage, the patient's general health, and the existence of particular molecular abnormalities. In order to give the best and most individualised treatment plan for each patient, treatment decisions are often determined using a multidisciplinary approach involving a team of oncologists, surgeons, radiation oncologists, and other specialists.

2.5 Challenges and limitations

Lung squamous cell carcinoma (LUSC) presents several challenges and limitations in its treatment, which can impact patient outcomes. Some of the key challenges and limitations associated with LUSC treatment include:

1. Drug Resistance: Like many other cancer types, LUSC may eventually grow resistant to many forms of therapy. As a result, chemotherapy, targeted therapy, or immunotherapy may no longer be effective in treating the tumours. The activation of alternative signalling pathways, modifications in the molecular pathways that medicines target, or genetic changes that encourage resistance can all be factors in the drug resistance mechanisms in LUSC. Drug resistance must be overcome, which is a substantial problem in the management of LUSC and necessitates the creation of novel treatment plans and combination methods.
2. Unfavourable side effects: Treatment options for LUSC, including as chemotherapy and radiation therapy, might have a negative influence on the quality of life of the patient. Chemotherapy can have a number of negative side effects, such as nausea, vomiting, exhaustion, hair loss, and an increased risk of infection. Radiation therapy may result in skin rashes, esophagitis, and radiation pneumonitis. Treatment interruptions or dose reductions may occasionally be necessary due to

unfavourable treatment effects, which may also necessitate supportive care measures.

3. Limited Options for Treatment of Metastatic or Advanced Disease: Treatment of advanced or metastatic LUSC is frequently difficult, primarily because there are few available treatments. LUSC has a lower prevalence of targetable mutations than lung adenocarcinoma, which has profited from the development of targeted medicines for particular genetic abnormalities. As a result, there aren't many targeted medicines that have been authorised especially for LUSC. This restricts the possibilities for treating patients with severe illness who have advanced after receiving conventional chemotherapy.
4. Lack of Predictive Biomarkers: For the best possible therapy options, it is essential to identify predictive biomarkers for LUSC. The prognostic utility of biomarkers in LUSC is still under investigation, despite the fact that some biomarkers, such as PD-L1 expression, have been used to direct treatment decisions. Finding trustworthy biomarkers to gauge patient response to various treatment modalities and direct decisions on individualised care continues to be a challenge.
5. Heterogeneity of LUSC: LUSC has significant molecular and histological heterogeneity, which may affect how each patient responds to treatment and how their case develops. It is difficult to create targeted medicines that are efficient for all LUSC patients due to this heterogeneity. It emphasises the necessity of gaining a deeper comprehension of the molecular subtypes of LUSC and the creation of specialised treatment strategies based on unique tumour characteristics.

2.6 Need of novel therapeutics

The challenges and limitations associated with the treatment of lung squamous cell carcinoma (LUSC) underscore the critical need for the development of novel therapeutic strategies and the exploration of alternative treatment approaches. Several factors contribute to this imperative:

1. Overcoming Drug Resistance: A major barrier to effective therapy is the formation of drug resistance in LUSC. The creation of novel therapeutic molecules that can circumvent or stop drug resistance processes is urgently needed to solve this problem. To reduce the possibility of resistance, this includes investigating new targets and creating combination medicines that can simultaneously target several pathways.
2. Targeted medicines: Although targeted medicines, such as lung adenocarcinoma, have revolutionised the treatment of several cancer types, the possibilities for targeted therapy in LUSC are constrained. Therefore, it is imperative to find and confirm fresh, targetable molecular changes that are unique to LUSC.
3. Optimisation of Immunotherapy: Immunotherapy, in particular immune checkpoint inhibitors, has demonstrated encouraging outcomes in the treatment of LUSC. However, not every patient responds to immunotherapy, emphasising the requirement to find prognostic indicators and create plans to increase the

effectiveness of immunotherapy in LUSC. To increase response rates and overcome resistance, this involves looking into combination strategies with additional immune modulators, targeted treatments, or chemotherapy.

4. The development of personalised medicine strategies catered to specific patients is essential for enhancing treatment outcomes in LUSC. In order to inform treatment choices and determine the most suitable therapeutic alternatives for each patient, this includes the integration of thorough genetic profiling and molecular characterisation. Real-time evaluation of treatment response and the formation of resistance can also be facilitated by the use of liquid biopsies and non-invasive monitoring techniques.
5. Clinical Trials: Stable clinical trials that assess the effectiveness and safety of novel therapy approaches are essential to the advancement of LUSC treatment. Participation in clinical trials gives patients access to cutting-edge treatments and helps generate data that could influence future paradigms of medical care. The creation and validation of new treatment modalities depend on promoting patient enrollment in clinical trials and facilitating interactions between researchers, doctors, and pharmaceutical corporations.

2.7 Tabernaemontana alternifolia

The Apocynaceae family of plants, known for their various medicinal effects, includes the plant species Tabernaemontana alternifolia. Tabernaemontana alternifolia, often known as "Christmasbush" or "Cape Jasmine," is indigenous to a number of places, including Africa, Asia, and Australia. Because of the pharmacological characteristics of this plant, it has a long history of usage as a traditional medicine in various cultures.

The therapeutic potential of Tabernaemontana alternifolia is enhanced by the presence of a variety of bioactive substances, such as alkaloids, flavonoids, terpenoids, and phenolic compounds. Science has investigated these bioactive chemicals' therapeutic properties and prospective applications in a range of medical ailments.

The Tabernaemontana alternifolia has historically been used to cure a number of illnesses, such as fever, inflammation, gastrointestinal problems, and respiratory problems. The bark, leaves, and roots of the plant are particularly prized for their therapeutic qualities.

Exploring Tabernaemontana alternifolia's pharmacological properties and potential therapeutic uses has been the subject of recent scientific research. The plant extracts have demonstrated cytotoxic and apoptotic properties against a variety of cancer cell lines, including lung squamous cell carcinoma (LUSC), and have demonstrated potential benefits as anticancer medicines. This has spurred interest in researching Tabernaemontana alternifolia's medicinal potential in the treatment of LUSC.

Additionally, the plant extracts have shown antibacterial, antioxidant, anti-inflammatory, and analgesic characteristics, suggesting they may be used to treat infectious diseases, ailments linked to oxidative stress, and pain management.

The variety of bioactive substances found in Tabernaemontana alternifolia provide a wide range of pharmacological effects, making it an interesting topic for further investigation. It is crucial to remember that although traditional uses and preliminary research suggest that it has medicinal promise, additional scientific studies, like as preclinical and clinical trials, are required to completely comprehend its efficacy, safety, and therapeutic processes.

2.8 Pharmacological activities of Tabernaemontana alternifolia

The potential therapeutic uses of Tabernaemontana alternifolia are facilitated by a broad spectrum of pharmacological actions. The bioactive substances found in Tabernaemontana alternifolia and their effects on numerous physiological processes have been studied in scientific investigations. The main pharmacological effects of Tabernaemontana alternifolia include the following:

1. Tabernaemontana alternifolia has demonstrated promising anticancer qualities. Different cancer cell lines, including lung squamous cell carcinoma (LUSC) cells, have shown cytotoxic effects and the ability to trigger apoptosis in response to plant extracts and isolated chemicals. Alkaloids, for example, which inhibit the growth, proliferation, and survival pathways of cancer cells, may be responsible for these anticancer actions.
2. Tabernaemontana alternifolia has been shown to have anti-inflammatory qualities, which have been linked to its bioactive components. Different diseases often include inflammation, and plant extracts have shown to have inhibitory effects on inflammatory mediators and indicators. This anti-inflammatory action points to its possible application in diseases including arthritis, inflammatory bowel disease, and other inflammatory disorders, which are characterised by excessive inflammation.
3. Antioxidant Activity: The development of many diseases is significantly influenced by oxidative stress, which is brought on by an imbalance between reactive oxygen species (ROS) and antioxidant defences. Researchers have discovered that Tabernaemontana alternifolia possesses antioxidant qualities that reduce oxidative stress by scavenging free radicals and preventing oxidative damage to cells and tissues. The antioxidant activity of Tabernaemontana alternifolia is influenced by the presence of phenolic compounds and flavonoids.
4. Against a variety of diseases, including bacteria, fungi, and viruses, Tabernaemontana alternifolia demonstrates antibacterial activity. Various fungal species, as well as Gram-positive and Gram-negative bacteria, have been shown to be inhibited by the plant extracts. Alkaloids, which have antimicrobial effects through interrupting cellular processes, are among the bioactive substances that may be responsible for these antimicrobial capabilities.

2.9 Previous studies

The medicinal potential of Tabernaemontana alternifolia and its bioactive components against different diseases, including cancer, has been investigated in pertinent studies and experiments. The following are some important conclusions drawn from studies and publications on the anticancer properties of Tabernaemontana alternifolia:

1. An investigation on the anticancer properties of Tabernaemontana alternifolia extracts against breast cancer cell lines was published in the journal Frontiers in Pharmacology. The outcomes demonstrated the extracts' potential as natural anticancer medicines by inhibiting cell proliferation and inducing apoptosis.
2. Researchers looked at the cytotoxic effects of alkaloids extracted from Tabernaemontana alternifolia against lung cancer cell lines in a different study that was published in the journal BMC Complementary and Alternative Medicine. The alkaloids showed considerable cytotoxicity and caused apoptosis in the cancer cells, showing that they have the potential to be used as therapeutics.

3. The Journal of Ethnopharmacology released a study that looked at how Tabernaemontana alternifolia extracts affected colorectal cancer cells' ability to proliferate. The extracts' promise as an all-natural treatment for colorectal cancer is suggested by the study's discovery that they suppressed cell growth and caused cell cycle arrest.
4. Prostate cancer cells were used in a study published in the journal Evidence-Based Complementary and Alternative Medicine to examine Tabernaemontana alternifolia extracts' anticancer properties. The extracts demonstrated inhibitory effects on cell viability and caused apoptosis in the cancer cells, showing their promise as a cutting-edge therapeutic strategy for the treatment of prostate cancer.

The potential of Tabernaemontana alternifolia and its bioactive substances in the treatment of different cancers is highlighted by these research.

2.10 Network pharmacology

Network pharmacology is an interdisciplinary approach that integrates network analysis, computational biology, and pharmacology to study the interactions between drugs, targets, and biological systems. It involves the construction of biological networks that represent the relationships between genes, proteins, pathways, and diseases, and utilizes computational methods to analyze and interpret these networks.

The advantages of network pharmacology in identifying complex relationships and molecular interactions within biological networks include:

1. Instead of concentrating on specific genes or targets, network pharmacology enables researchers to take the entire biological system into account. By recording the interactions and crosstalk between various system components, it offers a comprehensive viewpoint and sheds light on the general network behaviour.
2. Identification of Important Players: Network analysis aids in the discovery of important genes, proteins, and pathways that are essential for the development of disease or the response to treatment. Researchers can rank goals and paths for additional research by analysing the network architecture and centrality metrics.
3. Drug-Target Interaction Prediction: Network pharmacology computational approaches allow for the prediction of possible drug-target interactions. This aids in finding new therapeutic targets and adapting already-approved medications for use in different conditions.
4. Integration of Omics Data: Network pharmacology enables the integration of various omics data, such as transcriptomics, proteomics, and genomes, to provide a thorough understanding of the biological system. The underlying molecular pathways are better understood and possible biomarkers are found because to this integration.

Network pharmacology assists in the rational creation of novel medications by taking the network environment into account. It enables researchers to find medication combinations that target various network elements, thereby producing synergistic effects and better therapeutic results.

In conclusion, network pharmacology provides a potent method for comprehending the intricate relationships between biological systems and locating possible treatment targets and approaches. A thorough understanding of drug-target interactions is provided by its integration of network analysis and computational approaches, helping the discovery and development of novel therapeutic interventions.

CHAPTER 3: MATERIALS

3.1 TOOLS AND SOFTWARES

This research work worked with various open-source applications and databases including

IMPPAT https://cb.imsc.res.in/imppat/,

Venny 2.1 https://bioinfogp.cnb.csic.es/tools/venny/,

SwissTargetPrediction http://www.swisstargetprediction.ch/,

GEPIA2 http://gepia2.cancer-pku.cn/#index,

STRING https://string-db.org/,

Cytoscape https://cytoscape.org/,

Cystoscope's Molecular Complex Detection (MCODE) plug-in https://apps.cytoscape.org/apps/mcode,

Cytoscape's CYTOHUBBA plug-in https://apps.cytoscape.org/apps/CYTOHUBBA,

DAVID https://apps.cytoscape.org/apps/CYTOHUBBA,

Protein Data Bank https://www.rcsb.org/,

NCBI PubChem https://pubchem.ncbi.nlm.nih.gov/,

UNIPROT https://www.uniprot.org/,

BIOVIA Discovery Studio Visualizer https://discover.3ds.com/discovery-studio-visualizer-download,

AutoDock Vina https://vina.scripps.edu/, and

Open Babel GUI https://openbabel.org/docs/current/GUI/GUI.html

3.1.1**IMPPAT**

The IMPPAT 2.0 database features an easy-to-use online interface that provides users with vital information about Indian medicinal plants and their phytochemical qualities. The website used Bootstrap 4.1.3, an open-source CSS framework, as a base to construct this interface. Customizations were then done to the front end using internal HTML, PHP, CSS, jQuery scripts, and JavaScript.**doi: 10.1021/ACSOMEGA.3C00156, doi: 10.1093/bioinformatics/btv557**

3.1.2 Pubchem

The public chemical database PubChem is housed at the National Institutes of Health (NIH) in the United States. PubChem is a popular site for researchers, patent agents, and students, as well as millions of monthly users. Importantly, PubChem data is extensively used in machine learning and artificial intelligence projects. PubChem, a data aggregator, collects chemical data from thousands of sources. While the majority of the molecules in PubChem are siRNA, miRNA, lipids, carbohydrates, and biopolymers that have been chemically modified, it also includes other chemical substances such as siRNA and miRNA. This information is organised into numerous data sets, including Substance, Compound, BioAssay, Gene, Protein, Taxonomy, Pathway, Cell Line, and Patent. **doi: 10.1093/nar/gkaa971, doi: 10.1080/17460441.2016.1216967, doi: 10.1093/nar/gkv951, doi: 10.1016/j.jmb.2022.167514.**

3.1.3 Uniprot

The massive database UniProt provides information on proteins. UniProt provides access to protein sequences, functional annotations, structural features, interactions, and other data. UniProt also provides additional information and tools, such as UniProtKB/Swiss-Prot, UniProtKB terms, and cross-references to other databases, protein annotation tools, and more.**doi: 10.1093/nar/gky1049**

3.1.4 Swiss Target Prediction

The Swiss Institute of Bioinformatics (SIB) developed Swiss Target Prediction that forecasts potential targets or interactions for small molecules like drugs or chemical compounds. Calculations are made to determine how likely it is that a given chemical will bind to particular protein targets using a variety of approaches and algorithms.Using a ligand-based methodology, the Swiss Target Prediction programme compares a compound's chemical structure to a database of recognised ligands and their associated protein targets. Additionally, a target-based approach is used to identify probable binding sites.
interactions by comparing the chemical characteristics of the drug to those of known protein structures

.**doi: 10.1093/nar/gkz382.**

3.1.4 GEPIA2

A web-based programme called GEPIA2, or Gene Expression Profiling Interactive Analysis 2, exists. The Genotype-Tissue Expression (GTEx) studies on gene expression and the Cancer Genome Atlas (TCGA) data will be analysed and visualised. GEPIA2 can be used by users and researchers to look at the links and patterns of gene expression in both normal and cancerous tissues.A survival analysis option in GEPIA2 enables researchers to investigate the connection between gene expression and patient survival outcomes.**doi: 10.1093/nar/gkz430**

3.1.5 VENNY2.1

Venny 2.1 is a web-based used for making Venn diagrams. Venn diagrams are graphic representations of the relationships between numerous sets or groups of objects. They are frequently used in a range of areas, including biology, statistics, and data analysis, to visualise the overlaps and distinct components across several sets [55]. Venny 2.1's user-friendly interface makes it simple to build Venn diagrams with up to six groupings. **doi: 10.3390/cancers14102447**

3.1.6 STRING DB

STRING-DB (Search Tool for the Retrieval of Interacting Genes/Proteins) is a bioinformatics database and online resource that covers protein-protein interactions (PPIs), functional connections, and networks. STRING-DB creates a huge network of proven and anticipated protein interactions by combining data from multiple sources. **doi: 10.1038/s41598-023- 31413-1**

3.1.7 Cytoscape

An effective open-source software tool for visualising, analysing, and simulating complex networks is called Cytoscape. It is frequently used in systems biology and bioinformatics research to examine and grasp biological networks like gene regulatory networks, metabolic pathways, and protein-protein interactions.One of Cytoscape's main advantages is the enormous selection of plugins that it offers, which expand its functionality and enable users to do a variety of network analyses. Network clustering, pathway enrichment analysis, network topology analysis, and network motif recognition are just a few of the topics covered by these plugins. Additionally, Cytoscape enables users to combine and study other data sources, such as genomic annotations and gene expression data, with network data.**doi: 10.1007/978-981-19-0901-6_5**

3.1.8 BIOVIA Discovery Studio

An all-in-one software package for computational drug discovery and molecular modelling is called BIOVIA Discovery Studio. With regard to target selection and validation, virtual screening, ligand design, and protein-ligand interaction analysis, it offers a wide range of tools and features to assist researchers.In addition to molecular modelling, BIOVIA Discovery Studio has capabilities for virtual screening, which is the computational screening of sizable compound databases to find potential drug candidates. It offers a number of virtual screening approaches, including docking based on structure and similarity searches based on ligands, enabling researchers to quickly prioritise and choose compounds for additional research.The BIOVIA discovery Studio software suite as a whole integrates a number of computational tools and algorithms to support drug development efforts.

doi:10.2174/1389557520666201214101329.

3.1.9 Cytoscape MCODE

The MCODE (Molecular Complex Detection) Cytoscape plug-in is a powerful tool for identifying clusters or parts of a biological network that are closely connected to one another. It facilitates in the finding of probable functional modules or complexes in protein-protein interaction networks or other types of molecular networks [59].The MCODE plug-in is commonly used when analysing large-scale molecular networks to uncover physiologically relevant modules or complexes. It aids in the detection of regulating components of biological systems, potential protein complexes, and functional linkages.**doi: 10.1038/s41598-020-79235-9**

3.1.10 Cytoscape CYTOHUBBA

Cytoscape's CytoHubba plug-in is an effective tool for network analysis and detecting significant hubs or nodes within a biological network. It provides a number of methods and strategies for determining node centralities, ranking nodes based on their topological relevance, and detecting key nodes in the network [60]. The CytoHubba Cytoscape plug-in is often used in network biology research to find major nodes, hub proteins, or critical regulatory components within biological networks. It aids in understanding the network architecture and determining the functional and regulatory functions of specific components in biological systems. **doi: 10.1038/s41598-020-76024-2**

3.1.11 DAVID

DAVID (Database for Annotation, Visualisation, and Integrated Discovery) is a web-based bioinformatics application used for functional annotation and enrichment analysis of gene or protein lists. It provides a large variety of functional annotation tools and resources to help you understand the biological importance of a certain set of genes or proteins [61].DAVID is commonly used by genomic, transcriptomic, and proteomic researchers to get functional insights into their gene or protein lists. It aids in high-throughput data analysis, the identification of biological pathways, and the identification of underlying biological processes associated to the input genes or proteins.**doi: 10.1007/s12010-022-04170-6**

3.1.12 RCSB PDB

The RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) is a comprehensive and well-known tool for examining the three-dimensional structures of biological macromolecules. It provides users with access to a large library of complex biomolecular structures that have been experimentally determined. Through investigation, analysis, and visualisation, it provides a better understanding of the roles, interactions, and structural properties of protein and nucleic acid structures.**doi: 10.1002/pro.3730**

3.1.13 AutoDock VINA

To predict the affinities and binding patterns of small molecules (ligands) to protein targets, a well-known and widely used molecular docking programme called AutoDock Vina is utilised. It performs flexible docking simulations by fusing empirical scoring techniques with evolutionary algorithms.**doi: 10.1021/acs.jcim.1c00203**

3.1.14 Open Babel GUI

The Open Babel programme, a potent and adaptable toolkit for chemical informatics, computational chemistry, and molecular modeling, has a graphical user interface (GUI) called Open Babel. The Open Babel GUI offers a simple user interface for navigating and using Open Babel's features. **doi: 10.1142/S0219720020400119**

CHAPTER 4 METHODOLOGY

4.1 **Bioactive identification**:
Data on the phytonutrients present in the bark of Tabernaemontana alternifolia were gathered using the IMPPAT database. A carefully maintained collection of data on Indian Medicinal Plants, Phytochemistry, and Therapeutics is housed in the database known as IMPPAT. The IMPPAT IDs were recorded for future reference. All of the phytonutrients' ADME properties were investigated.

4.2 **Potential gene target prediction:**
The PUBCHEM database, supplied the distinctive identification code (Canonical SMILES) for these compounds, to identify the phytonutrients in Tabernaemontana alternifolia. The probable protein or molecular targets with which these phytonutrients might interact throughout the body were then predicted using Swiss Target Prediction, a webtool. As information sources, the studies' associated references are included.

4.3 Identification of differentially expressed genes (DEGs) of squamous cell lung cancer (LUSC):
The GEPIA2 web application provided a list of Differentially Expressed Genes (DEGs) for LUSC.

4.4 Identifying of intersection gene targets:
To find possible targets for genes that fight lung squamous cell carcinoma (LUSC). Using the venny2.1 online tool, we compared the gene targets linked to the active phytonutrients in Tabernaemontana alternifolia with the gene targets linked to LUSC. The overlapping genes were taken into consideration as prospective anti-LUSC gene targets by identifying the shared genes between these two groups using Venny 2.1.0.

4.5 protein-protein interaction analysis:
The STRING database was used to further analyse the putative anti-LUSC gene targets and investigate protein-protein interactions. The outcomes of the STRING

analysis of protein-protein interactions were subsequently exported as tab-separated values (tsv) files. The possible anti-LUSC core targets were then visualised and investigated using the Cytoscape programme once this file was submitted. The study only took into account targets that were unique to the Homo sapiens species and had a moderate confidence score of larger than 0.4.

4.6 prime targets identification using MCODE:

The MCODE plug-in for Cytoscape was used to locate the important elements in the protein-protein interaction network of possible anti-LUSC targets. For the purpose of finding molecular complexes in extensive protein interaction networks, this plug-in employs an automated technique. A degree cutoff of 2, a node score cutoff of 0.2, a k-core of 2, and a maximum depth of 100 were the parameters that were used for the analysis. The discovery of clusters across the whole network was made possible by these parameter settings.

4.7 core targets identification using CYTOHUBBA:

The top 10 targets were filtered using the "cytohubba" plugin in Cytoscape version 3.9.1. Based on different network analysis algorithms, this plugin is intended to identify significant nodes or targets within a network. The Degree, Maximum Neighbourhood Component (MNC), Maximal Clique Centrality (MCC), and Closeness approaches were applied. The core targets were found by identifying the point at which the targets from these four strategies intersected. The most important and central targets in the network of protein-protein interactions might be found using this method.

4.8 Network construction between the bioactive and the potential targets:

The interactions between the active phytonutrients of Tabernaemontana alternifolia and the LUSC-related prime and core targets were modelled using the Cytoscape software. The links and interactions between these elements are better understood through the network visualisation. The network offers a thorough perspective of the potential connections and effects in the context of LUSC by merging the data on phytonutrients and the indicated targets.

4.9 GO and KEGG ENRICHMENT analysis :

GO functional and KEGG pathway enrichment techniques were used to do additional analysis on the potential core targets for anti-LUSC. The targets' functional annotations and their connection to cellular processes, biological functions, and molecular processes were the main focus of this investigation. The top 10 enriched GO terms for biological process (BP), cellular component (CC), and molecular function (MF), as well as the top 30 enriched KEGG pathways, were found after the data were uploaded to a bioinformatics platform for analysis. An enrichment dot bubble plot was used to display the results. The Benjamini-Hochberg technique for multiple hypothesis testing was employed to control the false discovery rate (FDR), and a conventional hypergeometric test was utilised to establish statistical significance. An adjusted p-value of less than 0.05 was used in the analysis.

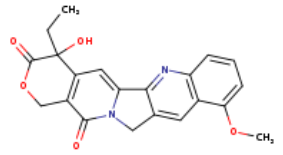
4.10 **Molecular docking :**

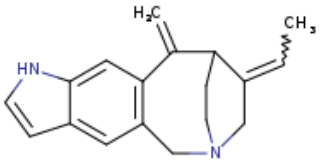
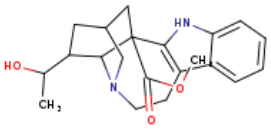
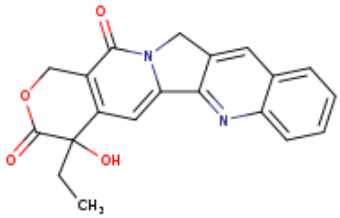
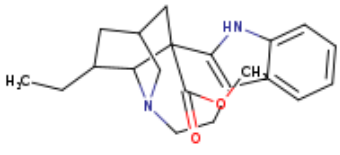
In this study, the top three active phytonutrients from Tabernaemontana alternifolia were compared to the top ten probable anti-LUSC core targets. The key targets' crystal structures (MMP9, KDR, MMP2, JAK2, NOS3, MET, KIT, PDGFRB, FGFR1, PARP1) were downloaded in PDB format from the RCSB Protein Data Bank (PDB). PDB IDs are 6ESM, 3WZE, 7XGJ, 8BAK, 1M9J, 2WD1, 4U0I, 3MJG, 4ZSA, and 7KK2 respectively. On the other hand, NCBI PubChem was used to obtain the chemical structures of the active phytonutrients (9-Methoxycamptothecin, Heyneanine, and Camptothecin).The crystal structures and phytonutrient structures were converted from PDB format to PDBQT file format using the Open Babel GUI software. Polar hydrogens were added into the crystal formations in place of the heteroatoms (water and other ligands). The Kollman partial charges were applied to these cleaned-up protein structures after they had been put into AutoDock Tools and saved in PDBQT format.Then, as ligands and macromolecules, respectively, the produced protein structures and phytonutrient structures in PDBQT format were employed and stored. The molecular docking process was carried out using AutoDock Vina. For blind docking, a grid box was made for each protein. The command prompt was used to create molecular docking programmes, and the results were then examined in terms of binding affinity. The docked complexes were visualised using BIOVIA Discovery Studio Visualizer to provide 2D and 3D images that show the binding contacts between the active phytonutrients and the target proteins.

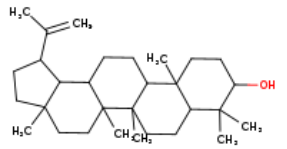
CHAPTER 5: RESULTS

The IMPPAT database lists 38 phytonutrients in the T. alternifolia plant. As demonstrated in Table 1, six of the active phytonutrients were discovered in the plant's bark.

TABLE 1.

Phytochemical	PubChem ID	IMPPAT ID	Structure
9-methoxycamptothecin	**123617**	**IMPHY012331**	

Pericalline	**6436240**	**IMPHY006461**	
Heyneanine	**15559731**	**IMPHY001591**	
Camptothecin	**24360**	**IMPHY002933**	
Coronaridine	**73489**	**IMPHY007011**	

Lupeol	**259846**	**IMPHY012473**	

3.2

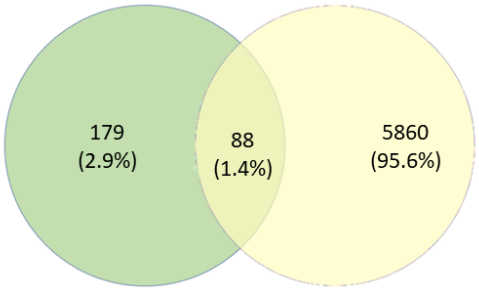
For the six active phytonutrients under study, the SwissTargetPrediction online database was used to find 401 possible gene targets with a probability less than 0. Based on computational techniques, this database offers predictions of the molecular targets that these phytonutrients may interact with. Based on the probability scores of the gene targets, those with a probability smaller than 0 received the most attention. These gene targets represent potential interactions between genes and the active phytonutrients.

3.3 **LUSC-related gene target**

Using the Gepia2 online tool, a total of 5960 gene targets associated with LUSC (lung squamous cell carcinoma) were discovered. The ANOVA differential method, which allows for the distinguishing of over-expressed and under-expressed genes, was used to determine the gene targets. This method specifically examines the levels of gene expression between LUSC samples and normal samples. The research concentrated on genes located on chromosomes that passed the q-value and log2FC (logarithm of fold change) thresholds of 0.01 and 1, respectively. These gene targets identify genes that may contribute to the onset or progression of LUSC and offer important information on the underlying molecular mechanisms of the condition.

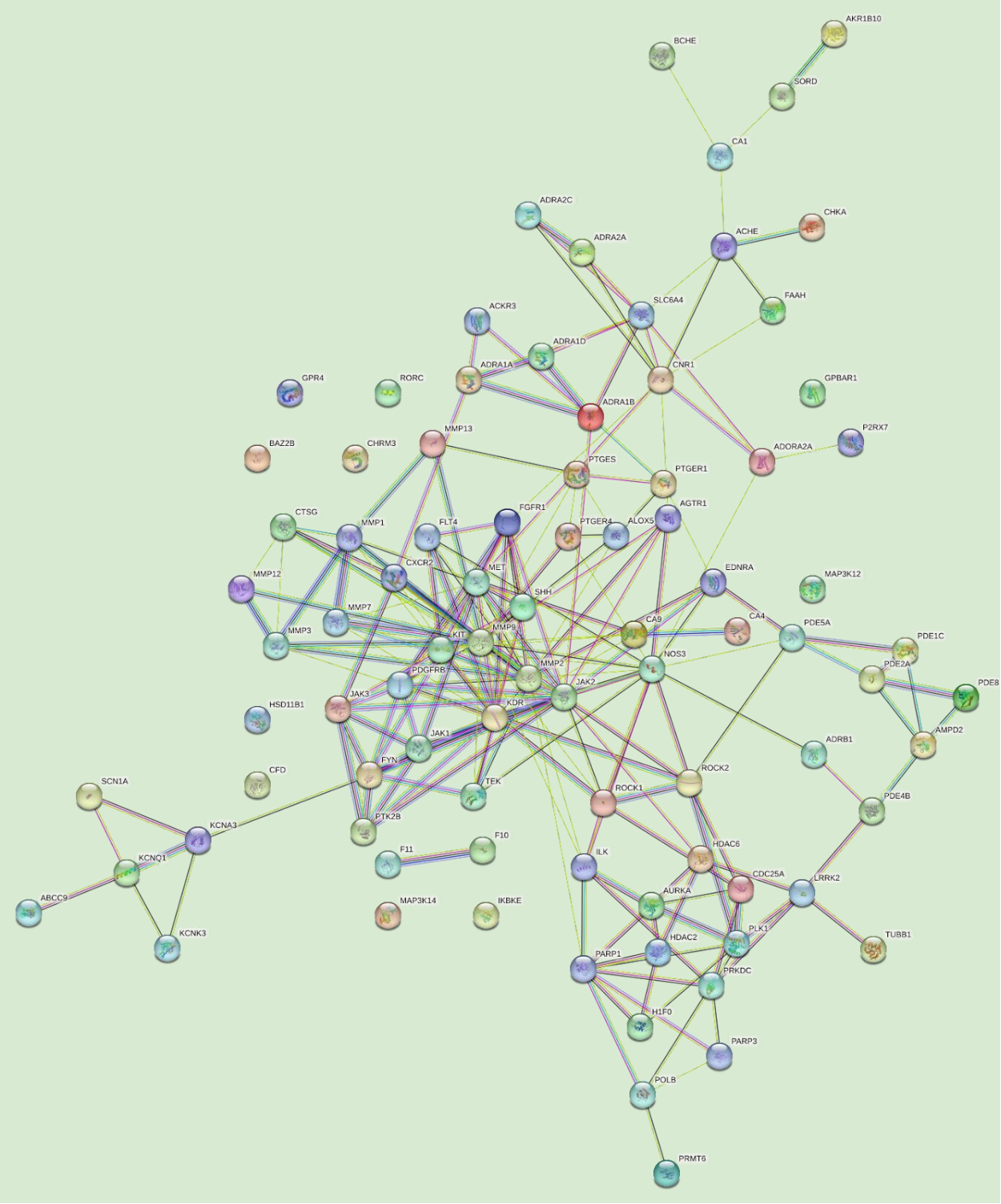
3.4 **Intersection gene targets analysis**

In accordance with Figure 1, the Venny 2.1 analysis showed that there were 88 gene targets that overlapped between the 401 potential gene targets of the active phytonutrients in Tabernaemontana alternifolia and the 5960 gene targets of LUSC. These 88 gene targets were chosen as prospective anti-LUSC gene targets. The overlap of the gene targets impacted by the phytonutrients and the gene targets linked to LUSC shows a possible relationship and raises the possibility that these shared gene targets contribute to the anti-LUSC actions of Tabernaemontana alternifolia's active phytonutrients.



3.5 **PPI network analysis**

Figure 2A illustrates the results of the STRING analysis of the protein-protein interaction (PPI) network, which revealed that the network included 88 nodes and 219 edges. The degree to which nodes in a network tend to cluster together was measured using the average local clustering coefficient, which was estimated to be 0.511. It was found that there were 4.98 average node degrees, or the average number of edges connecting each node. The network's observed interactions' statistical significance as measured by the PPI enrichment p-value, which is less than 1.0e-16, was discovered. In addition, the network contained 91 predicted edges.



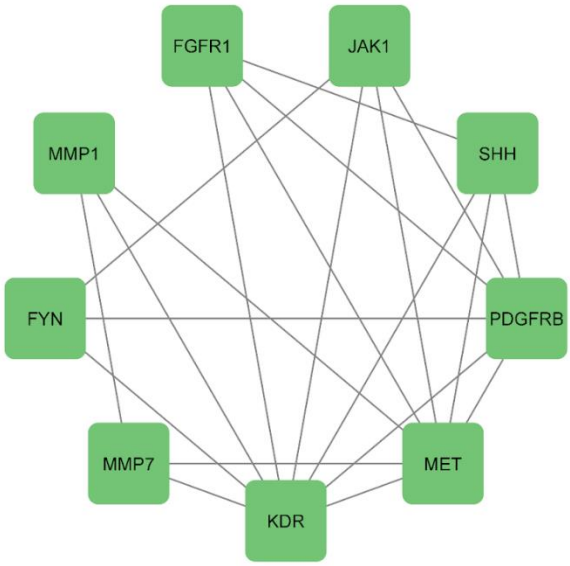
However, it was discovered that the PPI network contained 10 non-interacting nodes during the Cytoscape study. The resulting PPI network in Figure 2A has 78 nodes as a consequence. Every pair of nodes in the network had the characteristic path length of 2.473. Additional network statistics were calculated, including the diameter (6.0), which represents the longest shortest path

between any pair of nodes, the average number of neighbours (5.615), which represents the average degree of connectivity for the nodes, the clustering coefficient (0.224), which reflects the extent to which nodes tend to cluster together, and the network radius (0.036), which measures the proportion of possible edges that are present in the network.

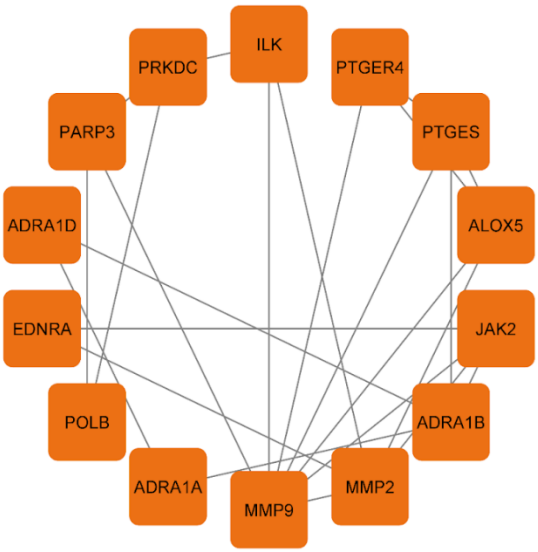
29 nodes were chosen from the network as the main anti-LUSC targets based on the degree centrality (DC) criterion, with a threshold of average value (5.6), as shown in Figure 4. The 29 nodes in Table 2 were prioritised according to their degree centrality scores and represent the primary anti-LUSC gene targets. The ordering of these nodes according to their degree centrality values is shown in the image as a bar graph.

3.6 Cluster network analysis

Using Cytoscape's MCODE plugin, cluster analysis was performed on the network made up of the 88 prospective targets for LUSC. The protein-protein interaction (PPI) network of the anti-LUSC primary targets was shown to contain two unique cluster networks. Figure 3 & 4 shows these cluster networks, which highlight the interconnection within each cluster and provide a visual picture of the clustering of the prospective targets.
The initial cluster network, which has 9 nodes and 21 edges, is shown in Figure 3A. The cluster network scored 5.250, demonstrating a high level of node interconnectivity within the cluster. Notably, a number of genes, including FGFR1, PDGFRB, MET, and KDR, have many gene targets and a degree centrality (DC) value of less than 4.667, showing their significance in the network.

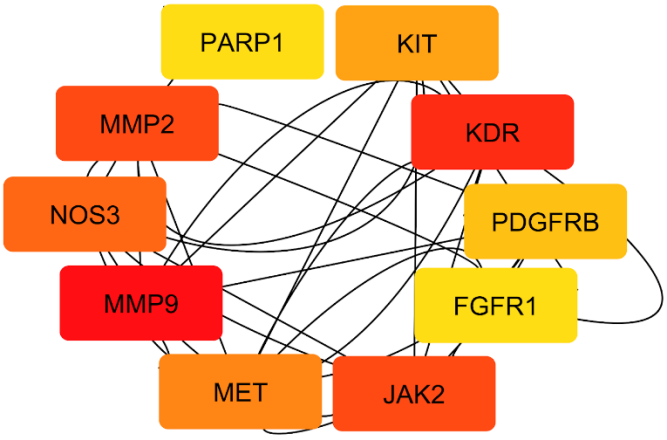


The second cluster network, shown in Figure 4, has 14 nodes and 22 edges. A score of 3.385 for the cluster network indicates a little lower degree of interconnectivity than the first cluster. Genes like MMP9, JAK2, and MMP2 show substantial connectivity with other nodes in this network. The importance of these genes within the network and their potential function as anti-LUSC targets are indicated by their degree centrality (DC) values, which are 3.143 for these genes.The second cluster network, shown in Figure 5B, has 14 nodes and 22 edges.



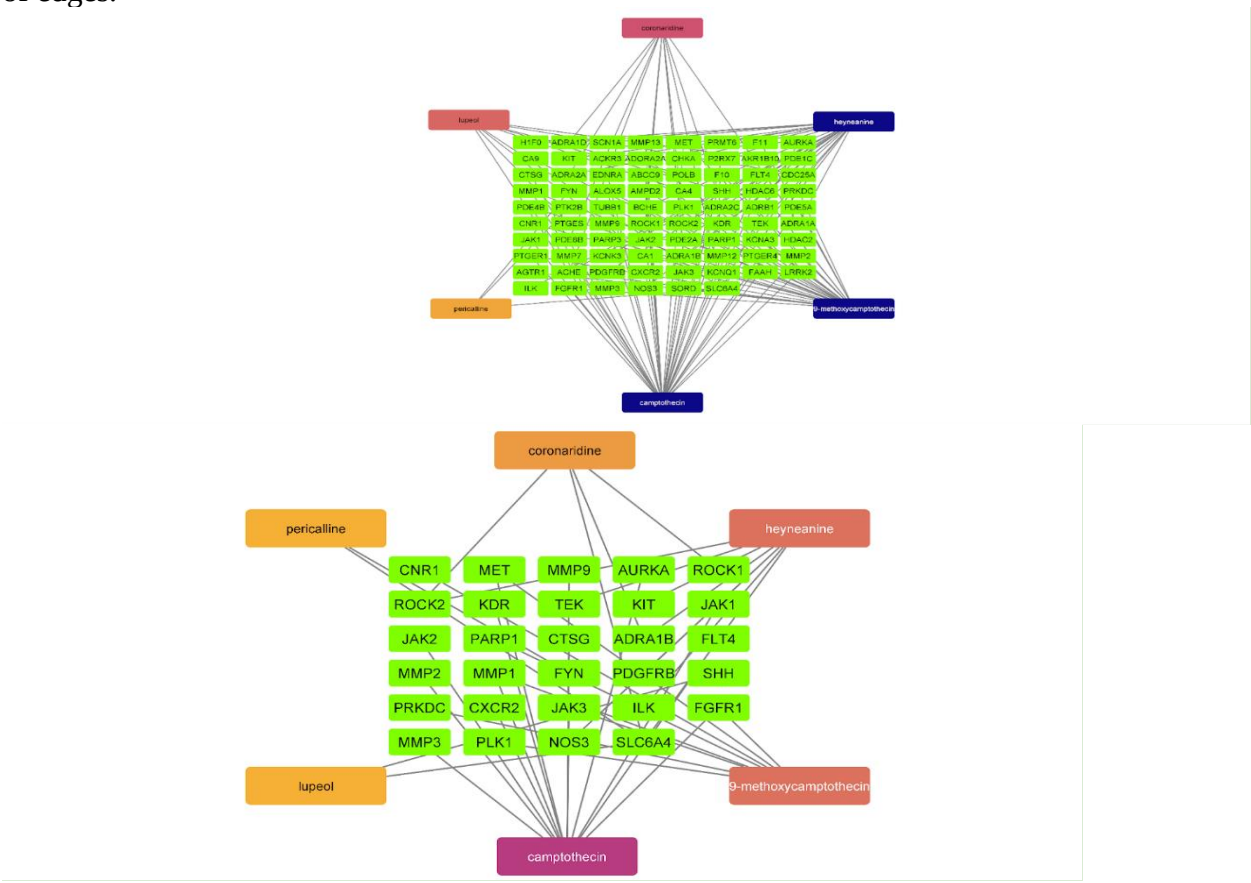
3.7 **Core targets screening**

The "cytohubba" plugin in Cytoscape 3.9.1 was used, and four distinct techniques were used to identify the key targets inside the network: Degree, Maximum Neighbourhood Component (MNC), Maximal Clique Centrality (MCC), and Closeness. Figure 6 displays the findings of this research and lists the top 10 core targets chosen by each technique. The relevance and centrality of these main targets inside the network, as determined by the particular approach used, were filtered.

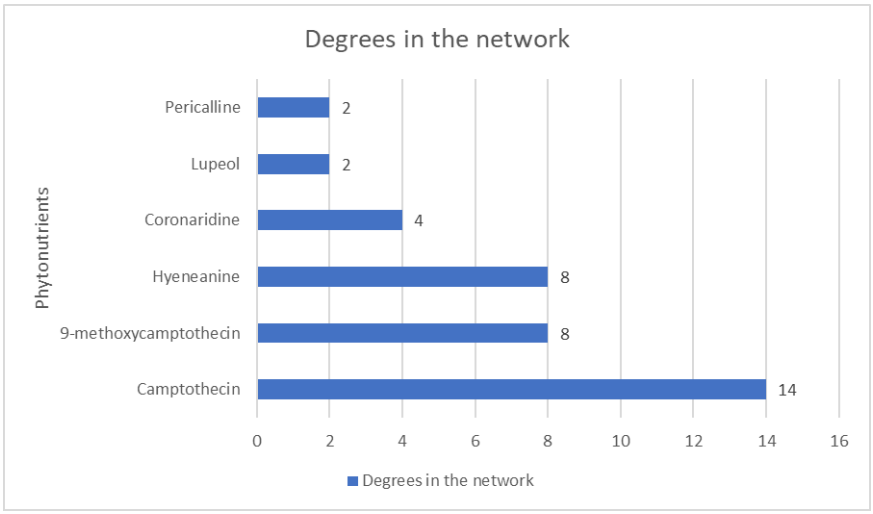


3.8 **Network of active phytonutrients and anti-PAAD targets**

The network shown in the figure represents the link between the active phytochemicals found in Tabernaemontana alternifolia and 78 putative anti-LUSC targets. The network is made up of 84 nodes and 114 edges that represent the interactions between the targets and phytonutrients. The network's diameter is one, meaning that the shortest path length between any two nodes is one. The radius is also one, signifying the network's minimum eccentricity. The network density is 0.010, which indicates the proportion of edges present relative to the maximum potential number of edges.

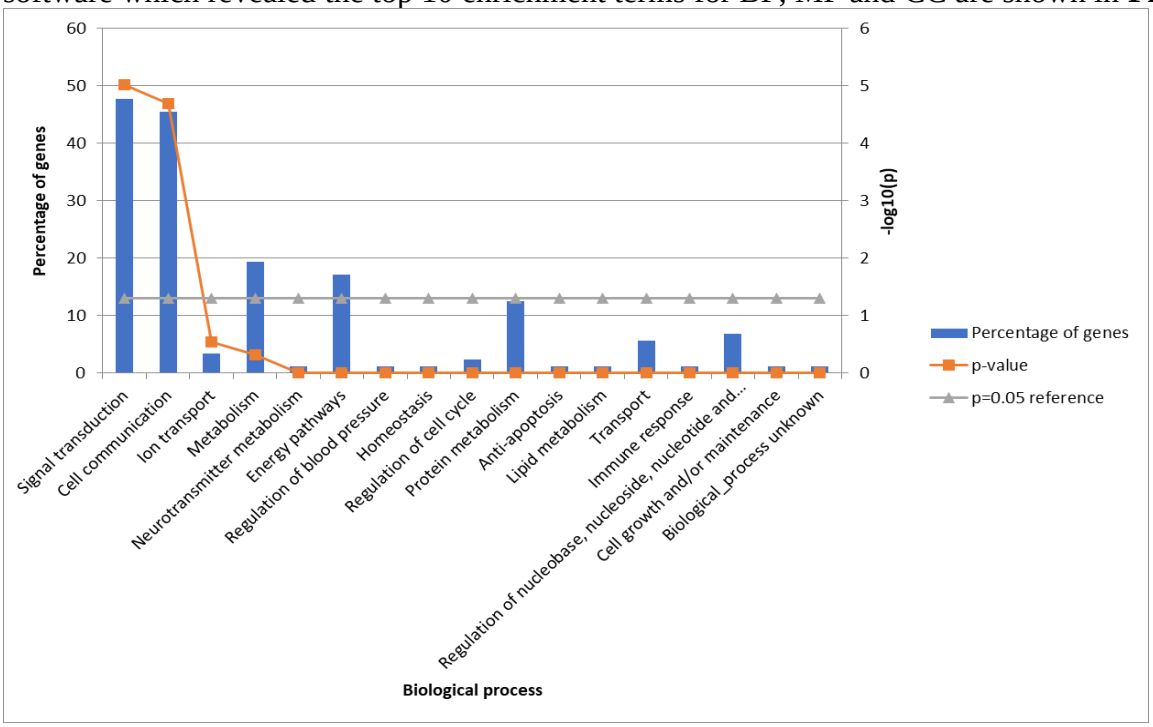


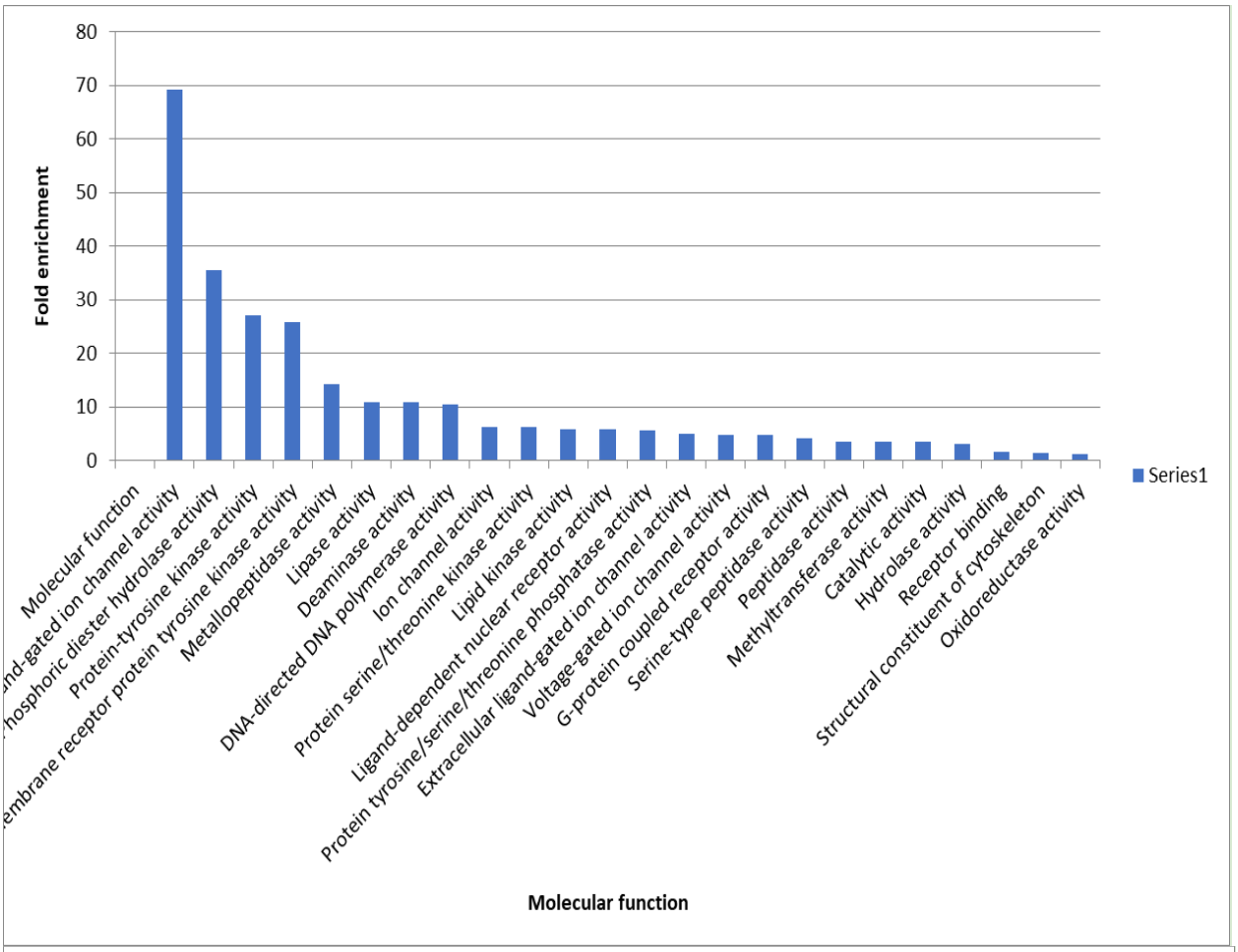
The average number of neighbours for each node is 2.714, demonstrating the network's average degree of connectedness. The characteristic path length is 1.000, reflecting the average number of edges along all pairs of nodes' shortest pathways. The clustering coefficient, which indicates how much nodes in a network tend to cluster together, is 0.000, indicating that there is no clustering in this network. The nodes in the illustration are coloured to signify their degree, with nodes becoming more purple as their degree grows, representing the amount of connections they have with other nodes.

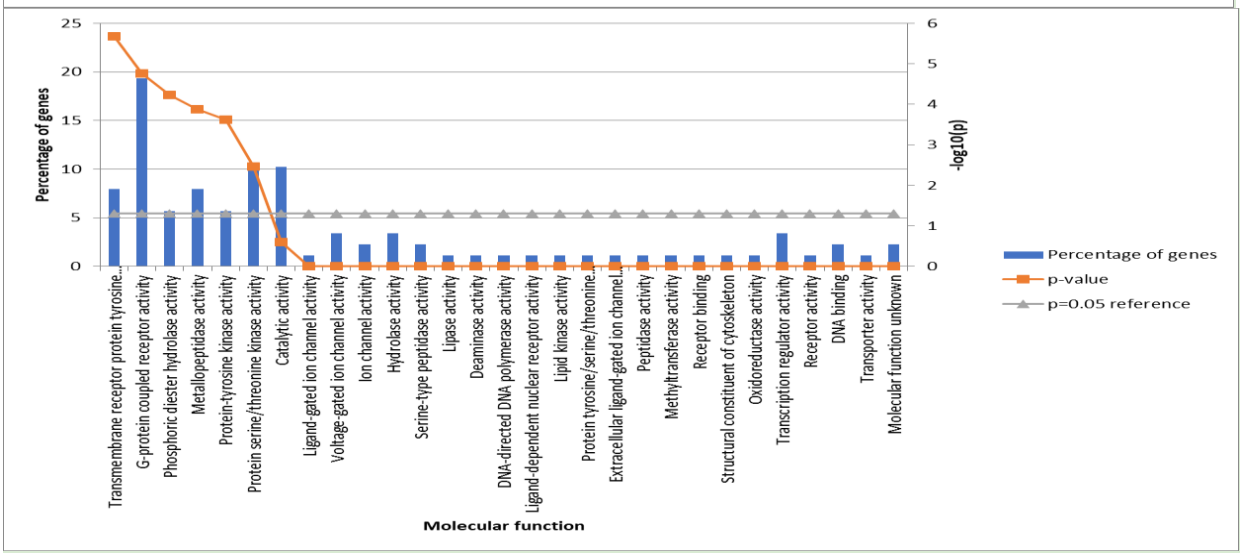


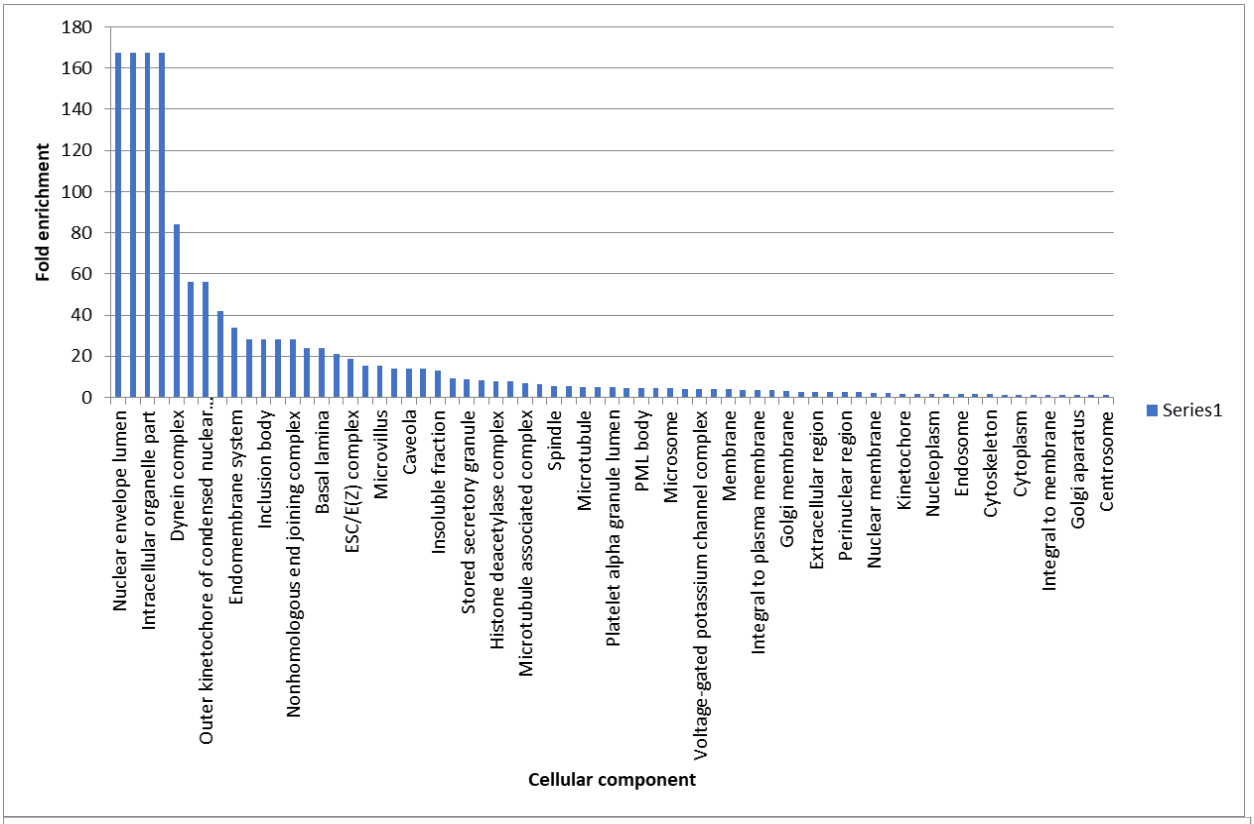
GO enrichment analysis

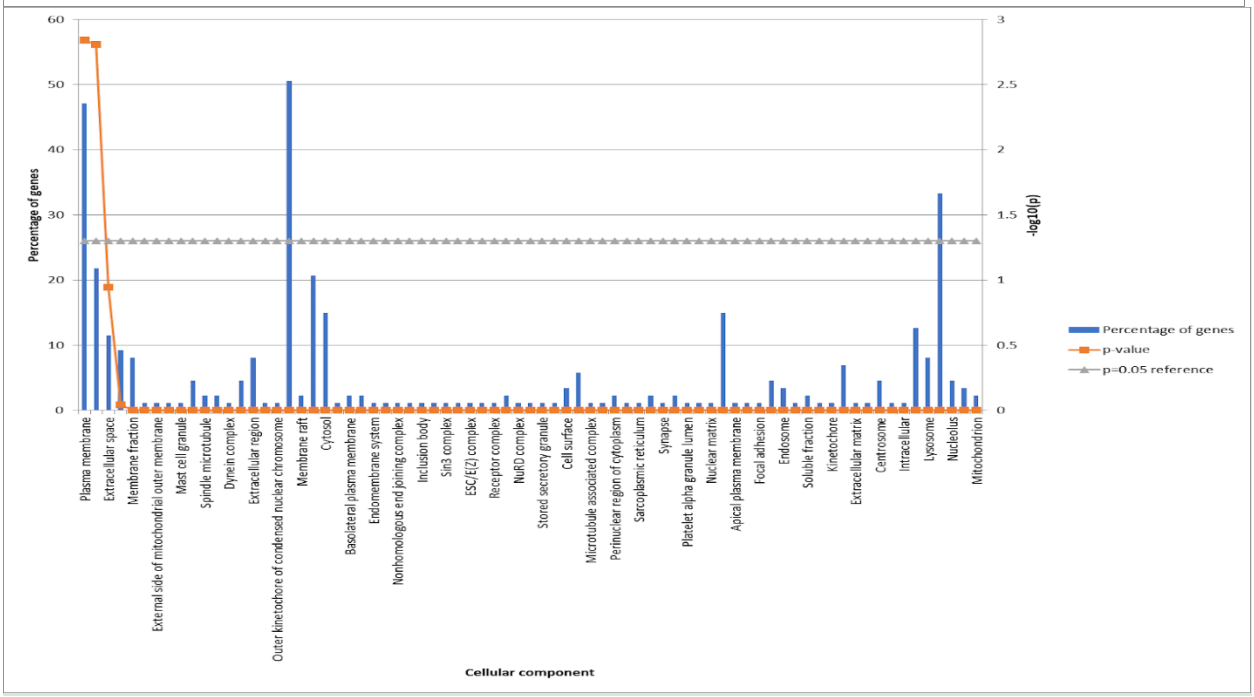
We further analysed the 88 potential anti-LUSC targets for GO enrichment analysis using Funrich software which revealed the top 10 enrichment terms for BP, MF and CC are shown in **Figure 9**.




Fold enrichment
80
70
60
50
40
30
20
10
0
Molecular function
Series1
Molecular function
nd-gated ion channel activity
hosphoric diester hydrolase activity
Protein-tyrosine kinase activity
embrane receptor protein tyrosine kinase activity
Metallopeptidase activity
Lipase activity
Deaminase activity
DNA-directed DNA polymerase activity
Ion channel activity
Protein serine/threonine kinase activity
Lipid kinase activity
Ligand-dependent nuclear receptor activity
Protein tyrosine/serine/threonine phosphatase activity
Extracellular ligand-gated ion channel activity
Voltage-gated ion channel activity
G-protein coupled receptor activity
Serine-type peptidase activity
Peptidase activity
Methyltransferase activity
Catalytic activity
Hydrolase activity
Receptor binding
Structural constituent of cytoskeleton
Oxidoreductase activity


Percentage of genes
-log10(p)
25
20
15
10
5
0
6
5
4
3
2
1
0
Molecular function
Transmembrane receptor protein tyrosine...
G-protein coupled receptor activity
Phosphoric diester hydrolase activity
Metallopeptidase activity
Protein-tyrosine kinase activity
Protein serine/threonine kinase activity
Catalytic activity
Ligand-gated ion channel activity
Voltage-gated ion channel activity
Ion channel activity
Hydrolase activity
Serine-type peptidase activity
Lipase activity
Deaminase activity
DNA-directed DNA polymerase activity
Ligand-dependent nuclear receptor activity
Lipid kinase activity
Protein tyrosine/serine/threonine...
Extracellular ligand-gated ion channel...
Peptidase activity
Methyltransferase activity
Receptor binding
Structural constituent of cytoskeleton
Oxidoreductase activity
Transcription regulator activity
Receptor activity
DNA binding
Transporter activity
Molecular function unknown
Percentage of genes
p-value
p=0.05 reference





The analysis showed that the gene targets related to BP were involved in various processes such as signal transduction, cell communication, homeostasis and neurotransmitter metabolism etc.
Gene targets in CC were primarily found in locations such as plasma membrane, extracellular space
Moreover, the enriched MF ontology was largely comprised of terms such as Transmembrane receptor protein tyrosine, GPCR, metalloprotein activity etc.

CHAPTER 6 : DISCUSSION

This research work aimed to explore the molecular targets, active phytonutrients, and molecular processes involved in the use of *T. alternifolia* for treating LUSC. 6 phytonutrients were identified and determined to be the active components of *T. alternifolia* shown in **Table 1.** The network findings revealed a synergistic effect of multiple anti-LUSC core targets and multiple key active phytonutrients in the *T. alternifolia's* bark in alleviating LUSC pathology.

The PPI network analysis exhibits that numerous genes, including MMP9, KDR, MMP2, JAK2, NOS3, MET, KIT, PDGFRB, FGFR1, PARP1, etc., are implicated in the pathogenicity of LUSC and also in the anti-LUSC effects of *T. alternifolia* bark's key active phytonutrients (**FIGURES 3B, & 4) e**xtracellular matrix proteins, which are essential for tissue repair and remodelling, are broken down by an enzyme called MMP9 & MMP2 https://doi.org/10.3390/cells7100167 , also referred to as matrix metalloproteinase-9 & matrix metalloproteinase-2 respectively. They have been discovered to be over-expressed in tumor tissues when LUSC is present as opposed to healthy lung tissues https://doi.org/10.1038/s41416-020-0742-9 . According to studies, MMP9 & MMP2 may be involved in the angiogenesis and development of the tumour as well as the invasion and metastasis of LUSC cells https://doi.org/10.1111/jcmm.17464 . The receptor proteins dysregulation has been associated with the onset and development of numerous cancer types, including LUSC. These proteins involves FGFR1, MET (hepatocyte growth factor receptor), KIT, PDGFRB & KDR (VEGF2). However, the precise function of KIT in LUSC is still unclear, and more investigation is required to ascertain if KIT could be a suitable therapeutic target for this particular malignancy. A number of signalling pathways, including the PI3K/Akt and MAPK/ERK pathways, which are involved in the survival, proliferation, and migration of cancer cells, can be activated by these receptor proteins**.** They have been found to be over-expressed in LUSC and may hasten the disease's development by promoting angiogenesis, invasion, migration, and proliferation **Chang, L. S. (2019). *The NUP98 Gene as a Potential Modifier of NF2-Associated Tumors*. NATIONWIDE CHILDREN'S HOSPITAL**. Specifically, KDR, MET and PDGFRB are linked to poor prognosis of LUSC, Several downstream signalling pathways, including the JAK/STAT system, which is essential for controlling cell proliferation, differentiation, and survival, are thought to be activated by JAK2. Most of the LUSC tumours contain JAK2 mutations and overexpression, which are linked to a poor prognosis https://doi.org/10.1016/j.biopha.2023.114452 . JAK2 has been demonstrated to have a role in boosting angiogenesis, which is necessary for tumour development and metastasis https://doi.org/10.3389/fonc.2022.1023177 , in addition to its direct effects on cancer cells. The NOS3 enzyme, sometimes referred to as endothelial nitric oxide synthase, is essential for producing nitric oxide (NO) in the endothelial cells that line blood arteries. The control of blood pressure, angiogenesis, and immunological response are just a few examples of the physiological processes in which nitric oxide plays a key role as a strong vasodilator and signalling molecule https://doi.org/10.1016/j.ccr.2023.215052 . The function of NOS3 in LUSC is not entirely known. According to certain research, NOS3 may limit tumour growth in LUSC by preventing cell proliferation and encouraging apoptosis https://doi.org/10.1002/bab.1909 . However, additional research has revealed that NOS3 may accelerate the growth and progression of LUSC by boosting

angiogenesis and tumour cell invasion https://doi.org/10.1038/s42003-021-02470-x . Poly(ADP-ribose) polymerase 1, or PARP1, is an enzyme that contributes to genomic stability and DNA repair. Studies have revealed that through encouraging tumour cell survival and proliferation, PARP1 may contribute to the onset and progression of LUSC https://doi.org/10.1038/s41598-020-77284-8 . This may happen because PARP1-mediated signalling pathways such the PI3K/AKT pathway, which can encourage cell growth and survival, are activated. According to certain theories, PARP1 has a role in LUSC cells' reaction to DNA damage https://doi.org/10.1016/j.dnarep.2019.102651 . When DNA damage occurs, PARP1 may be turned on to speed up DNA repair. In contrast, PARP1 activation in LUSC cells may actually encourage DNA damage and genomic instability https://doi.org/10.1016%2Fj.dnarep.2019.102651 , which may eventually result in the growth of cancer.

CHAPTER 7 : CONCLUSION

The study showed the phytochemicals from Tabernaemontana alternifolia may have therapeutic benefits on lung squamous cell carcinoma (LUSC). By suppressing LUSC cell proliferation and triggering apoptosis, the active phytonutrients from Tabernaemontana alternifolia's bark shown promising anti-LUSC effects. These phytochemicals also addressed important biochemical pathways implicated in the pathogenesis of LUSC, such as cell cycle regulation, DNA repair, proliferation, and angiogenesis. They also reduced the expression of cancer-promoting elements such MMP9, MMP2, KDR, MET, FGFR1, PDGFRB, JAK2, NOS3, and PARP1. The results indicate that the phytochemicals from Tabernaemontana alternifolia may one day be used to treat LUSC.

CHAPTER 8 : FUTURE PROSPECTUS

More preclinical research is required to confirm the efficacy and safety of Tabernaemontana alternifolia's phytochemicals in the treatment of LUSC. Studies using cell culture and animal models can offer insightful information about their mechanisms of action and potential negative consequences.

Clinical trials: Strict clinical trials should be carried out to assess the efficacy and safety of phytochemicals from Tabernaemontana alternifolia as a therapy for LUSC in people. These studies can define suitable dosage regimens and offer proof for their therapeutic application.

Combination therapies may improve treatment outcomes for LUSC patients by examining the synergistic interactions of Tabernaemontana alternifolia's phytochemicals with already available chemotherapeutic medicines or targeted therapies. Combination strategies may increase effectiveness, combat medication resistance, and reduce side effects.

Mechanistic studies: Additional research is required to determine the precise molecular processes by which the phytochemicals in Tabernaemontana alternifolia exert their anticancer properties in LUSC. This information can assist discover specific molecular targets for drug development and maximise their therapeutic potential.

Evaluation of the safety profile and potential negative consequences of the phytochemicals in Tabernaemontana alternifolia should include thorough toxicity tests. For their clinical application, it will be essential to comprehend their pharmacokinetics, biodistribution, and long-term toxicity.

Overall, the network pharmacology study offers insightful information on the therapeutic potential of the phytochemicals found in Tabernaemontana alternifolia for the treatment of LUSC. To promote their development as innovative treatment medicines for this aggressive kind of lung cancer, additional study and clinical trials are required.

APPENDICES – CONFERENCE DETAILS

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Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma: a network pharmacology study

Abstract - With a high incidence and death rate, squamous cell lung cancer (LUSC) is a serious issue for the world's health. Drug toxicity and medication resistance continue to be major obstacles in the treatment of LUSC. Finding new therapeutic drugs is therefore urgently needed for the management of LUSC. Natural alkaloids, which comes from the *Tabernaemontana alternifolia* plant of Apocynaceae family, has shown promising anticancer benefits against many cancer forms, including LUSC. Molecular processes of *T. alternifolia's* phtochemicals in LUSC haven't been fully understood, yet. In this work, we looked at their potential as a treatment for LUSC by focusing on its molecular targets. To identify the molecular targets and pathways of them for the treatment of LUSC, we undertook network pharmacology analysis. Our in-silico docking studies using AutoDock vina showed that 9-methoxycamptothecin, camptothecin and heyneanine can reduce LUSC cell growth and trigger apoptosis. Importantly, they suppressed genes related to proliferation, angiogenesis, DNA repair, and cell cycle control. They also prevented the expression of important oncogenic factors such as MMPs, KDR and MET, etc. These findings imply that several molecular pathways can be targeted by *T. alternifolia's* phtochemicals to treat LUSC. The molecular mechanisms underpinning their anticancer actions in LUSC are significantly clarified by this work. New therapeutic approaches for the treatment of LUSC may be developed as a result of these findings. To validate the safety and effectiveness of *T. alternifolia's* phtochemicals as a possible treatment drug for LUSC, more preclinical and clinical trials are required.

Chapter 1 Introduction

As one of the most common forms of cancer and the major cause of cancer-related deaths worldwide, lung cancer is one of the most prevalent forms. It is estimated that 1.8 million people worldwide die from lung cancer every year. There are over 2 million lung cancer diagnoses worldwide each year.https://doi.org/10.5114/wo.2021.103829

Worldwide, squamous cell carcinoma (LUSC) is the second most likely histological subtype of lung cancer and the leading cause of death and morbidity. https://doi.org/10.1016/j.molmed.2019.04.012

LUSC treatment and detection have improved tremendously in recent years, but the prognosis remains dismal, with a low survival rate and frequent recurrences. **Siegel RL, Miller KD, Jemal A. 2019. Cancer statistics, 2019. Cancer J Clin. 69:7–34** 10.3322/caac.21551

Previous research has uncovered numerous changed genes and genomic pathways as well as the complicated genetic landscape of LUSC. However, few targetable driver mutations have so far been identified, in striking contrast to lung adenocarcinoma, and targeted treatments for LUSC continue to be

ineffective. Treatment for LUSC has been transformed by immunotherapy, which is now recognised as the new standard of care.
10.1038/s41388-021-01723-7

Radiation treatment, chemotherapy, and surgery are further therapies for lung LUSC. However, the effectiveness of these treatments is constrained, and they frequently have detrimental side effects. This emphasises the necessity for cutting-edge and efficient treatment modalities to increase lung cancer patients' chances of survival and quality of life.

Traditional medicine has used natural remedies made from plants to treat a variety of illnesses, including cancer. Modern medicinal compounds that have been demonstrated to have anti-cancer properties have been derived from a variety of plants. A plant species found in Asia and Africa is T. alternifolia, also known as tree jasmine. In conventional medicine, it has been used to treat a wide range of illnesses, such as cancer, inflammation, and fever.
https://doi.org/10.3390/plants10020313

Recent research has shown that T. alternifolia extracts have anti-cancer potential against a variety of cancer types, including lung cancer. It has been shown that these extracts can decrease cell proliferation and trigger apoptosis in a variety of cancer cell lines.
https://doi.org/10.3390/plants10020313

The intricate interactions between medications and their targets in biological systems are the focus of network pharmacology, which blends network biology, systems biology, and pharmacology.https://doi.org/10.1016%2FB978-0-12-801814-9.00005-2

Network pharmacology has become a potent technique for identifying new therapeutic targets and the mechanisms that regulate the

actions of natural compounds in recent years. The molecular mechanisms underlying the therapeutic advantages of natural compounds can be investigated using network pharmacology, which can also be utilised to find prospective therapeutic targets and drug development routes. Recently, network pharmacology has gained popularity as a tool in cancer research for identifying novel therapeutic targets and creating fresh anti-cancer medications. 10.1016/j.tips.2021.11.004

1.1 OBJECTIVES

To treat lung squamous cell carcinoma (LUSC), determine the molecular targets and pathways that Tabernaemontana alternifolia's phytochemicals interact.

Examine the impact of active phytonutrients from Tabernaemontana alternifolia on the development and apoptosis of LUSC.

Analyse the effects of the phytochemicals in Tabernaemontana alternifolia on gene and pathway expression for proliferation, angiogenesis, DNA repair, and cell cycle control of LUSC.

Review of literature

Lung squamous cell carcinoma (LUSC) develops from the squamous cells that line the lungs' airways. It is one of the two primary subtypes of NSCLC, along with lung adenocarcinoma, which is the other subtype. About 25 to 30 percent of all instances of lung cancer are LUSC.**https://doi.org/10.1038/s41467-021-22801-0**

The bronchi and bigger airways of the lungs are lined with squamous cells, which are flat, scale-like cells. These cells develop into LUSC when they go through a cancerous transformation. Smoking exposes lung tissue to carcinogens that can result in genetic changes and abnormalities in the squamous cells, which can lead to the development of LUSC.**Montserrat Sanchez-Cespedes, Steven A. Ahrendt, Steven Piantadosi, Rafael Rosell, Maria Monzo, Li Wu, William H. Westra,**

Steven C. Yang, Jin Jen, David Sidransky; Chromosomal Alterations in Lung Adenocarcinoma from Smokers and Nonsmokers[1]. *Cancer Res* 2 February 2001; 61 (4): 1309–1313.

The most common LUSC symptom is a centrally positioned lung tumour that frequently obstructs the airways. Symptoms such as coughing, chest pain, wheezing, and recurrent respiratory infections may be present. LUSC has the potential to metastasis, or spread, to other parts of the body, such as the lymph nodes, distant organs, and bones, like other types of lung cancer.

Prevalence

Due to its high incidence and fatality rates, lung squamous cell carcinoma (LUSC) is a serious health concern for people all over the world. It makes up between 25 to 30 percent of all instances of non-small cell lung cancer (NSCLC), making it one of the most prevalent kinds of lung cancer. About 85% of lung cancer cases are NSCLC, making LUSC a significant contributor to the overall burden of lung cancer.**https://doi.org/10.1016/j.semcancer.2020.07.009**

Geographically, LUSC prevalence varies, with higher rates seen in some places.**https://doi.org/10.1038/ng.3891** It is more common in developing nations, particularly in areas with high smoking rates and tobacco consumption. However, LUSC continues to be a worldwide problem that has an impact on people everywhere.

Age, gender, and smoking all have an impact on the incidence rates of LUSC. Most occurrences of LUSC are caused by current or previous smokers, making smoking the biggest risk factor. Other risk factors that affect the development of LUSC include exposure to secondhand smoke, environmental toxins, and workplace dangers including asbestos and radon.

LUSC has a significant impact on world health, primarily because of its high death rates. The most common cause of cancer-related fatalities in both men and women is lung cancer, particularly LUSC. LUSC typically has a poor prognosis than other forms of lung cancer, because it is frequently discovered at an advanced stage when curative treatment options are few.

The death rates linked to LUSC emphasise the pressing need for better methods of detection, prevention, and treatment. Despite improvements in medical science and therapeutic approaches, the overall survival rate for LUSC remains low. The high fatality rates are a result of the aggressive nature of the disease as well as the fact that there are few viable treatments available for cases in their advanced stages.

A thorough strategy that combines public health initiatives to lessen tobacco use, support early identification and screening programmes, and improve treatment choices is needed to address the global burden of LUSC. To improve patients outcome of LUSC, further research is required to better understand the underlying mechanisms of LUSC development, find novel therapeutic targets, and create cutting-edge treatment modalities.

Significance

The relationship of lung squamous cell carcinoma (LUSC) with a number of variables that contribute to its aggressive behaviour and treatment difficulties is what gives LUSC its clinical significance. These elements underline the necessity of giving LUSC specific consideration in clinical practise and research.

1. The majority of instances of LUSC are seen in smokers who are currently smoking or former smokers. LUSC is significantly related with smoking. The genetic mutations and anomalies that the toxins in tobacco smoke can cause in the squamous cells lining the airways can result in LUSC. The association between smoking

and LUSC highlights the significance of tobacco control policies and programmes for quitting smoking in lowering the prevalence of this illness.

2.

The LUSC are aggressive in nature and have a tendency to metastasize early. It frequently manifests as a central tumour that blocks the airways, causing symptoms including coughing, chest pain, and breathing problems. Its dismal prognosis is a result of LUSC's early dissemination to local lymph nodes and distant organs. Because LUSC is aggressive, early identification and treatment are essential for enhancing patient outcomes.

Compared to other kinds of lung cancer, LUSC presents unique challenges. Another non-small cell lung cancer subtype, lung adenocarcinoma, responds to targeted therapy more successfully than LUSC does. Since LUSC is frequently discovered in an advanced stage, curative surgical approaches are less effective. LUSC is frequently treated with chemotherapy and radiation therapy, however the effectiveness of these therapies may change according on the disease's stage. Further complicating therapeutic techniques is the aggressive behaviour of LUSC, which raises the risk of disease recurrence and therapy resistance.

LUSC has a lower prevalence of targetable genetic abnormalities than lung adenocarcinoma, which commonly harbours actionable mutations. As a result, people with LUSC have less access to certain targeted medicines. Research is now being done on discovering new therapeutic targets and creating individualised therapy plans that are specific to LUSC.

Pathways involved:

The deregulation of important signalling pathways is a major factor in enhancing tumour development, survival, and metastasis in lung squamous cell carcinoma (LUSC). The PI3K/Akt/mTOR pathway, the MAPK/ERK route, and the Notch signalling pathway are among the many pathways that are frequently changed in LUSC, leading to its pathophysiology. For the purpose of locating possible therapeutic targets and creating cutting-edge therapeutic approaches, it is crucial to comprehend these dysregulated pathways.

1. PI3K/Akt/mTOR Pathway Cell growth, proliferation, survival, and metabolism are all governed by this pathway. This pathway is frequently dysregulated in LUSC, which increases cell survival and proliferation. The PI3K/Akt/mTOR pathway can be activated by genetic changes, such as activating mutations in PIK3CA (encoding the catalytic subunit of PI3K) or loss of tumour suppressor PTEN (a negative regulator of the process). This pathway's activation encourages cell cycle progression, prevents apoptosis, and boosts angiogenesis, which aids in the development of tumours and their resistance to treatment.
2.
3. MAPK/ERK Pathway: The MAPK/ERK pathway controls cell proliferation, survival, and differentiation as well as the transmission of extracellular signals to the nucleus. Dysregulation of this pathway is frequently seen in LUSC. The MAPK/ERK pathway can be activated by mutations or amplification of receptor tyrosine kinases (RTKs), including EGFR and FGFR. Additionally, abnormal activation of this pathway may result from abnormalities in downstream signalling molecules like KRAS. In LUSC, MAPK/ERK signalling activation encourages cell proliferation, invasion, and metastasis.
4.
5.
6. Notch Signaling Pathway: The Notch signaling pathway plays a crucial role in cell fate determination, differentiation, and proliferation. Dysregulation of the Notch pathway is frequently observed in LUSC. Abnormal activation of Notch signaling, through mutations or overexpression of Notch receptors (Notch1-4) or downstream effectors, promotes cell survival, proliferation, and epithelial-to-mesenchymal transition (EMT) in LUSC. Notch signaling is also implicated in maintaining cancer stem cells and contributing to therapeutic resistance in LUSC.

These dysregulated signaling pathways in LUSC are interconnected and often cross-talk with each other, forming a complex network of molecular interactions. The activation of these pathways promotes tumor progression, metastasis, and resistance to therapy. Targeting these pathways has emerged as a promising therapeutic approach in LUSC. Several targeted agents and inhibitors against components of these pathways are being investigated in preclinical and clinical studies.

Current treatment

Lung squamous cell carcinoma (LUSC) is typically treated using a multimodal approach that combines different treatment modalities based on the stage of the disease and individual patient characteristics. The current treatment modalities for LUSC include surgery, radiation therapy, chemotherapy, targeted therapy, and immunotherapy. Here is an overview of each modality:

1. Surgery: Surgery plays a central role in the treatment of early-stage LUSC. The main surgical approach is called lobectomy, which involves the removal of the affected lobe of the lung. In some cases, pneumonectomy (removal of the entire lung) or segmentectomy (removal of a smaller portion of the lung) may be performed. Lymph node dissection or sampling is also carried out to assess the spread of cancer. Surgical resection aims to remove the tumor and any nearby lymph nodes to achieve complete tumor removal.

2. Radiation Therapy: Radiation therapy uses high-energy X-rays or other forms of radiation to kill cancer cells and shrink tumors. It can be used in combination with surgery or as the primary treatment for locally advanced LUSC that cannot be surgically removed. Radiation therapy may be delivered externally (external beam radiation) or internally through the placement of radioactive sources (brachytherapy). It is also employed for palliative purposes to alleviate symptoms and improve quality of life in advanced-stage LUSC.

3. Chemotherapy: Chemotherapy is a systemic treatment that uses drugs to kill cancer cells throughout the body. It is typically administered either before surgery (neoadjuvant chemotherapy) to shrink tumors and facilitate surgical resection, or after surgery (adjuvant chemotherapy) to destroy any remaining cancer cells. In advanced or metastatic LUSC, chemotherapy may be used as the primary treatment to control the disease. Combination chemotherapy regimens, such as platinum-based drugs (cisplatin or carboplatin) in combination with other agents (e.g., paclitaxel, gemcitabine), are commonly employed.

4. Targeted Therapy: Targeted therapy involves using drugs that specifically target molecular alterations or genetic mutations driving the growth and survival of cancer cells. In LUSC, targeted therapies are typically utilized for tumors with specific mutations, such as EGFR mutations or FGFR alterations. However, targeted therapies are more commonly used in lung adenocarcinoma than in LUSC, as LUSC has a lower frequency of targetable genetic alterations.

5. Immunotherapy: Immunotherapy has revolutionized the treatment of advanced-stage lung cancer, including LUSC. Immune checkpoint inhibitors, such as drugs targeting PD-1 (programmed cell death protein 1) or PD-L1 (programmed death-ligand 1), help the immune system recognize and attack cancer cells. Immunotherapy has shown significant efficacy in a subset of LUSC patients, particularly those with high PD-L1 expression.

The choice of treatment for LUSC is based on a number of variables, such as the disease's stage, the patient's general health, and the existence of particular molecular abnormalities. In order to give the best and most individualised treatment plan for each patient, treatment decisions are often determined using a multidisciplinary approach involving a team of oncologists, surgeons, radiation oncologists, and other specialists.

Challenges and limitations

Lung squamous cell carcinoma (LUSC) presents several challenges and limitations in its treatment, which can impact patient outcomes. Some of the key challenges and limitations associated with LUSC treatment include:

1. Drug Resistance: Like many other cancer types, LUSC may eventually grow resistant to many forms of therapy. As a result, chemotherapy, targeted therapy, or immunotherapy may no longer be effective in treating the tumours. The activation of alternative signalling pathways, modifications in the molecular pathways that medicines target, or genetic changes that encourage resistance can all be factors in the drug resistance mechanisms in LUSC. Drug resistance must be overcome, which is a substantial problem in the management of LUSC and necessitates the creation of novel treatment plans and combination methods.
2.
3.
4. Unfavourable side effects: Treatment options for LUSC, including as chemotherapy and radiation therapy, might have a negative influence on the quality of life of the patient. Chemotherapy can have a number of negative side effects, such as nausea, vomiting, exhaustion, hair loss, and an increased risk of infection. Radiation therapy may result in skin rashes, esophagitis, and radiation pneumonitis. Treatment interruptions or dose reductions may occasionally be necessary due to unfavourable treatment effects, which may also necessitate supportive care measures.
5.
6.
7. Limited Options for Treatment of Metastatic or Advanced Disease: Treatment of advanced or metastatic LUSC is frequently difficult, primarily because there are few available treatments. LUSC has a lower prevalence of targetable mutations than lung adenocarcinoma, which has profited from the development of targeted medicines for particular genetic abnormalities. As a result, there aren't many targeted medicines that have been authorised especially for LUSC. This restricts the possibilities for treating patients with severe illness who have advanced after receiving conventional chemotherapy.
8.
9.
10. Lack of Predictive Biomarkers: For the best possible therapy options, it is essential to identify predictive biomarkers for LUSC. The prognostic utility of biomarkers in LUSC is still under investigation, despite the fact that some biomarkers, such as PD-L1 expression, have been used to direct treatment decisions. Finding trustworthy biomarkers to gauge patient response to various treatment modalities and direct decisions on individualised care continues to be a challenge.

11.
12.
13. Heterogeneity of LUSC: LUSC has significant molecular and histological heterogeneity, which may affect how each patient responds to treatment and how their case develops. It is difficult to create targeted medicines that are efficient for all LUSC patients due to this heterogeneity. It emphasises the necessity of gaining a deeper comprehension of the molecular subtypes of LUSC and the creation of specialised treatment strategies based on unique tumour characteristics.
14.
15.

NEED OF NOVEL THERAPEUTICS

The challenges and limitations associated with the treatment of lung squamous cell carcinoma (LUSC) underscore the critical need for the development of novel therapeutic strategies and the exploration of alternative treatment approaches. Several factors contribute to this imperative:

1. Overcoming Drug Resistance: A major barrier to effective therapy is the formation of drug resistance in LUSC. The creation of novel therapeutic molecules that can circumvent or stop drug resistance processes is urgently needed to solve this problem. To reduce the possibility of resistance, this includes investigating new targets and creating combination medicines that can simultaneously target several pathways.
2. Targeted medicines: Although targeted medicines, such as lung adenocarcinoma, have revolutionised the treatment of several cancer types, the possibilities for targeted therapy in LUSC are constrained. Therefore, it is imperative to find and confirm fresh, targetable molecular changes that are unique to LUSC.
3.
4.
5. Optimisation of Immunotherapy: Immunotherapy, in particular immune checkpoint inhibitors, has demonstrated encouraging outcomes in the treatment of LUSC. However, not every patient responds to immunotherapy, emphasising the requirement to find prognostic indicators and create plans to increase the effectiveness of immunotherapy in LUSC. To increase response rates and overcome resistance, this involves looking into

combination strategies with additional immune modulators, targeted treatments, or chemotherapy.

6.
7.
8. The development of personalised medicine strategies catered to specific patients is essential for enhancing treatment outcomes in LUSC. In order to inform treatment choices and determine the most suitable therapeutic alternatives for each patient, this includes the integration of thorough genetic profiling and molecular characterisation. Real-time evaluation of treatment response and the formation of resistance can also be facilitated by the use of liquid biopsies and non-invasive monitoring techniques.
9. Clinical Trials: Stable clinical trials that assess the effectiveness and safety of novel therapy approaches are essential to the advancement of LUSC treatment. Participation[49] in clinical trials gives patients access to cutting-edge treatments and helps generate data that could influence future paradigms of medical care. The creation and validation of new treatment modalities depend on promoting patient enrollment in clinical trials and facilitating interactions between researchers, doctors, and pharmaceutical corporations.
10.
11.

Tabernaemontana alternifolia

The Apocynaceae family of plants, known for their various medicinal effects, includes the plant species Tabernaemontana alternifolia. Tabernaemontana alternifolia, often known as "Christmasbush" or "Cape Jasmine," is indigenous to a number of places, including Africa, Asia, and Australia. Because of the pharmacological characteristics of this plant, it[21] has a long history of usage as a traditional medicine in various cultures.

The therapeutic potential of Tabernaemontana alternifolia is enhanced by the presence of a variety of bioactive substances, such as alkaloids, flavonoids, terpenoids, and phenolic compounds. Science has investigated these bioactive chemicals' therapeutic properties and prospective applications in a range of medical ailments.

The Tabernaemontana alternifolia has historically been used to cure a number of illnesses, such as fever, inflammation,

gastrointestinal problems, and respiratory problems. The bark, leaves, and roots of the plant are particularly prized for their therapeutic qualities.

Exploring Tabernaemontana alternifolia's pharmacological properties and potential therapeutic uses has been the subject of recent scientific research. The plant extracts have demonstrated cytotoxic and apoptotic properties against a variety of cancer cell lines, including lung squamous cell carcinoma (LUSC), and have demonstrated potential benefits as anticancer medicines. This has spurred interest in researching Tabernaemontana alternifolia's medicinal potential in the treatment of LUSC.

Additionally, the plant extracts have shown antibacterial, antioxidant, anti-inflammatory, and analgesic characteristics, suggesting they may be used to treat infectious diseases, ailments linked to oxidative stress, and pain management.

The variety of bioactive substances found in Tabernaemontana alternifolia provide a wide range of pharmacological effects, making it an interesting topic for further investigation. It is crucial to remember that although traditional uses and preliminary research suggest that it has medicinal promise, additional scientific studies, like as preclinical and clinical trials, are required to completely comprehend its efficacy, safety, and therapeutic processes.

pharmacological activities of Tabernaemontana alternifolia

The potential therapeutic uses of Tabernaemontana alternifolia are facilitated by a broad spectrum of pharmacological actions. The bioactive substances found in Tabernaemontana alternifolia and their effects on numerous physiological processes have been studied in scientific investigations. The main pharmacological effects of Tabernaemontana alternifolia include the following:

1. Tabernaemontana alternifolia has demonstrated promising anticancer qualities. Different cancer cell lines, including lung squamous cell carcinoma (LUSC) cells, have shown

cytotoxic effects and the ability to trigger apoptosis in response to plant extracts and isolated chemicals. Alkaloids, for example, which inhibit the growth, proliferation, and survival pathways of cancer cells, may be responsible for these anticancer actions.

2. Tabernaemontana alternifolia has been shown to have anti-inflammatory qualities, which have been linked to its bioactive components. Different diseases often include inflammation, and plant extracts have shown to have inhibitory effects on inflammatory mediators and indicators. This anti-inflammatory action points to its possible application in diseases including arthritis, inflammatory bowel disease, and other inflammatory disorders, which are characterised by excessive inflammation.
3.
4.
5. Antioxidant Activity: The development of many diseases is significantly influenced by oxidative stress, which is brought on by an imbalance between reactive oxygen species (ROS) and antioxidant defences. Researchers have discovered that Tabernaemontana alternifolia possesses antioxidant qualities that reduce oxidative stress by scavenging free radicals and preventing oxidative damage to cells and tissues. The antioxidant activity of Tabernaemontana alternifolia is influenced by the presence of phenolic compounds and flavonoids.
6. Against a variety of diseases, including bacteria, fungi, and viruses, Tabernaemontana alternifolia demonstrates antibacterial activity. Various fungal species, as well as Gram-positive and Gram-negative bacteria, have been shown to be inhibited by the plant extracts. Alkaloids, which have antimicrobial effects through interrupting cellular processes, are among the bioactive substances that may be responsible for these antimicrobial capabilities.
7.

Previous studies

The medicinal potential of Tabernaemontana alternifolia and its bioactive components against different diseases, including cancer, has been investigated in pertinent studies and experiments. The following are some important conclusions drawn from studies and publications on the anticancer properties of Tabernaemontana alternifolia:

An investigation on the anticancer properties of Tabernaemontana alternifolia extracts against breast cancer cell lines was published in the journal Frontiers in Pharmacology. The outcomes demonstrated the extracts' potential as natural anticancer medicines by inhibiting cell proliferation and inducing apoptosis.

Researchers looked at the cytotoxic effects of alkaloids extracted from Tabernaemontana alternifolia against lung cancer cell lines in a different study that was published in the journal BMC Complementary and Alternative Medicine. The alkaloids showed considerable cytotoxicity and caused apoptosis in the cancer cells, showing that they have the potential to be used as therapeutics.

The Journal of Ethnopharmacology released a study that looked at how Tabernaemontana alternifolia extracts affected colorectal cancer cells' ability to proliferate. The extracts' promise as an all-natural treatment for colorectal cancer is suggested by the study's discovery that they suppressed cell growth and caused cell cycle arrest.

Prostate cancer cells were used in a study published in the journal Evidence-Based Complementary and Alternative Medicine to examine Tabernaemontana alternifolia extracts' anticancer properties. The extracts demonstrated inhibitory effects on cell viability and caused apoptosis in the cancer cells, showing their promise as a cutting-edge therapeutic strategy for the treatment of prostate cancer.

The potential of Tabernaemontana alternifolia and its bioactive substances in the treatment of different cancers is highlighted by these research.

Network pharmacology

Network pharmacology is an interdisciplinary approach that integrates network analysis, computational biology, and pharmacology to study the interactions between drugs, targets, and biological systems. It involves the construction of biological

networks that represent the relationships between genes, proteins, pathways, and diseases, and utilizes computational methods to analyze and interpret these networks.

The advantages of network pharmacology in identifying complex relationships and molecular interactions within biological networks include:

1. **Instead of concentrating on specific genes or targets, network pharmacology enables researchers to take the entire biological system into account. By recording the interactions and crosstalk between various system components, it offers a comprehensive viewpoint and sheds light on the general network behaviour.**

1. **Identification of Important Players: Network analysis aids in the discovery of important genes, proteins, and pathways that are essential for the development of disease or the response to treatment. Researchers can rank goals and paths for additional research by analysing the network architecture and centrality metrics.**
2. **Drug-Target Interaction Prediction: Network pharmacology computational approaches allow for the prediction of possible drug-target interactions. This aids in finding new therapeutic targets and adapting already-approved medications for use in different conditions.**
3.

Integration of Omics Data: Network pharmacology enables the integration of various omics data, such as transcriptomics, proteomics, and genomes, to provide a thorough understanding of the biological system. The underlying molecular pathways are better understood and possible biomarkers are found because to this integration.

Network pharmacology assists in the rational creation of novel medications by taking the network environment into account. It enables researchers to find medication combinations that target various network elements, thereby producing synergistic effects and better therapeutic results.

In conclusion, network pharmacology provides a potent method for comprehending the intricate relationships between biological systems and locating possible treatment targets and approaches. A thorough understanding of drug-target interactions is provided by its integration of network analysis and computational approaches, helping the discovery and development of novel therapeutic interventions.

Chapter 2 Materials and Methodology

Softwares and tools

2.1 This research work worked with various open-source applications and databases including IMPPAT https://cb.imsc.res.in/imppat/, Venny 2.1 https://bioinfogp.cnb.csic.es/tools/venny/, SwissTargetPrediction http://www.swisstargetprediction.ch/, GEPIA2 http://gepia2.cancer-pku.cn/#index, STRING https://string-db.org/, Cytoscape https://cytoscape.org/, Cystoscope's Molecular Complex Detection (MCODE) plug-in https://apps.cytoscape.org/apps/mcode, Cytoscape's CYTOHUBBA plug-in https://apps.cytoscape.org/apps/CYTOHUBBA, DAVID https://apps.cytoscape.org/apps/CYTOHUBBA, Protein Data Bank https://www.rcsb.org/, NCBI PubChem https://pubchem.ncbi.nlm.nih.gov/, UNIPROT https://www.uniprot.org/, BIOVIA Discovery Studio Visualizer https://discover.3ds.com/discovery-studio-visualizer-download, AutoDock Vina https://vina.scripps.edu/, and Open Babel GUI https://openbabel.org/docs/current/GUI/GUI.html

Softwares and tools

2.1.1 IMPPAT

The IMPPAT 2.0 database features an easy-to-use online interface that provides users with vital information about Indian medicinal plants and their phytochemical qualities. The website used Bootstrap 4.1.3, an open-source CSS framework, as a base to construct this interface. Customizations were then done to the front end using internal HTML, PHP, CSS, jQuery scripts, and JavaScript.**doi: 10.1021/ACSOMEGA.3C00156, doi: 10.1093/bioinformatics/btv557**

2.1.2 Pubchem

The public chemical database PubChem is housed at the National Institutes of Health (NIH) in the United States. PubChem is a popular site for researchers, patent agents, and students, as well as millions of monthly users. Importantly, PubChem data is extensively used in machine learning and artificial intelligence projects. PubChem, a data aggregator, collects chemical data from thousands of sources. While the majority of the molecules in PubChem are siRNA, miRNA, lipids, carbohydrates, and biopolymers that have been chemically modified, it also includes other chemical substances such as siRNA and miRNA. This information is organised into numerous data sets, including Substance, Compound, BioAssay, Gene, Protein, Taxonomy, Pathway, Cell Line, and Patent. **doi: 10.1093/nar/gkaa971, doi: 10.1080/17460441.2016.1216967, doi: 10.1093/nar/gkv951, doi: 10.1016/j.jmb.2022.167514.**

2.1.3 uniprot

The massive database UniProt provides information on proteins. UniProt provides access to protein sequences, functional annotations, structural features, interactions, and other data. UniProt also provides additional information and tools, such as UniProtKB/Swiss-Prot, UniProtKB terms, and cross-references to other databases, protein annotation tools, and more.**doi: 10.1093/nar/gky1049**

2.1.4 swiss target prediction
The Swiss Institute of Bioinformatics (SIB) developed Swiss Target Prediction that forecasts potential targets or interactions for small molecules like drugs or chemical compounds. Calculations are made to determine how likely it is that a given chemical will bind to particular protein targets using a variety of approaches and algorithms.Using a ligand-based methodology, the Swiss Target Prediction programme compares a compound's chemical structure to a database of recognised ligands and their associated protein targets. Additionally, a target-based approach is used to identify probable binding sites.
interactions by comparing the chemical characteristics of the drug to those of known protein structures

.**doi: 10.1093/nar/gkz382.**

2.1.4 GEPIA2
A web-based programme called GEPIA2, or Gene Expression Profiling Interactive Analysis 2, exists. The Genotype-Tissue Expression (GTEx) studies on gene expression and the Cancer Genome Atlas (TCGA) data will be analysed and visualised. GEPIA2 can be used by users and researchers to look at the links and patterns of gene expression in both normal and cancerous tissues.A survival analysis option in GEPIA2 enables researchers to investigate the connection between gene expression and patient survival outcomes.**doi: 10.1093/nar/gkz430**

2.1.5 VENNY2.1
Venny 2.1 is a web-based used for making Venn diagrams. Venn diagrams are graphic representations of the relationships between numerous sets or groups of objects.
They are frequently used in a range of areas, including biology, statistics, and data analysis, to visualise the overlaps and distinct components across several sets [55].
Venny 2.1's user-friendly interface makes it simple to build Venn diagrams with up to six groupings. **doi: 10.3390/cancers14102447**

2.1.6 STRING DB

STRING-DB (Search Tool for the Retrieval of Interacting Genes/Proteins) is a bioinformatics database and online resource that covers protein-protein interactions (PPIs), functional connections, and networks. STRING-DB creates a huge network of

proven and anticipated protein interactions by combining data from multiple sources. **doi: 10.1038/s41598-023- 31413-1**

2.1.7 cytoscape

An effective open-source software tool for visualising, analysing, and simulating complex networks is called Cytoscape. It is frequently used in systems biology and bioinformatics research to examine and grasp biological networks like gene regulatory networks, metabolic pathways, and protein-protein interactions.One of Cytoscape's main advantages is the enormous selection of plugins that it offers, which expand its functionality and enable users to do a variety of network analyses. Network clustering, pathway enrichment analysis, network topology analysis, and network motif recognition are just a few of the topics covered by these plugins. Additionally, Cytoscape enables users to combine and study other data sources, such as genomic annotations and gene expression data, with network data.**doi: 10.1007/978-981-19-0901-6_5**

BIOVIA Discovery Studio

An all-in-one software package for computational drug discovery and molecular modelling is called BIOVIA Discovery Studio. With regard to target selection and validation, virtual screening, ligand design, and protein-ligand interaction analysis, it offers a wide range of tools and features to assist researchers.In addition to molecular modelling, BIOVIA Discovery Studio has capabilities for virtual screening, which is the computational screening of sizable compound databases to find potential drug candidates. It offers a number of virtual screening approaches, including docking based on structure and similarity searches based on ligands, enabling researchers to quickly prioritise and choose compounds for additional research.The BIOVIA discovery Studio software suite as a whole integrates a number of computational tools and algorithms to support drug development efforts.

doi:10.2174/1389557520666201214101329.

2.1.9 cytoscape MCODE

The MCODE (Molecular Complex Detection) Cytoscape plug-in is a powerful tool for identifying clusters or parts of a biological network that are closely connected to one another. It facilitates in the finding of probable functional modules or complexes in protein-protein interaction networks or other types of molecular networks [59].The MCODE plug-in is commonly used when analysing large-scale molecular networks to uncover physiologically relevant modules or complexes. It aids in the detection of regulating components of biological systems, potential protein complexes, and functional linkages.**doi: 10.1038/s41598-020-79235-9**

2.1.10 Cytoscape CYTOHUBBA

Cytoscape's CytoHubba plug-in is an effective tool for network analysis and detecting significant hubs or nodes within a biological network. It provides a number of methods and

strategies for determining node centralities, ranking nodes based on their topological relevance, and detecting key nodes in the network [60]. The CytoHubba Cytoscape plug-in is often used in network biology research to find major nodes, hub proteins, or critical regulatory components within biological networks. It aids in understanding the network architecture and determining the functional and regulatory functions of specific components in biological systems. **doi: 10.1038/s41598-020-76024-2**

2.1.11 DAVID

DAVID (Database for Annotation, Visualisation, and Integrated Discovery) is a web-based bioinformatics application used for functional annotation and enrichment analysis of gene or protein lists. It provides a large variety of functional annotation tools and resources to help you understand the biological importance of a certain set of genes or proteins [61].DAVID is commonly used by genomic, transcriptomic, and proteomic researchers to get functional insights into their gene or protein lists. It aids in high-throughput data analysis, the identification of biological pathways, and the identification of underlying biological processes associated to the input genes or proteins.**doi: 10.1007/s12010-022-04170-6**

2.1.12 PDB

The RCSB PDB (Research Collaboratory for Structural Bioinformatics Protein Data Bank) is a comprehensive and well-known tool for examining the three-dimensional structures of biological macromolecules. It provides users with access to a large library of complex biomolecular structures that have been experimentally determined. Through investigation, analysis, and visualisation, it provides a better understanding of the roles, interactions, and structural properties of protein and nucleic acid structures.**doi: 10.1002/pro.3730**

2.1.13 AutoDock VINA

To predict the affinities and binding patterns of small molecules (ligands) to protein targets, a well-known and widely used molecular docking programme called AutoDock Vina is utilised. It performs flexible docking simulations by fusing empirical scoring techniques with evolutionary algorithms.**doi: 10.1021/acs.jcim.1c00203**

2.1.14 Open Babel GUI

The Open Babel programme, a potent and adaptable toolkit for chemical informatics, computational chemistry, and molecular modeling, has a graphical user interface (GUI) called Open Babel. The Open Babel GUI offers a simple user interface for navigating and using Open Babel's features. **doi: 10.1142/S0219720020400119**

2.2 Bioactive identification: Data on the phytonutrients present in the bark of Tabernaemontana alternifolia were gathered using the IMPPAT database. A carefully maintained collection of data on Indian Medicinal Plants, Phytochemistry, and Therapeutics is housed in the database known as IMPPAT. The IMPPAT IDs were recorded for future reference. All of the phytonutrients' ADME properties were investigated.

2.3

The PUBCHEM database, supplied the distinctive identification code (Canonical SMILES) for these compounds, to identify the phytonutrients in Tabernaemontana alternifolia.

The probable protein or molecular targets with which these phytonutrients might interact throughout the body were then predicted using Swiss Target Prediction, a webtool. As information sources, the studies' associated references are included.

2.4

The GEPIA2 web application provided a list of Differentially Expressed Genes (DEGs) for LUSC.

2.5

to find possible targets for genes that fight lung squamous cell carcinoma (LUSC). Using the venny2.1 online tool, we compared the gene targets linked to the active phytonutrients in Tabernaemontana alternifolia with the gene targets linked to LUSC. The overlapping genes were taken into consideration as prospective anti-LUSC gene targets by identifying the shared genes between these two groups using Venny 2.1.0.

2.6

The STRING database was used to further analyse the putative anti-LUSC gene targets and investigate protein-protein interactions. The outcomes of the STRING analysis of protein-protein interactions were subsequently exported as tab-separated values (tsv) files. The possible anti-LUSC core targets were then visualised and investigated using the Cytoscape programme once this file was submitted. The study only took into account targets that were unique to the Homo sapiens species and had a moderate confidence score of larger than 0.4.

2.7

The MCODE plug-in for Cytoscape was used to locate the important elements in the protein-protein interaction network of possible anti-LUSC targets. For the purpose of finding molecular complexes in extensive protein interaction networks, this plug-in employs an automated technique. A degree cutoff of 2, a node score cutoff of 0.2, a k-core of 2, and a maximum depth of 100 were the parameters that were used for the analysis. The discovery of clusters across the whole network was made possible by these parameter settings.

2.8

The top 10 targets were filtered using the "cytohubba" plugin in Cytoscape version 3.9.1. Based on different network analysis algorithms, this plugin is intended to identify significant nodes or targets within a network. The Degree, Maximum Neighbourhood Component (MNC), Maximal Clique Centrality (MCC), and Closeness approaches were applied. The core targets were found by identifying the point at which the targets from these four strategies intersected. The most important and central targets in the network of protein-protein interactions might be found using this method.

2.9

The interactions between the active phytonutrients of Tabernaemontana alternifolia and the LUSC related prime and core targets were modelled using the Cytoscape software. The links and interactions between these elements are better understood through the network visualisation. The network offers a thorough perspective of the potential connections and effects in the context of LUSC by merging the data on phytonutrients and the indicated targets.

2.10

GO functional and KEGG pathway enrichment techniques were used to do additional analysis on the potential core targets for anti-LUSC. The targets' functional annotations and their connection to cellular processes, biological functions, and molecular processes were the main focus of this investigation. The top 10 enriched GO terms for biological process (BP), cellular component (CC), and molecular function (MF), as well as the top 30 enriched KEGG pathways, were found after the data were uploaded to a bioinformatics platform for analysis. An enrichment dot bubble plot was used to display the results. The Benjamini-Hochberg technique for multiple hypothesis testing was employed to control the false discovery rate (FDR), and a conventional hypergeometric test was utilised to establish statistical significance. An adjusted p-value of less than 0.05 was used in the analysis.

2.11

In this study, the top three active phytonutrients from Tabernaemontana alternifolia were compared to the top ten probable anti-LUSC core targets. The key targets' crystal structures (MMP9, KDR, MMP2, JAK2, NOS3, MET, KIT, PDGFRB, FGFR1, PARP1) were downloaded in PDB format from the RCSB Protein Data Bank (PDB). PDB IDs are 6ESM, 3WZE, 7XGJ, 8BAK, 1M9J, 2WD1, 4U0I, 3MJG, 4ZSA, and 7KK2 respectively. On the other hand, NCBI PubChem was used to obtain the chemical structures of the active phytonutrients (9-Methoxycamptothecin, Heyneanine, and Camptothecin).

The crystal structures and phytonutrient structures were converted from PDB format to PDBQT file format using the Open Babel GUI software. Polar hydrogens were added into the crystal formations in place of the heteroatoms (water and other ligands). The Kollman

partial charges were applied to these cleaned-up protein structures after they had been put into AutoDock Tools and saved in PDBQT format.

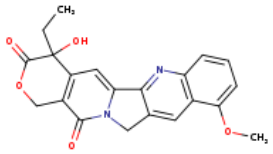
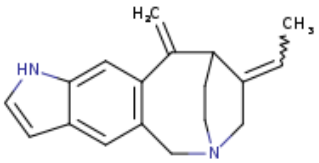
Then, as ligands and macromolecules, respectively, the produced protein structures and phytonutrient structures in PDBQT format were employed and stored. The molecular docking process was carried out using AutoDock Vina. For blind docking, a grid box was made for each protein. The command prompt was used to create molecular docking programmes, and the results were then examined in terms of binding affinity.

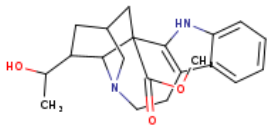
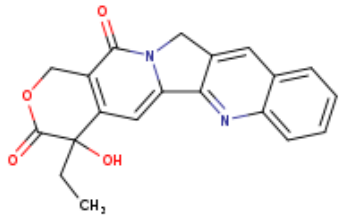
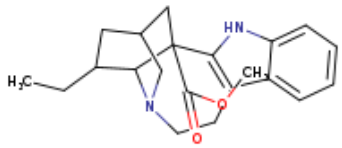
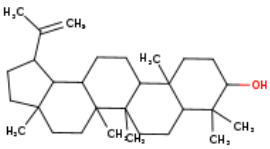
The docked complexes were visualised using BIOVIA Discovery Studio Visualizer to provide 2D and 3D images that show the binding contacts between the active phytonutrients and the target proteins.

Chapter 3 Result

The IMPPAT database lists 38 phytonutrients in the T. alternifolia plant. As demonstrated in Table 1, six of the active phytonutrients were discovered in the plant's bark.

TABLE 1.

Phytochemical	PubChem ID	IMPPAT ID	Structure
9-methoxycamptothecin	**123617**	**IMPHY012331**	
Pericalline	**6436240**	**IMPHY006461**	

Heyneanine	**15559731**	**IMPHY001591**	
Camptothecin	**24360**	**IMPHY002933**	
Coronaridine	**73489**	**IMPHY007011**	
Lupeol	**259846**	**IMPHY012473**	

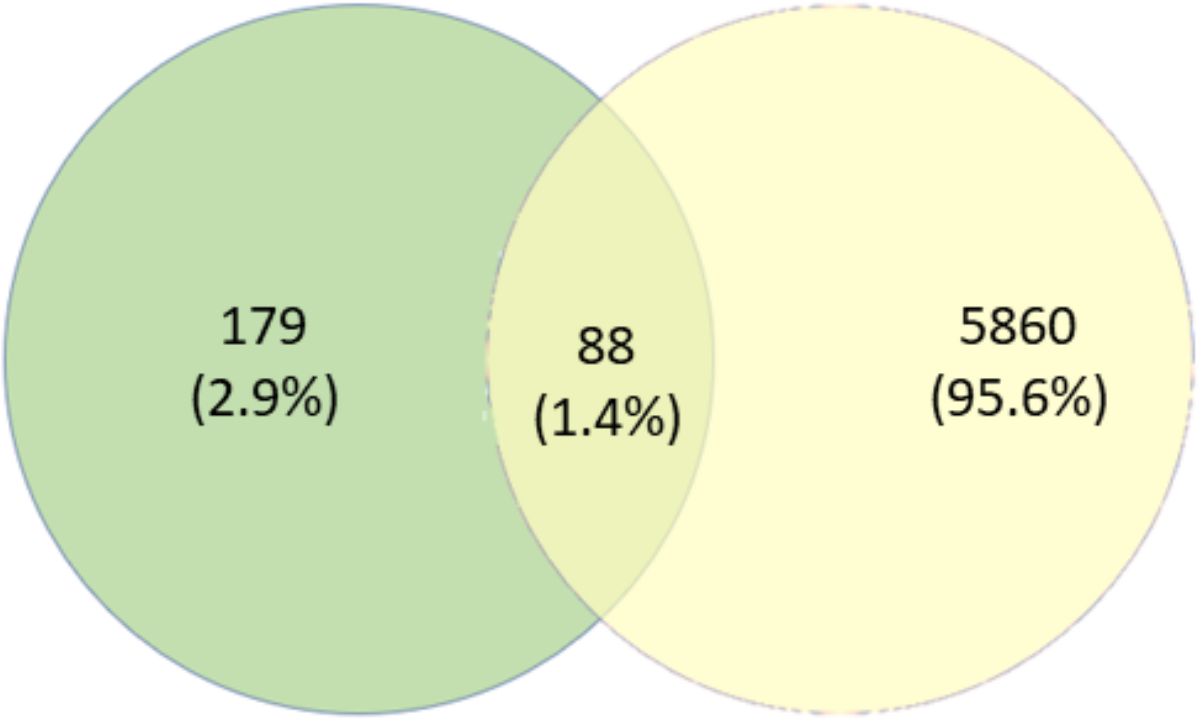
For the six active phytonutrients under study, the SwissTargetPrediction online database was used to find 401 possible gene targets with a probability less than 0. Based on computational techniques, this database offers predictions of the molecular targets that these phytonutrients may interact with. Based on the probability scores of the gene targets, those with a probability smaller than 0 received the most attention. These gene targets represent potential interactions between genes and the active phytonutrients.

3.3

Using the Gepia2 online tool, a total of 5960 gene targets associated with LUSC (lung squamous cell carcinoma) were discovered. The ANOVA differential method, which allows for the distinguishing of over-expressed and under-expressed genes, was used to determine the gene targets. This method specifically examines the levels of gene expression between LUSC samples and normal samples. The research concentrated on genes located on chromosomes that passed the q-value and log2FC (logarithm of fold change) thresholds of 0.01 and 1, respectively. These gene targets identify genes that may contribute to the onset or progression of LUSC and offer important information on the underlying molecular mechanisms of the condition.

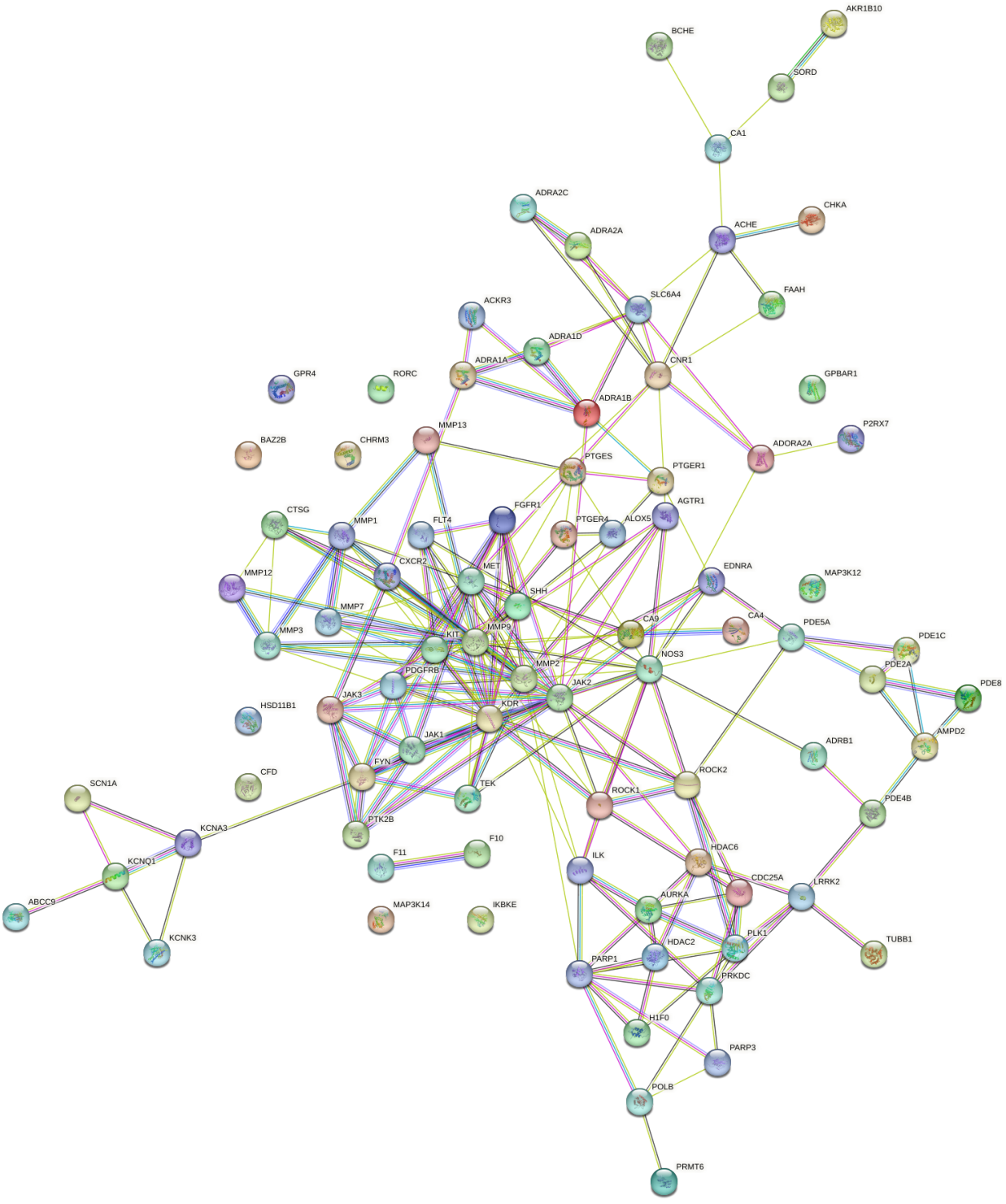
3.4

In accordance with Figure 1, the Venny 2.1 analysis showed that there were 88 gene targets that overlapped between the 401 potential gene targets of the active phytonutrients in Tabernaemontana alternifolia and the 5960 gene targets of LUSC. These 88 gene targets were chosen as prospective anti-LUSC gene targets. The overlap of the gene targets impacted by the phytonutrients and the gene targets linked to LUSC shows a possible relationship and raises the possibility that these shared gene targets contribute to the anti-LUSC actions of Tabernaemontana alternifolia's active phytonutrients.



3.5

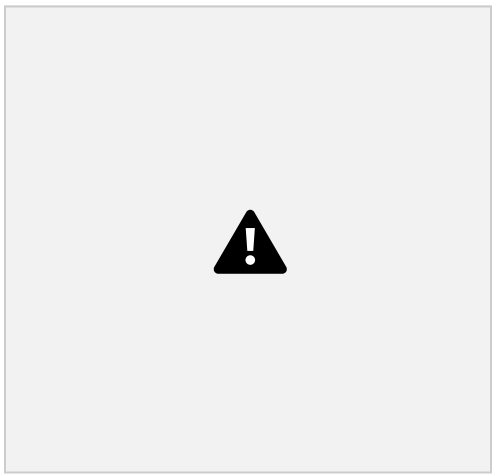
Figure 2A illustrates the results of the STRING analysis of the protein-protein interaction (PPI) network, which revealed that the network included 88 nodes and 219 edges. The degree to which nodes in a network tend to cluster together was measured using the average local clustering coefficient, which was estimated to be 0.511. It was found that there were 4.98 average node degrees, or the average number of edges connecting each node. The network's observed interactions' statistical significance as measured by the PPI enrichment p-value, which is less than 1.0e-16, was discovered. In addition, the network contained 91 predicted edges.



However, it was discovered that the PPI network contained 10 non-interacting nodes during the Cytoscape study. The resulting PPI network in Figure 2A has 78 nodes as a consequence. Every pair of nodes in the network had the characteristic path length of 2.473. Additional network statistics were calculated, including the diameter (6.0), which represents the longest shortest path

between any pair of nodes, the average number of neighbours (5.615), which represents the average degree of connectivity for the nodes, the clustering coefficient (0.224), which reflects the extent to which nodes tend to cluster together, and the network radius (0.036), which measures the proportion of possible edges that are present in the network.

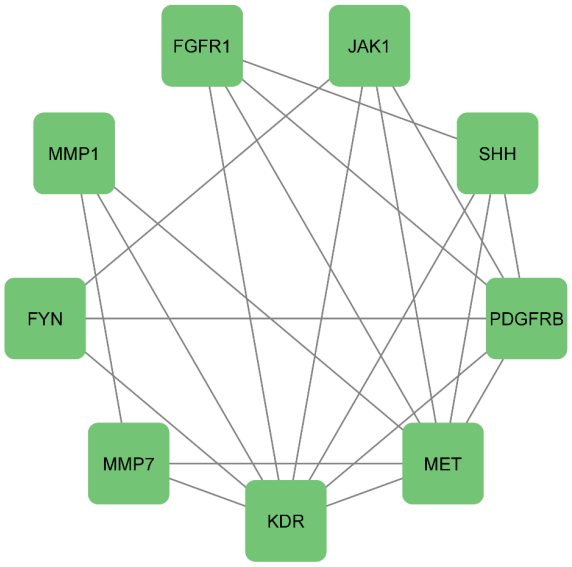
29 nodes were chosen from the network as the main anti-LUSC targets based on the degree centrality (DC) criterion, with a threshold of average value (5.6), as shown in Figure 4. The 29 nodes in Table 2 were prioritised according to their degree centrality scores and represent the primary anti-LUSC gene targets. The ordering of these nodes according to their degree centrality values is shown in the image as a bar graph.



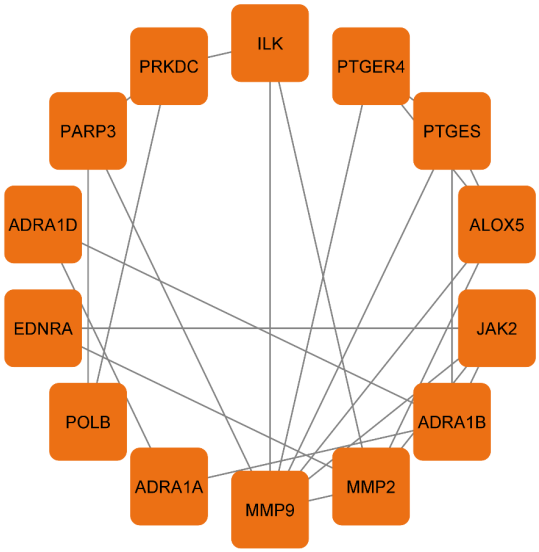
3.6

Using Cytoscape's MCODE plugin, cluster analysis was performed on the network made up of the 88 prospective targets for LUSC. The protein-protein interaction (PPI) network of the anti-LUSC primary targets was shown to contain two unique cluster networks. Figure 3 & 4 shows these cluster networks, which highlight the interconnection within each cluster and provide a visual picture of the clustering of the prospective targets.

The initial cluster network, which has 9 nodes and 21 edges, is shown in Figure 3A. The cluster network scored 5.250, demonstrating a high level of node interconnectivity within the cluster. Notably, a number of genes, including FGFR1, PDGFRB, MET, and KDR, have many gene targets and a degree centrality (DC) value of less than 4.667, showing their significance in the network.



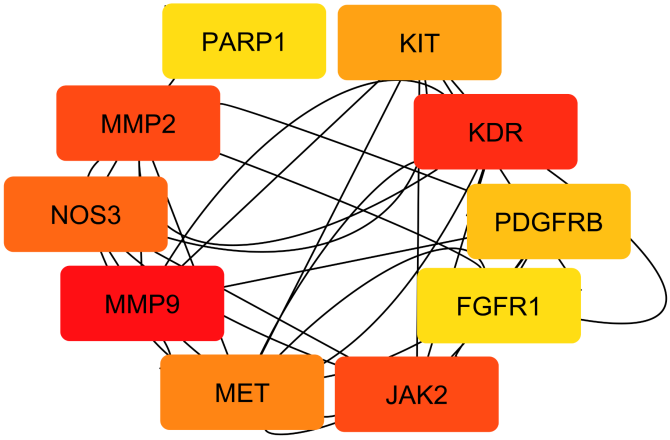
The second cluster network, shown in Figure 4, has 14 nodes and 22 edges. A score of 3.385 for the cluster network indicates a little lower degree of interconnectivity than the first cluster. Genes like MMP9, JAK2, and MMP2 show substantial connectivity with other nodes in this network. The importance of these genes within the network and their potential function as anti-LUSC targets are indicated by their degree centrality (DC) values, which are 3.143 for these genes.The second cluster network, shown in Figure 5B, has 14 nodes and 22 edges.



3.7

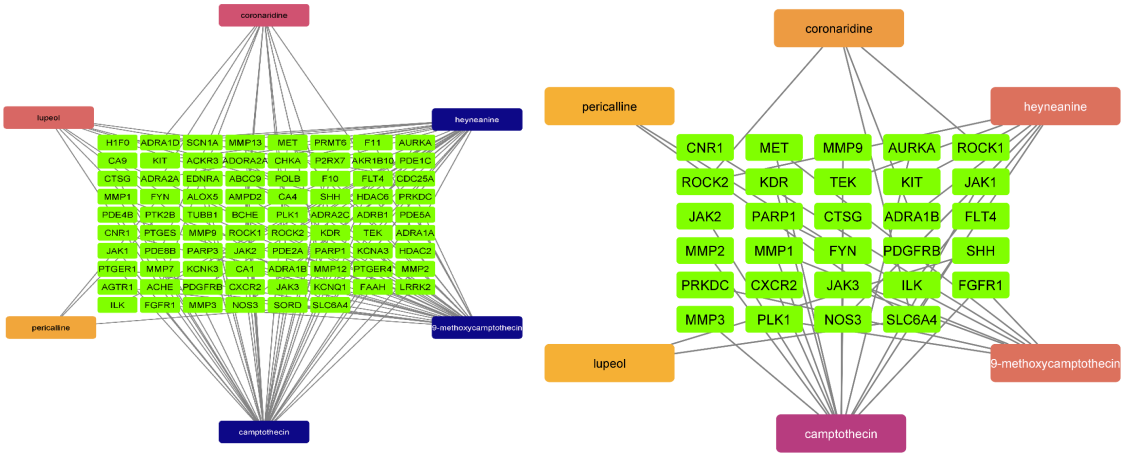
The "cytohubba" plugin in Cytoscape 3.9.1 was used, and four distinct techniques were used to identify the key targets inside the network: Degree, Maximum Neighbourhood Component (MNC), Maximal Clique Centrality (MCC), and Closeness. Figure 6 displays the findings of this research and lists the top 10 core targets chosen by each technique. The relevance and centrality

of these main targets inside the network, as determined by the particular approach used, were filtered.



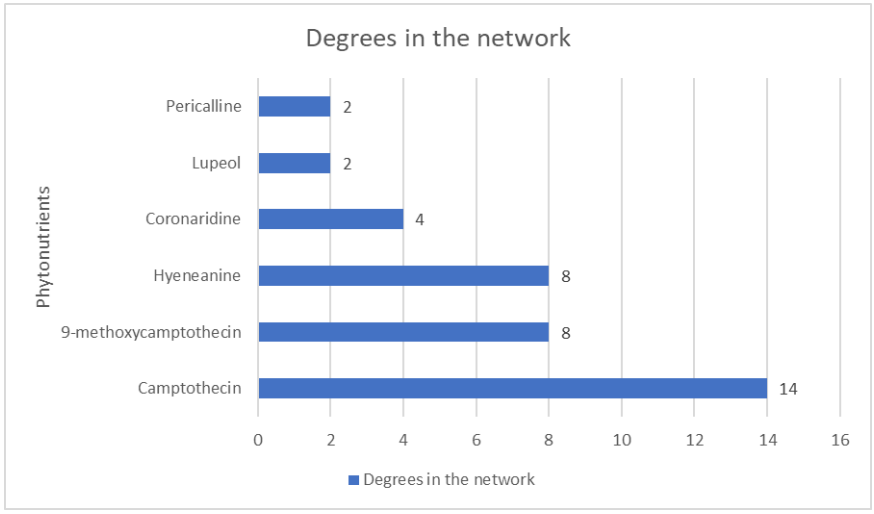
3.8

The network shown in the figure represents the link between the active phytochemicals found in Tabernaemontana alternifolia and 78 putative anti-LUSC targets. The network is made up of 84 nodes and 114 edges that represent the interactions between the targets and phytonutrients. The network's diameter is one, meaning that the shortest path length between any two nodes is one. The radius is also one, signifying the network's minimum eccentricity. The network density is 0.010, which indicates the proportion of edges present relative to the maximum potential number of edges.



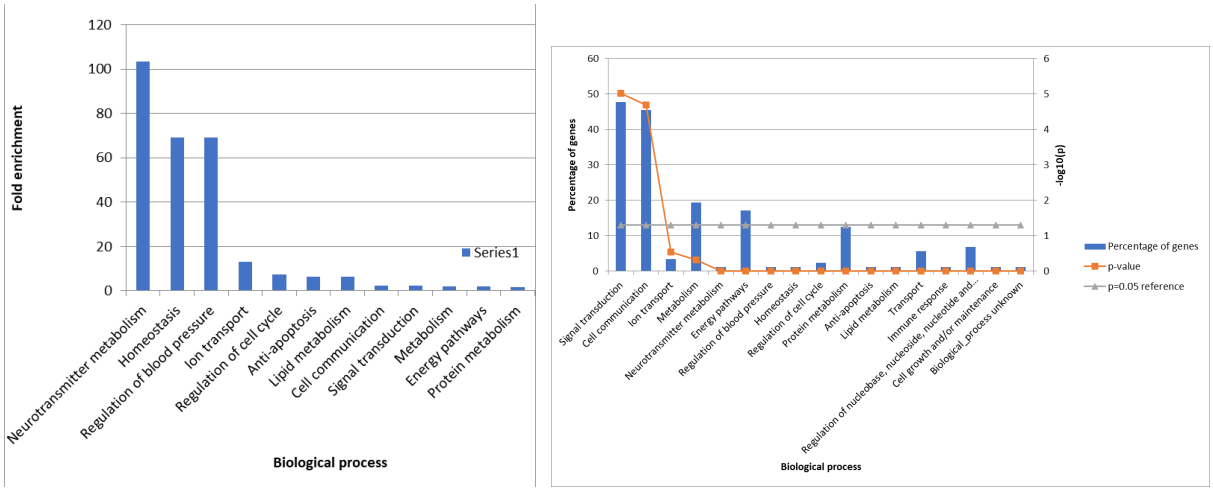
The average number of neighbours for each node is 2.714, demonstrating the network's average degree of connectedness. The characteristic path length is 1.000, reflecting the average number of edges along all pairs of nodes' shortest pathways. The clustering coefficient, which indicates how much nodes in a network tend to cluster together, is 0.000, indicating that there is no clustering in this network. The nodes in the illustration are coloured to signify their

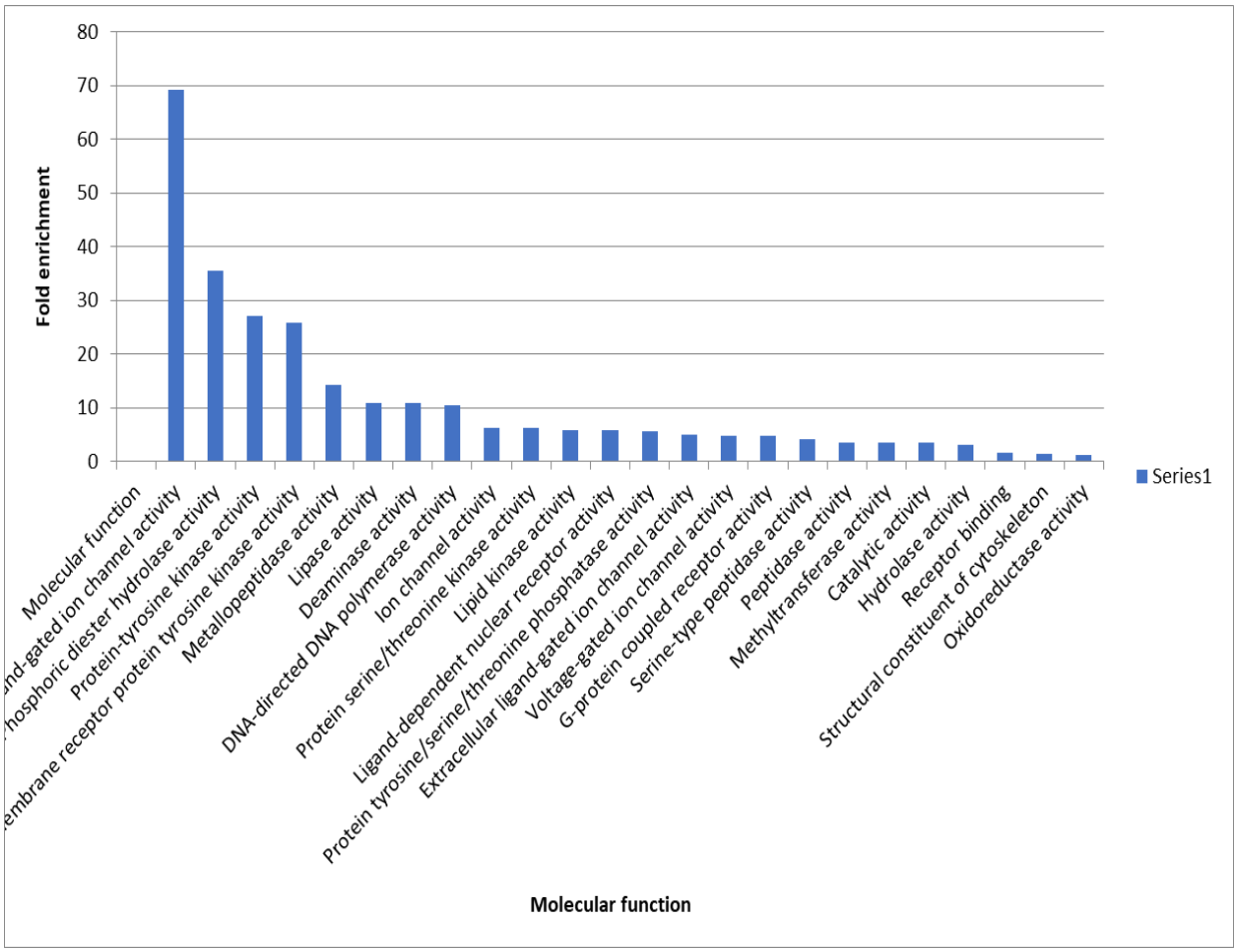
degree, with nodes becoming more purple as their degree grows, representing the amount of connections they have with other nodes.

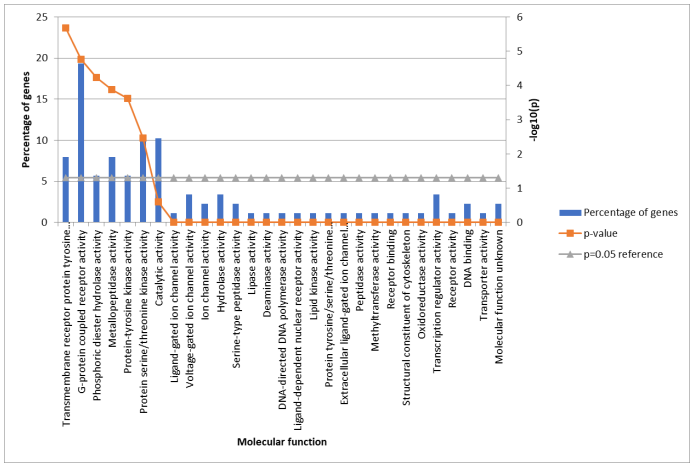


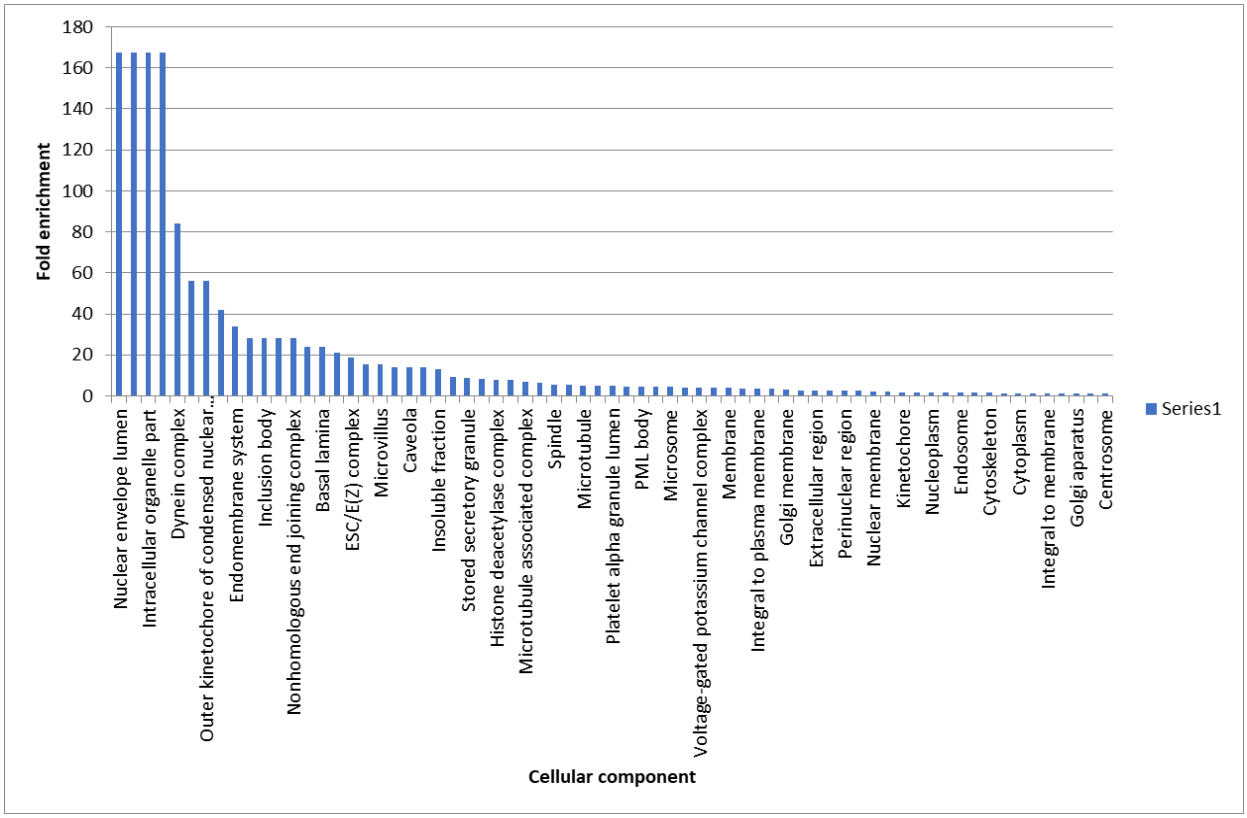
GO enrichment analysis

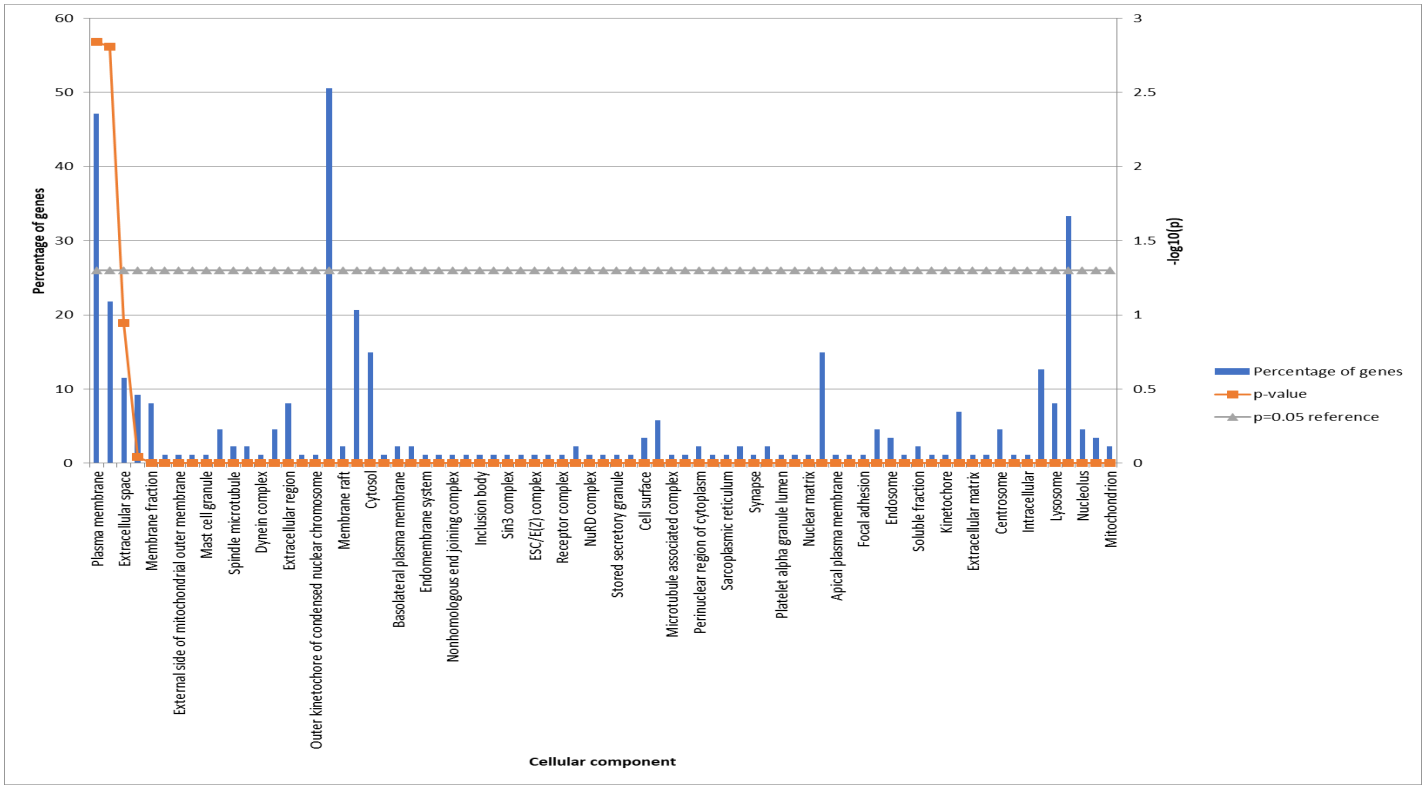
We further analysed the 88 potential anti-LUSC targets for GO enrichment analysis using Funrich software which revealed the top 10 enrichment terms for BP, MF and CC are shown in **Figure 9**.




Fold enrichment
80
70
60
50
40
30
20
10
0
Molecular function
Series1
Molecular function
nd-gated ion channel activity
hosphoric diester hydrolase activity
Protein-tyrosine kinase activity
embrane receptor protein tyrosine kinase activity
Metallopeptidase activity
Lipase activity
Deaminase activity
DNA-directed DNA polymerase activity
Ion channel activity
Protein serine/threonine kinase activity
Lipid kinase activity
Ligand-dependent nuclear receptor activity
Protein tyrosine/serine/threonine phosphatase activity
Extracellular ligand-gated ion channel activity
Voltage-gated ion channel activity
G-protein coupled receptor activity
Serine-type peptidase activity
Peptidase activity
Methyltransferase activity
Catalytic activity
Hydrolase activity
Receptor binding
Structural constituent of cytoskeleton
Oxidoreductase activity


Percentage of genes
-log10(p)
25
20
15
10
5
0
6
5
4
3
2
1
0
Molecular function
Percentage of genes
p-value
p=0.05 reference
Transmembrane receptor protein tyrosine...
G-protein coupled receptor activity
Phosphoric diester hydrolase activity
Metallopeptidase activity
Protein-tyrosine kinase activity
Protein serine/threonine kinase activity
Catalytic activity
Ligand-gated ion channel activity
Voltage-gated ion channel activity
Ion channel activity
Hydrolase activity
Serine-type peptidase activity
Lipase activity
Deaminase activity
DNA-directed DNA polymerase activity
Ligand-dependent nuclear receptor activity
Lipid kinase activity
Protein tyrosine/serine/threonine...
Extracellular ligand-gated ion channel...
Peptidase activity
Methyltransferase activity
Receptor binding
Structural constituent of cytoskeleton
Oxidoreductase activity
Transcription regulator activity
Receptor activity
DNA binding
Transporter activity
Molecular function unknown


Fold enrichment
180
160
140
120
100
80
60
40
20
0
Nuclear envelope lumen
Intracellular organelle part
Dynein complex
Outer kinetochore of condensed nuclear...
Endomembrane system
Inclusion body
Nonhomologous end joining complex
Basal lamina
ESC/E(Z) complex
Microvillus
Caveola
Insoluble fraction
Stored secretory granule
Histone deacetylase complex
Microtubule associated complex
Spindle
Microtubule
Platelet alpha granule lumen
PML body
Microsome
Voltage-gated potassium channel complex
Membrane
Integral to plasma membrane
Golgi membrane
Extracellular region
Perinuclear region
Nuclear membrane
Kinetochore
Nucleoplasm
Endosome
Cytoskeleton
Cytoplasm
Integral to membrane
Golgi aparatus
Centrosome
Cellular component
Series1


Percentage of genes
60
50
40
30
20
10
0
3
2.5
2
1.5
1
0.5
0
-log10(p)
Plasma membrane
Extracellular space
Membrane fraction
External side of mitochondrial outer membrane
Mast cell granule
Spindle microtubule
Dynein complex
Extracellular region
Outer kinetochore of condensed nuclear chromosome
Membrane raft
Cytosol
Basolateral plasma membrane
Endomembrane system
Nonhomologous end joining complex
Inclusion body
Sin3 complex
ESC/E(Z) complex
Receptor complex
NuRD complex
Stored secretory granule
Cell surface
Microtubule associated complex
Perinuclear region of cytoplasm
Sarcoplasmic reticulum
Synapse
Platelet alpha granule lumen
Nuclear matrix
Apical plasma membrane
Focal adhesion
Endosome
Soluble fraction
Kinetochore
Extracellular matrix
Centrosome
Intracellular
Lysosome
Nucleolus
Mitochondrion
Cellular component
Percentage of genes
p-value
p=0.05 reference

The analysis showed that the gene targets related to BP were involved in various processes such as signal transduction, cell communication, homeostasis and neurotransmitter metabolism etc.
Gene targets in CC were primarily found in locations such as plasma membrane, extracellular space
Moreover, the enriched MF ontology was largely comprised of terms such as

Chapter 4 discussion

This research work aimed to explore the molecular targets, active phytonutrients, and molecular processes involved in the use of *T. alternifolia* for treating LUSC. 6 phytonutrients were identified and determined to be the active components of *T. alternifolia* shown in **Table 1.** The network findings revealed a synergistic effect of multiple anti-LUSC core targets and multiple key active phytonutrients in the *T. alternifolia's* bark in alleviating LUSC pathology.

The PPI network analysis exhibits that numerous genes, including MMP9, KDR, MMP2, JAK2, NOS3, MET, KIT, PDGFRB, FGFR1, PARP1, etc., are implicated in the pathogenicity of LUSC and also in the anti-LUSC effects of *T. alternifolia* bark's key active phytonutrients (**FIGURES 3B, & 4) e**xtracellular matrix proteins, which are essential for tissue repair and remodelling, are broken down by an enzyme called MMP9 & MMP2 https://doi.org/10.3390/cells7100167 , also referred to as matrix metalloproteinase-9 & matrix metalloproteinase-2 respectively. They have been discovered to be over-expressed in tumor tissues when LUSC is present as opposed to healthy lung tissues https://doi.org/10.1038/s41416-020-0742-9 . According to studies, MMP9 & MMP2 may be involved in the angiogenesis and development of the tumour as well as the invasion and metastasis of LUSC cells https://doi.org/10.1111/jcmm.17464 . The receptor proteins dysregulation has been associated with the onset and development of numerous cancer types, including LUSC. These proteins involves FGFR1, MET (hepatocyte growth factor receptor), KIT, PDGFRB & KDR (VEGF2). However, the precise function of KIT in LUSC is still unclear, and more investigation is required to ascertain if KIT could be a suitable therapeutic target for this particular malignancy. A number of signalling pathways, including the PI3K/Akt and MAPK/ERK pathways, which are involved in the survival, proliferation, and migration of cancer cells, can be activated by these receptor proteins**.** They have been found to be over-expressed in LUSC and may hasten the disease's development by promoting angiogenesis, invasion, migration, and proliferation **Chang, L. S. (2019). *The NUP98 Gene as a Potential Modifier of NF2-Associated Tumors*. NATIONWIDE CHILDREN'S HOSPITAL**. Specifically, KDR, MET and PDGFRB are linked to poor prognosis of LUSC, Several downstream signalling pathways, including the JAK/STAT system, which is essential for controlling cell proliferation,

differentiation, and survival, are thought to be activated by JAK2. Most of the LUSC tumours contain JAK2 mutations and overexpression, which are linked to a poor prognosis https://doi.org/10.1016/j.biopha.2023.114452 . JAK2 has been demonstrated to have a role in boosting angiogenesis, which is necessary for tumour development and metastasis https://doi.org/10.3389/fonc.2022.1023177 , in addition to its direct effects on cancer cells. The NOS3 enzyme, sometimes referred to as endothelial nitric oxide synthase, is essential for producing nitric oxide (NO) in the endothelial cells that line blood arteries. The control of blood pressure, angiogenesis, and immunological response are just a few examples of the physiological processes in which nitric oxide plays a key role as a strong vasodilator and signalling molecule https://doi.org/10.1016/j.ccr.2023.215052 . The function of NOS3 in LUSC is not entirely known. According to certain research, NOS3 may limit tumour growth in LUSC by preventing cell proliferation and encouraging apoptosis https://doi.org/10.1002/bab.1909 . However, additional research has revealed that NOS3 may accelerate the growth and progression of LUSC by boosting angiogenesis and tumour cell invasion https://doi.org/10.1038/s42003-021-02470-x . Poly(ADP-ribose) polymerase 1, or PARP1, is an enzyme that contributes to genomic stability and DNA repair. Studies have revealed that through encouraging tumour cell survival and proliferation, PARP1 may contribute to the onset and progression of LUSC https://doi.org/10.1038/s41598-020-77284-8 . This may happen because PARP1-mediated signalling pathways such the PI3K/AKT pathway, which can encourage cell growth and survival, are activated. According to certain theories, PARP1 has a role in LUSC cells' reaction to DNA damage https://doi.org/10.1016/j.dnarep.2019.102651 . When DNA damage occurs, PARP1 may be turned on to speed up DNA repair. In contrast, PARP1 activation in LUSC cells may actually encourage DNA damage and genomic instability https://doi.org/10.1016%2Fj.dnarep.2019.102651 , which may eventually result in the growth of cancer.

Chapter 5 conclusion

The study showed the phytochemicals from Tabernaemontana alternifolia may have therapeutic benefits on lung squamous cell carcinoma (LUSC). By suppressing LUSC cell proliferation and triggering apoptosis, the active phytonutrients from Tabernaemontana alternifolia's bark shown promising anti-LUSC effects. These phytochemicals also addressed important biochemical pathways implicated in the pathogenesis of LUSC, such as cell cycle regulation, DNA repair, proliferation, and angiogenesis. They also reduced the expression of cancer-promoting elements such MMP9, MMP2, KDR, MET, FGFR1, PDGFRB, JAK2, NOS3, and PARP1. The results indicate that the phytochemicals from Tabernaemontana alternifolia may one day be used to treat LUSC.

Chapter 6 Future Propectus

More preclinical research is required to confirm the efficacy and safety of Tabernaemontana alternifolia's phytochemicals in the treatment of LUSC. Studies using cell culture and animal models can offer insightful information about their mechanisms of action and potential negative consequences.

Clinical trials: Strict clinical trials should be carried out to assess the efficacy and safety of phytochemicals from Tabernaemontana alternifolia as a therapy for LUSC in people. These studies can define suitable dosage regimens and offer proof for their therapeutic application.

Combination therapies may improve treatment outcomes for LUSC patients by examining the synergistic interactions of Tabernaemontana alternifolia's phytochemicals with already available chemotherapeutic medicines or targeted therapies. Combination strategies may increase effectiveness, combat medication resistance, and reduce side effects.

Mechanistic studies: Additional research is required to determine the precise molecular processes by which the phytochemicals in Tabernaemontana alternifolia exert their anticancer properties in LUSC. This information can assist discover specific molecular targets for drug development and maximise their therapeutic potential.

Evaluation of the safety profile and potential negative consequences of the phytochemicals in Tabernaemontana alternifolia should include thorough toxicity tests. For their clinical application, it will be essential to comprehend their pharmacokinetics, biodistribution, and long-term toxicity.

Overall, the network pharmacology study offers insightful information on the therapeutic potential of the phytochemicals found in Tabernaemontana alternifolia for the treatment of LUSC. To promote their development as innovative treatment medicines for this aggressive kind of lung cancer, additional study and clinical trials are required.

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CANDIDATE'S DECLARATION

I Anjali Sinha, Roll Number: 2K21/MSCBIO/62 student of M.Sc. Biotechnology, hereby declare that the project dissertation titled - **"Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma : a Network Pharmacology Study"** which is submitted by me to the Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is original and not copied from any source with proper citation. This work has not previously formed the basis for the award of any degree, Diploma Associateship, fellowship or other similar title or recognition.

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CERTIFICATE

I hereby certify that the Project Dissertation titled "**Exploring the therapeutic potential of Tabernaemontana alternifolia bark for lung squamous cell carcinoma : a Network Pharmacology Study**" which is submitted by Anjali Sinha, Roll No.: 2K21/MSCBIO/62, Department of Biotechnology, Delhi Technological University, Delhi in partial fulfillment of the requirement for the award of the degree of Master of Science, is a record of the project work carried out by the student under my supervision. To the best of my knowledge, this work has not been submitted in part or full for any Degree or Diploma to the University or elsewhere.

Place: Delhi
Date: 30/05/2023

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