Isolation and Characterization of Trypsin Inhibitors from the seeds of *Artocarpus hirsutus* Lam. and *Garcinia gummi-gutta* (L.) Roxb. and studies on their effects on Skin cancer (A431) and Colon cancer (HT29) Cell lines

Thesis Submitted to
the University of Calicut in partial fulfilment of
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DOCTOR OF PHILOSOPHY IN BIOCHEMISTRY

By

CHANCHITHA CHANDRAN

Under the Guidance of

Dr. Gayathri Devi. D.

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CERTIFICATE

This is to certify that this thesis entitled entitled "**Isolation and Characterization of Trypsin Inhibitors from the seeds of *Artocarpus hirsutus* Lam. and *Garcinia gummi-gutta* (L.) Roxb.and studies on their effects on Skin cancer (A431) and Colon cancer (HT29) Cell lines**" is a bonafide research work done by **Ms. Chanchitha Chandran.,** under my supervision and guidance in the Department of Life Sciences, University of Calicut, for the award of the degree of Doctor of Philosophy in Biochemistry, under the faculty of Science of the University of Calicut. I also certify that the same has not been submitted for any other degree diploma or associateship in any other University.

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Date:

DECLARATION

I hereby declare that the work presented in the thesis entitled **"Isolation and Characterization of Trypsin Inhibitors from the seeds of *Artocarpus hirsutus* Lam. and *Garcinia gummi-gutta* (L.) Roxb.and studies on their effects on Skin cancer (A431) and Colon cancer (HT29) Cell lines"** submitted to the University of Calicut, as partial fulfillment of the requirement for the award of the degree of Doctor of Philosophy in Biochemistry, is original and carried out by me under the supervision of Dr. Gayathri Devi D., Department of Life Sciences, University of Calicut. This has not been submitted earlier either in part or full for any degree or diploma of any university. All corrections, as suggested by the examiners have been incorporated into this thesis.

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Date:

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Introduction

1.1 Proteases and their inhibitors

The performance of proteins in biology is exceptionally magnificent and fiddly, since one can perceive that most of the enzymes as well as their regulators are all proteins. Proteases are enzymes that catalyse proteolysis, which breaks down proteins into smaller polypeptides or single amino acids and encourages the synthesis of new protein products. They are also called peptidases, proteinases, or proteolytic enzymes. Research on proteases has long emphasised their fundamental functions as sharp attackers involved in protein destruction. Proteases play important roles in the processing of cellular information; the regulation of numerous proteins' destiny, location, activity, production, transduction, amplification of signalling molecules and the modulation of protein-protein interactions. The global market for industrial enzymes is dominated by proteases, which accounts for 59% of the total (Deng *et al*., 2010).

Protease-inhibiting molecules, often known as antiproteases, prevent the action of proteases (enzymes that aid the breakdown of proteins). There are numerous proteins that act as protease inhibitors (PIs) in nature. PIs are frequently found in both plants and animals. They prevent insects from ingesting seeds and have a variety of other functions, such as influencing animals' blood clots. Following evidence that both synthetic and natural inhibitors lower two-stage carcinogenesis and breast cancer, PIs were the subject of extensive research as chemopreventive medications. PIs are unique in that they inhibit the growth of cancer in multiple ways, including the suppression of metastasis, oxygen radicals, and oncogenes. In communities that consume foods containing them, epidemiologic data

supports their protective action against major human malignancies. They are just beginning to be used on humans under supervision. The value of lentils and other seeds which are sources of PIs was discovered through epidemiology. Bowman-Birk inhibitors (BBI) are a type of PIs that has received significant attention in the treatment of a range of conditions, especially in various type of cancer prevention. Soybeans, wheat, peanuts, chickpeas, etc. have been discovered to be good sources of PIs.

Many PIs were reported to be useful in the treatment of cardiovascular diseases, osteoporosis, neurological dysfunctions and inflammatory disease conditions (Srikanth *et al*., 2016). The malignancies of the colon, skin, mouth, lung, liver and oesophagus are responding favourably to this novel discovery. Both the type of protease that they inhibit and their mode of action can be used to categorise PIs. The use of PIs, which can lessen tumour cells' capacity for invasion and metastasis, is suggested by the involvement of proteases in cancer. Overexpression of trypsin may lead to various disease conditions. The activation of trypsin in the pancreas as a result of proteolytic cleavage of trypsinogen can set off a chain of events that result in pancreatic self-digestion and pancreatitis. A lack of transfer of trypsin and other digestive enzymes from the pancreas is one of the symptoms of the autosomal recessive disease cystic fibrosis. This causes meconium ileus, a condition characterised by intestinal obstruction (ileus) caused by excessively thick meconium, which is normally broken down by trypsin and other proteases and passed in faeces. Trypsin, a well-known digesting enzyme produced by the pancreas, has also been discovered in other tissues and malignancies,

most notably colorectal cancer. Trypsin encourages growth, invasion, and metastasis and is implicated in the development of colorectal cancer.

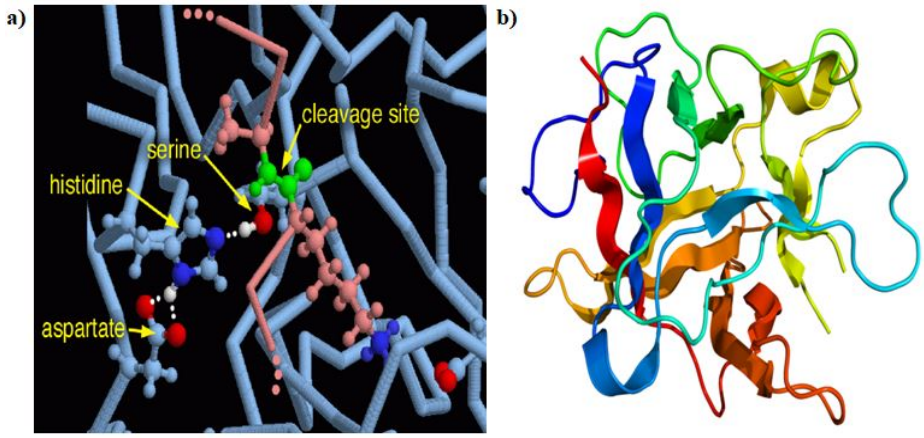
1.2 Trypsin and Trypsin Inhibitors

Trypsin (Fig.1.1.a) is a digestive enzyme largely produced by pancreatic acinar cells that facilitates the digestion of food, and the small intestine contains the trypsin enzyme. Trypsin belongs to peptidase family S1 (Enzyme 3.4.21.4) and the enzyme class Hydrolases (EC 3.-.-. - Hydrolases). EC 3.4.-.- Acting on peptide bonds (peptidases), EC 3.4.21. - Serine endopeptidases. Plants, fungus, and other microorganisms can all be used to make it. Trypsin is a medium-sized globular protein with a molecular weight of around 25kDa, made from the inactive form of trypsinogen. Trypsin is given to people who are weak in digestive enzymes. It is employed in a variety of biotechnological procedures. It is an endopeptidase that cleaves peptides from lysine and arginine amino acid residues on the C-terminal side. Trypsin is mostly produced for commercial purposes from the pancreas of cattle. Trypsin proteolysis or trypsinization is the term used to describe the process of digesting or treating proteins using trypsin. Trypsin expression has also been observed in epithelial cells of the skin, oesophagus, stomach, small intestine, colon, lung, kidney, liver, bile ducts, leukocytes, spleen and neuronal cells. Trypsin significantly promotes the growth of human colon cancer cells *in vitro*. Low nanomolar concentrations of trypsin stimulate the mitogenic effect, which is mediated by PAR-2, a G

protein-coupled receptor with seven transmembrane domains. Trypsin and probably other serine proteases operating on PAR-2 may represent new crucial signalling proteins in the regulation of colon cancer growth because PAR-2 expression and trypsin's mitogenic impact are seen in a variety of colon cancer cell lines (Darmoul *et al*., 2001).

A trypsin inhibitor (TI) is a protein and a category of serine protease inhibitor (serpin) that inhibits the activation and catalytic processes of proteins to lower the biological activity of trypsin. The soybean-derived Kunitz-type trypsin inhibitor (Fig.1.1.b) is an example for a trypsin inhibitor from plant source. It has 181 amino acid residues and two disulfide bridges (Cys39-Cys86 and Cys136-Cys145). It was characterized by Kunitz in 1947(Kunitz, 1947 a, b).

Figure 1.1 Trypsin and Kunitz Soybean Trypsin Inhibitor



a) Trypsin, b) Kunitz-type trypsin inhibitor. (Copy right- link addresses are mentioned below)

- https://encrypted-tbn2. gstatic. com/images?q=tbn:ANd9GcRX3CVfxYU68l3sWQ7omVwWNlTJ-Dhkpv86WyY7CdK3k4Zzncis
- https://upload. wikimedia. org/wikipedia/commons/thumb/8/8a/1TIE. png/220px-1TIE. png

1.3 Plants as medicinal sources

The use of medicinal plants by humans is not new; it has been done for thousands of years. Ancient man relied heavily on green plants for his daily medicinal needs. These plant species are regarded as a rich source of components for the production and formulation of medications. Additionally, plants are essential to the growth of cultures throughout history all across the world. Herbal medicines have a bright future with over 500, 000 plants on the globe and the majority of their potential medical benefits have not yet been explored. Several plants are regarded as significant sources of nutrients and as a result, they are suggested for their medicinal potential. Based on different aspects employed, the categorization of medicinal plants is organised differently.

Medicinal plants' storage organs such as their roots, leaves, flowers, seeds and other portions are arranged according to their bioactive ingredients. They can benefit in treatment of diseases. Humanity has exploited higher plants as a source of medications for many thousands of years. However, the significance of plants as a source of drug raw materials has significantly declined with the introduction of modern medicine, synthetic pharmaceuticals and antibiotics.

Different plant seeds selected for the study were *Artocarpus hirsutus*, *Bauhinia acuminata*, *Garcinia gummi-gutta*, *Syzygium cumini*

and *Thevetia neriifolia*. The huge edible fruit of the genus *Artocarpus* is prized for its high nutritional content. *Artocarpus heterophyllus, Artocarpus altilis, Artocarpus hirsutus, Artocarpus lakoocha* and *Artocarpus camansi* are some of the major species that belong to this genus. It is a tropical tree species that is endemic to India. It is most common in Kerala, although is also found in Maharashtra, Karnataka and Tamilnadu. The fruit has a similar structure to the jackfruit. The seeds are small, 1-2cm sized, white and ovoid in shape. Trees can be grown by grafting or seeds and these usually flower from December to January. The fruits ripen in May and June. The storage components found in seeds which are heterogenous storage reserves, comprise a variety of soluble carbohydrates, starch polymers, storage proteins and lipids. Proteolytic enzyme activity in beans increases over the first seven days of seed germination (Gepstin and Han 1980); also noted the disappearance of PIs which are proteins by nature. The seed part is enriched with proteins.

Bauhinia acuminata is an indigenous flowering shrub that can be seen in tropical Southeast Asia. Within the family Leguminosae, the genus Bauhinia is a vast and diverse group of tropical and subtropical evergreen plants, with about 300 species. Many cultures throughout the world use the plant for ethno-botanical purposes. The Javanese use the roots to treat colds and coughs, and the Indians use the decoction of leaves and bark to treat asthma. With benzopyrene as the cancer-causing agent, Sebastian (2022) examined *B. acuminta*'s anti-cancer activity in a lung cancer model in C57BL/6 mice. Four different dosages of *B.acuminata* (very low, low, mid and high) were

administered to animals that had been given disease-inducing substances. Tumor size and volume decreased in a dosage-dependent manner at high doses. Numerous experimental studies have demonstrated the antibacterial activity of plants from the genus Bauhinia, particularly against pathogenic fungus and bacteria, which are commonly employed in folk medicine to treat infectious disorders. The crude extract of the seed from *B.acuminata* L. showed significant antibacterial activity against gram positive and gram negative bacteria. Research showed that the gram positive *Bacillus subtilis* and gram negative *Pseudomonas aeruginosa* showed sensitivity to the crude extract of the *B.acuminata* seed (Phansri *et al*., 2011).

Garcinia gummi-gutta is a small to medium-sized tree belonging to the Guttiferae family. It has a rounded crown and horizontal or drooping branches. The tree is often found in the evergreen woods of the Western Ghats, from Konkan south to Travancore, and in the forests of the Nilgiris up to an altitude of 6000ft. The fruit contains six to eight seeds surrounded by a succulent aril. It blooms in the hot season and in the wet season, the fruits ripen. Southeast Asian origin *Garcinia gummi-gutta* is also known as Malabar tamarind or simply "Garcinia." The dried fruit has a long history of use in Southeast Asia as a food preservative, flavouring agent and carminative. It is widely used in developed nations as an ingredient in dietary supplements for weight loss. Indians in Maharashtra, coastal Karnataka, Kerala and Goa utilise garcinia as an essential culinary ingredient and as an acidulant for curries. The fruit is covered in yellowish, delicious pulp and has three to eight big seeds inside.

Syzygium cumini, also known as *Eugenia cumini* or *Syzygium jambolanum* belongs to the Myrtaceae family. Other common names include Indian Blackberry, Black Plum, Jamblang, Jamun, Java Plum and Jambul. These trees are now common throughout the Asian subcontinent, Eastern Africa, South America, Madagascar and the United States of America, where they have naturalised in Florida and Hawaii. The tree only bears fruits once a year and the taste of the berries is sweetish-sour. The ripe fruits are used to make wine, preserves, squash, jellies and health beverages. In addition to being utilised as food, the tree's seeds and all of its other parts are also used to treat a number of illnesses, the most significant of which being diabetes mellitus. Its seeds have the potential to play a role in controlling diabetes mellitus. They also contain a lot of other phytochemicals and are reasonably high in protein (6.3-8.5%) (Binita Kumari *et al*., 2017).

An evergreen shrub or small tree, *Thevetia neriifolia* (*Thevetia peruviana, Cascabela thevetia*) typically grows to a height of 3 to 8 m. The linear, 13-15cm long leaves are grouped in spirals. Flowers are funnel-shaped, bright yellow and spirally twisted. The fruits have a diameter of 4-5cm, are fairly spherical and are slightly meaty. Initially green, the fruits turn black as they ripen. A nut that is longitudinally and transversally split is found inside each fruit. The milky fluid is present in all plant sections. These plants contain a range of cardiac glycosides including nerifolin, thevetin A, thevetin B and oleandrin, and all parts are poisonous. Oleander consumption causes hyperkalemia, nausea, vomiting, abdominal pain, diarrhoea and

disrhythmias. Some research has proved the efficacy of the ethanolic leaf extract with its antibacterial, cytotoxic and antidiarrheal properties. Some poisoning symptoms include tachycardia, arrhythmia, paralysis, ataxia and confusion. These symptoms point to serious cardiac, neuromotor and mental malfunctions as well.

1.4 Protein Purification from Plant Sources

The goal of protein purification is to separate one or a small number of proteins from a complicated mixture, typically a group of cells, tissues, or entire organisms. The definition of the function, structure and interactions of the target protein depends on its purification. Differences in size, physical and chemical characteristics, biological activity and binding affinity are commonly used for separation. The homogeneity can be probed by techniques like SDS-PAGE, Reverse zymography, MALDI-TOF, etc. The commonly used methods for protein purification comprise of processes like ammonium sulphate precipitation, ion exchange chromatography, size exclusion chromatography (gel filtration chromatography), etc.

1.5 Cancer

In India, non-communicable diseases like cancer are becoming serious public health issues. In terms of the largest percentage of cancer cases, India comes in third place among all countries. By the end of the next five years, cancer incidences in India are expected to increase by 12%, according to the Indian Council of Medical Research (ICMR). According to the National Cancer Registry programme data,

about 13 lakh people in India experience cancer each year. According to annual figures released by the American Cancer Society, the chance of dying from cancer in the United States has dropped during the past 28 years (ACS). When compared to its peak rate in 1991 and 2019 for which data was available, the combined cancer death rate for men and women dropped by 32% (Annual statistics reported by the American Cancer Society (ACS). After heart disease, cancer continues to be the second most prevalent cause of death in the US. In the US, there were 1.9 million new cancer diagnoses and 609,360 deaths from cancer or its complications in 2022. This works out to around 1,670 fatalities each day. In India, 14, 61,427 incident cases of cancer were reported in 2022. According to available data crude rate of cancer incidence in India is 100.4 per 100,000 and one in nine people are likely developing cancer at some point in their lives (Sathish kumar *et al*., 2022).

Cancer can be characterised as the uncontrolled growth of cells. Growth may become out of control when a cell or set of cells' programming is compromised. Chronic irritability, cigarettes, smoke, dust, radioactive substances, age, sex, race and heredity are a few variables that can change the code. We must be aware of the elements we can control, even though many of these factors are beyond our control. Cancer prevention is unquestionably preferable to cancer treatment. Programmed cell death or apoptosis is a process that begins with the cell receiving an internal signal (such as DNA damage) or an external signal (such as an extracellular death ligand). Rounding up of the cell; retraction of pseudopodes; pyknosis (loss of cellular volume), chromatin condensation, karyorrhexis (nuclear fragmentation), plasma

membrane blebbing and disintegration of the cell into apoptotic bodies are common biochemical reactions that occur during signaling pathways.

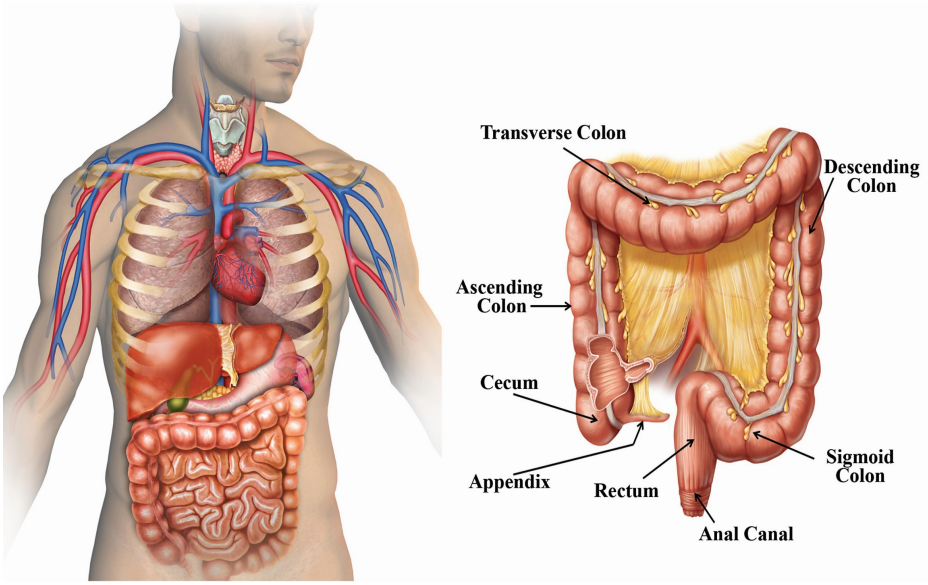
Apoptosis aids in the prevention of cancer. Apoptosis can be blocked for a variety of reasons, which can result in uncontrolled cell division and the significant expansion of a tumour. The majority of anticancer drug-activated signalling pathways eventually lead to the activation of the caspase family of cysteine proteases, which function as common death effector molecules in many types of cell death. (Thornberry *et al*., 1998; Los *et al*., 1999; Hengartner *et al*., 2000).

1.6 Colon Cancer

Colon (Fig.1.2) the first segment of the large intestine is a muscular tube divided in to four sections and measure around 5 feet (1.5m) in length and 2 inches (5cm) in diameter. The first section is the 'ascending colon' which starts on the right side of the belly, above the cecum (a pouch where undigested food is taken from the small intestine). The second segment, the 'transverse colon', which spans the body from right to left, is referred to as the proximal, or right, colon when combined with the ascending colon. The third section, the descending colon, is situated on the left. The descending colon, the fourth and last section of the colon, named the 'sigmoid colon', is commonly referred to as the distal, or left, colon because of its 'S' form. Following passage through the small intestine, the colon absorbs water and salt from the leftover food material (small bowel). The rectum, the last 6inches (15cm) of the digestive tract receives the waste

matter that is left behind after passing through the colon. It is kept there until it goes through the anus. When having a bowel movement, sphincter-like muscles around the anus prevent stool from coming out until they relax.

Figure 1.2 Colon



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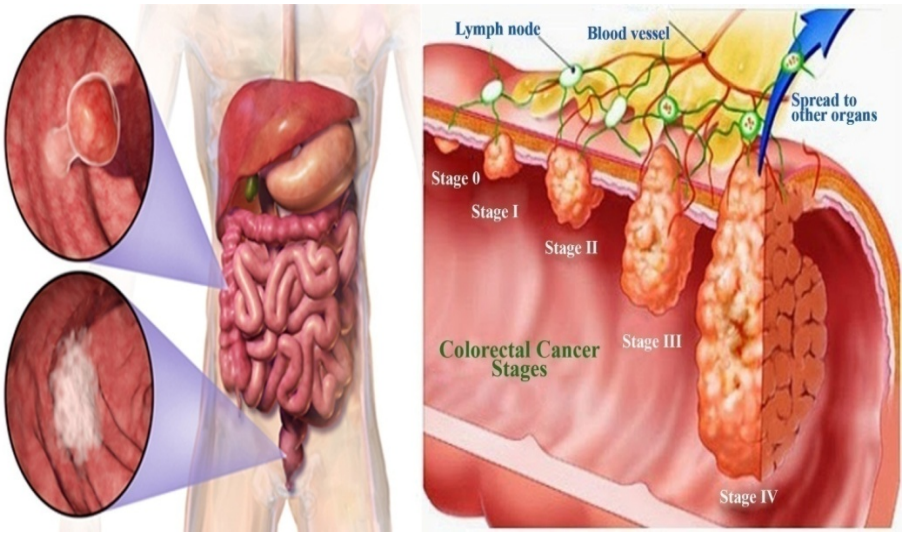
What causes colorectal cancer to develop is a polyp in the colon or rectum. The majority of colorectal cancers begin as tumours on the rectum or colon's inner lining. Polyps are the name for these growths. While not all polyps develop into cancer; some can do so over time (often many years). The type of polyp will determine whether it develops into cancer. There are various kinds of polyps.

Adenomatous polyps can occasionally develop in to malignancy. Hence adenomas are referred to as pre-cancerous conditions. Tubular, villous and tubulovillous are the three different forms of adenomas. Hyperplasic and inflammatory polyps are more frequent, but they are typically not precancerous. Larger (greater than 1cm) hyperplasic polyps may necessitate more frequent colorectal cancer screening. Due to their increased risk of colorectal cancer, sessile serrated polyps (SSP) and typical serrated adenomas (TSA) are frequently treated as adenomas.

Additional elements that can enhance the likelihood that a polyp contains cancer or raise a person's chance of acquiring colorectal cancer include: finding polyps larger than 1cm, finding more than three polyps and finding dysplasia in the polyp after it has been removed. Another precancerous condition is dysplasia. It indicates that there is a region in a polyp, the lining of the colon, or the rectum where the cells appear abnormal but have not yet developed into cancer. If cancer develops in a polyp, it may eventually spread to the colon or rectum's wall. There are several layers to the colon and rectum's walls. The mucosa, the deepest layer, is where colorectal cancer begins and can spread through any or all of the other layers (Fig.1.3). Cancer cells may develop blood vessels or lymphatic vessels (tiny channels that carry away waste and fluid) after they are embedded in the wall. They can then go to neighbouring lymph nodes or to other regions of the body that are far away. The depth to which a colorectal cancer penetrates the wall and if it has progressed outside the colon or rectum determine the stage (extent of dissemination) of the disease.

Figure 1.3 Colon cancer



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- https://www. southlakegeneralsurgery. com/wp-content/uploads/2019/12/colon-cancer-stages. jpg)
- https://upload. wikimedia. org/wikipedia/commons/thumb/1/12/Blausen_0246_ColorectalCancer. png/800px-Blausen_0246_ColorectalCancer. png)

According to the predictions of WHO's (World Health Organization) mortality database, which was received from the Australian Bureau of Statistics (ABS) the mortality rate for colon cancer would go down while it would go up for rectal cancer. While it can affect anyone at any age, colon cancer is more common in older adults. Polyps, which are small, noncancerous (benign) cell clusters that develop inside the colon, are the most common precursors to colon cancer. Over time, some of these polyps can develop into colon cancer. Colon cancer or colorectal cancer, a term that combines colon cancer with rectal cancer originates in the rectum. Colorectal cancer (CRC) is

one of the world's most dangerous and common cancers, accounting for almost 881,000 cancer-related deaths in 2018 (Xie *et al*., 2020).

For many years, surgery and chemotherapy were the treatment of choice for cancer patients. If colon cancer is limited to the gut, it can be treatable and often a curable disease. Surgery is the most common treatment option and it cures around half of the patients. Recurrence after surgery is a big issue and it is frequently the cause of death. Targeted therapy is a new treatment option that has successfully extended overall life in individuals with CRC. Although the specific etiology of colon cancer is unknown, several risk factors, such as nutrition, cigarette use and excessive alcohol intake are significantly connected to the disease. For instance, alcohol usage contributed to more than 1, 60,000 new cases of colorectal cancer in 2020, or 8% of all cases of the disease recorded in 2020. A person's risk of developing at least six more malignancies, including breast and liver cancer, is increased by drinking alcohol. People with specific genetic cancer syndromes, as well as those with a family background of colorectal cancer, are at an increased risk of acquiring the disease. The chance of developing colorectal cancer can be lowered with diets high in fish, fruits and vegetables, exercise and intentional weight loss.

There are different types of Colon cancer. Adenocarcinoma, the most prevalent kind of colorectal cancer, is one of many different subtypes. Other varieties include colorectal lymphoma, gastrointestinal stromal tumours and carcinoid tumours.

1.6.1 Gastrointestinal Stromal Tumors (GIST)

According to the ACS, it is estimated that between 4, 000 and 6, 000 new cases of GIST are detected in the country each year. It usually takes these tumours some time to get big enough to start producing symptoms.

1.6.2 Hereditary Colon Cancer

Approximately 5% of individuals with colorectal cancer have inherited gene variations (mutations) that can result in family cancer syndromes and make them more likely to get the illness. Peutz-Jeghers syndrome (PJS) or Hamartoma, Lynch syndrome (hereditary non-polyposis colorectal cancer or HNPCC) and familial adenomatous polyposis (FAP) are the most prevalent inherited syndromes associated with colorectal malignancies, although other less frequent syndromes can also raise the risk of developing the disease.

1.6.3 Peutz-Jeghers syndrome (PJS)

A specific kind of polyp called a hamartoma or Peutz-Jeghers syndrome (PJS) forms in the gastrointestinal tract. This syndrome is passed down from one's parents and is brought on by gene mutations (STK11). It carries an elevated risk of colorectal cancer as well as breast, ovarian, pancreatic and other cancers. If colorectal cancer develops in people with PJS, it typically does so earlier in life than the average case.

1.6.4 Familial adenomatous polyposis (FAP)

The APC (Adenomatous polyposis coli) gene alterations that a person inherits from their parents are the cause of FAP. FAP is responsible for about 1% of all colorectal malignancies.

1.6.5 Turcot syndrome

Turcot syndrome is a condition where there is a change (mutation) in the APC gene, a gene that inhibits the development of tumours. Defects in the APC gene can potentially lead to FAP and it is categorised by some experts as a kind of familial adenomatous polyposis (FAP). It is an extremely uncommon hereditary condition and it raises the chance of brain or spinal cord cancers and produces tiny growths (polyps) in the intestines. Two types of Turcot syndroms are there. They are Type I (True) and Type II Turcot syndrome. Type I is an autosomal recessive trait that runs in the family. This indicates that the gene alterations (mutations) that cause Turcot syndrome were passed down from both the parents. The MLH1 and PMS2 genes are most likely altered (mutated) in type I Turcot syndrome development. Type II is an autosomal dominant characteristic that is inherited. This implies that the gene mutation causing Turcot syndrome only has to be passed down from one of the parents.

1.6.6 Colorectal adenocarcinoma

The prefix "adeno" means "glands" in Greek. A form of cancer known as "carcinoma" develops in epithelial cells, which cover both the interior and exterior surfaces of the body. Adenocarcinomas form

at the end of the colon or the lining of the large intestine. They frequently begin in the inner lining and move to different layers. The symptoms of colorectal adenocarcinoma include abdominal pain and tenderness, changes in bowel habit such as diarrhoea or constipation, blood in stool, thin stool, and unexplained weight loss.

Adenocarcinoma is the most prevalent kind of colorectal cancer. The cells lining the large intestine are where rectal and colon adenocarcinoma in the digestive system develops. Ninety five percent of all cases of colorectal cancer are adenocarcinomas of the colon and rectum. These adenocarcinomas often begin as polyps, which is a growth of tissue. A particular kind of polyp known as an adenoma may turn cancerous. During a normal colonoscopy, polyps are frequently removed before they develop in to cancer. Adenocarcinoma has two different subtypes. They are mucinous adenocarcinoma and signet ring cell adenocarcinoma.

Mucus makes up about 60% of mucinous adenocarcinomas. Because of the mucus, cancer cells may spread and become more aggressive than in typical adenocarcinomas. Mucinous adenocarcinomas account for 10% to 15% of all rectal and colon adenocarcinomas. Approximately 1% of colon tumours are signet ring cell adenocarcinomas. Signet ring cell Adenocarcinoma is a type of aggressive cancer that gets its name from how it looks under a microscope. It can be more challenging to cure. The treatments for Adenocarcinoma include Surgery, Chemotherapy, Radiation therapy and Targeted therapy.

1.6.7 Primary colorectal lymphomas

This cancer arises in the lymphatic system, especially in cells known as lymphocytes and is a subtype of non-Hodgkin's lymphoma. White blood cells called lymphocytes aid the body in battling illnesses. Lymph nodes, bone marrow, spleen, thymus and the digestive tract are just a few of the organs where lymphoma can manifest itself. About 5% of lymphomas and 0.5% of all colorectal malignancies are primary colorectal lymphomas. This specific kind of colorectal cancer typically manifests later in life and is more prevalent in men.

1.6.8 Leiomyosarcomas

Leiomyosarcoma is a different type of sarcoma that basically means "cancer of the smooth muscle." The three layers of leiomyosarcoma-affected muscle found in the colon and rectum cooperate to move waste through the digestive tract. This uncommon subtype accounts for about 0.1% of all cases of colorectal cancer. Standard procedures for diagnosis typically involve a biopsy, blood testing and imaging studies. Surgery to remove the tumour is frequently the first step in treatment. Chemotherapy and radiation therapy are additional treatment possibilities.

1.6.9 Carcinoid Tumors

Neuro endocrine cells, a type of nerve cell that aids in the regulation of hormone production, are where carcinoid tumours form. These tumours are part of the neuroendocrine tumour subgroup of malignancies (NETs). Slow growing carcinoid tumour cells can

form in the lungs and or gastrointestinal tract. They represent 50% of all small intestinal tumours and 1% of all colorectal cancers, respectively. Gastrointestinal carcinoid tumours can be found in various ways depending on where they grow. For instance, an appendix tumour might only be discovered after it has caused appendicitis, at which point the appendix is removed. During routine examinations, rectal tumours may be found. Blood tests, urine tests, imaging scans, endoscopies and colonoscopies are only a few of the techniques performed to identify carcinoid tumours in the gastrointestinal system.

1.6.10 Colon and rectal melanomas

These are most frequently linked to skin cancer, but they can occur elsewhere, even in the colon or rectum, or they can move from the initial melanoma site to the GI tract. According to studies found in BMJ Case Reports, melanomas make up about 1% to 3% of all malignancies that arise in the digestive system. Due to their rarity, it is unclear how melanomas in the colon form. The diagnosis may include a biopsy and additional tests to identify whether the cancer originated in the colon or rectum or spread from another part of the body. Treatments include surgery, immunotherapy, radiation therapy and chemotherapy.

1.6.11 Colorectal squamous cell carcinoma

Less than 1% of colorectal cancer cases are primary squamous cell carcinomas of the colon and rectum, which are extremely

uncommon and it typically affects the oesophagus or anal canal. It was first noted by Herxheimer in 1907 and has been described as a tumour in which the squamous and glandular components are both malignant and able to metastasize.

1.7 Skin Cancer

Skin serves as a barrier between the human body and the outside world. It functions as a barrier to shield the body from UV radiation, toxins and infections. The epidermis is the skin's topmost layer (Fuchs *et al*., 2002). Different types of cells can be found in the epidermis, including keratinocytes, dendritic melanocytes, Merkel cells and Langerhans cells. The connective tissue beneath the dermis contains memory T-cells, mast cells, and dermal dendritic cells that can deliver antigens (Kupper *et al*., 2004: Dominique *et al*., 2010). Skin cancers are malignancies that develop on the surface of the skin. There are mainly two types of skin cancer.

1. Melanoma: Melanoma is a type of cancer that appears in the melanocytes of the skin.

2. Non melanoma: Any skin cancer that develops in the basal, squamous or Merkel cells is referred to as nonmelanoma skin cancer.

With more than four million cases of basal cell carcinoma identified each year in the United States, it is the most prevalent type of cancer.

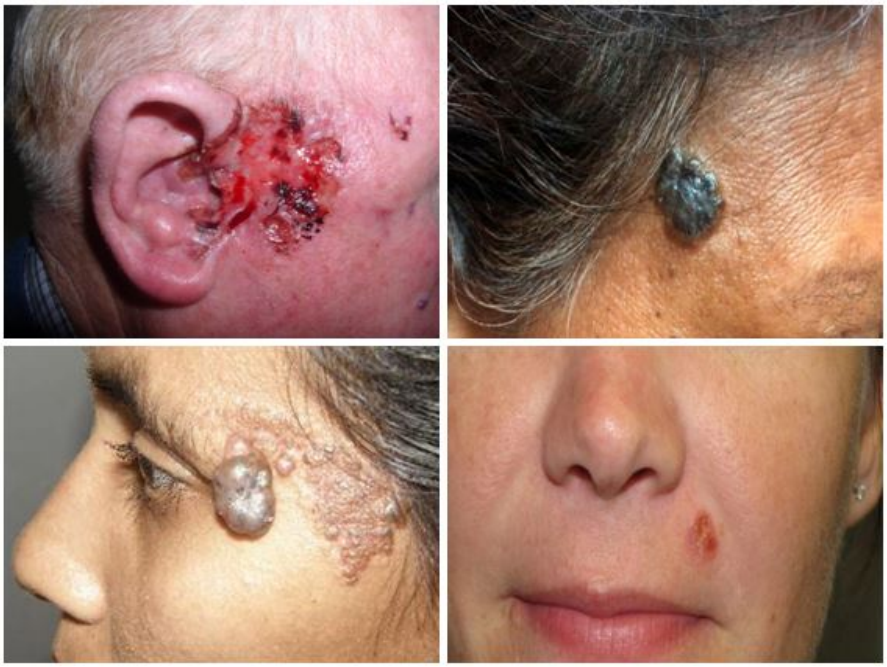
Basal-cell skin cancer (BCC) and squamous-cell skin cancer (SCC) are very rare skin cancers and are known as nonmelanoma skin cancers. BCC advances slowly and can harm surrounding tissue. It usually manifests as a painless elevated region of skin that is glossy but has little blood vessels running through it. Squamous-cell skin cancer has a higher proclivity for spreading. It shows up as a hard lump with a scaly surface, but it can also turn into an ulcer. Melanomas are the most severe of all cancers. A mole that has altered in size, form or colour, with irregular edges, multi-coloured, itchy or bleeds, is a sign. Approximately 2,000 Americans are diagnosed with the uncommon form of skin cancer known as Merkel cell carcinoma each year. The Merkel cell carcinoma is called an "Aggressive Carcinoma" because the likelihood of it spreading from the skin to another area of the body is high and it can recur after treatment. SEB (Surrogate Endpoint Biomarker) is crucial in determining the extent of a person's skin cancer. Cryosurgery, immunomodulation with imiquimod, 5FU, photodynamic treatment, and radiation are a few examples of new molecular therapeutic techniques for treating skin cancer.

1.7.1 Basal cell carcinoma

One of the three types of cells that make up the top layer of skin, basal cells is where this cancer develops. Basal cell carcinoma (Fig.1.4) can have a wide range of appearance; some examples include-A pink or white spot, an active sore, an oily bump, a scar, rounded growth that is elevated, a growth that oozes, bleeds, itches or crusts. They often affect the head and neck first, then the trunk and

extremeties. Although basal cell carcinoma seldom spreads and it is typically successfully treated, delay in care can result in problems.

Figure 1.4 Basal cell carcinoma



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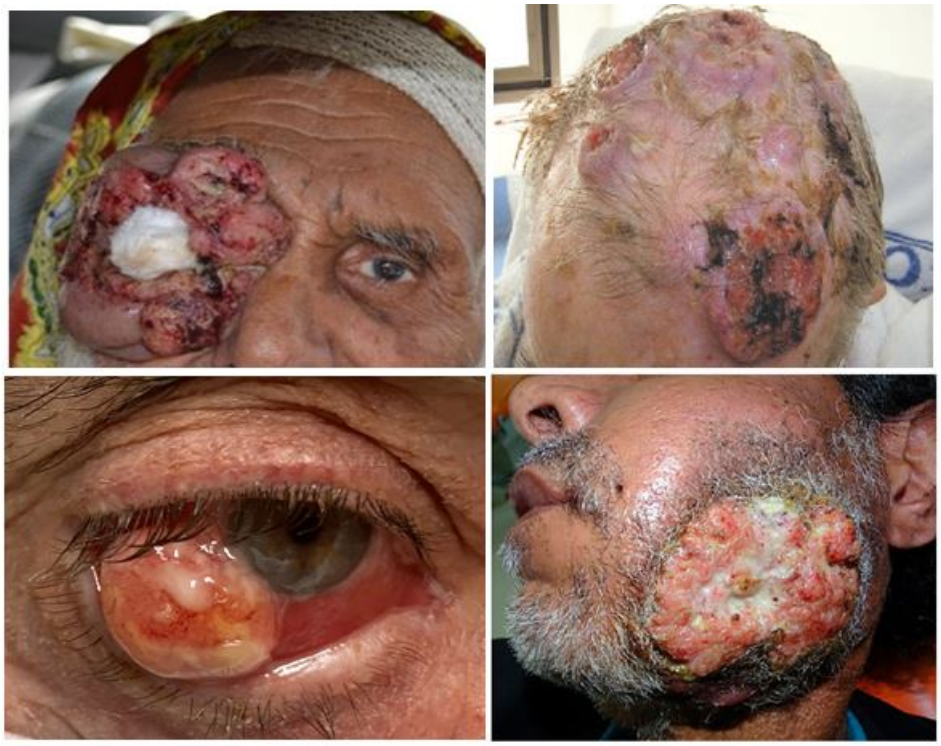
- https://commons. wikimedia. org/w/index. php?title=File:Basalioma. jpg&oldid=528388718.
- https://casereports. bmj. com/content/casereports/2016/bcr-2015-214166/F1. large. jpg
- https://stratumskin. com. sg/wp-content/uploads/elementor/thumbs/Basal-Cell-Cancer-prch01ma5e7cnyl3q5lik65f6lmtit2fo2nrjustts. jpg

1.7.2 Squamous cell carcinoma

In the flat squamous cells close to the skin's surface, squamous cell carcinoma (Fig.1.5) forms. Contrary to basal cell carcinoma, which typically develops on skin that has been exposed to the sun,

squamous cell carcinoma can also appear around the genital region and other parts of the body that have not been exposed to the sun. It may be seen as red scaly patch, skin that has growth and become tough, wart and increased growth combined with depression. Although squamous cell carcinoma is frequently seen and typically extremely treatable, it is crucial to get medical attention when any abnormalities in the skin appear. If treatment is postponed this type of skin cancer has the potential to spread and worsen.

Figure 1.5 Squamous cell carcinoma



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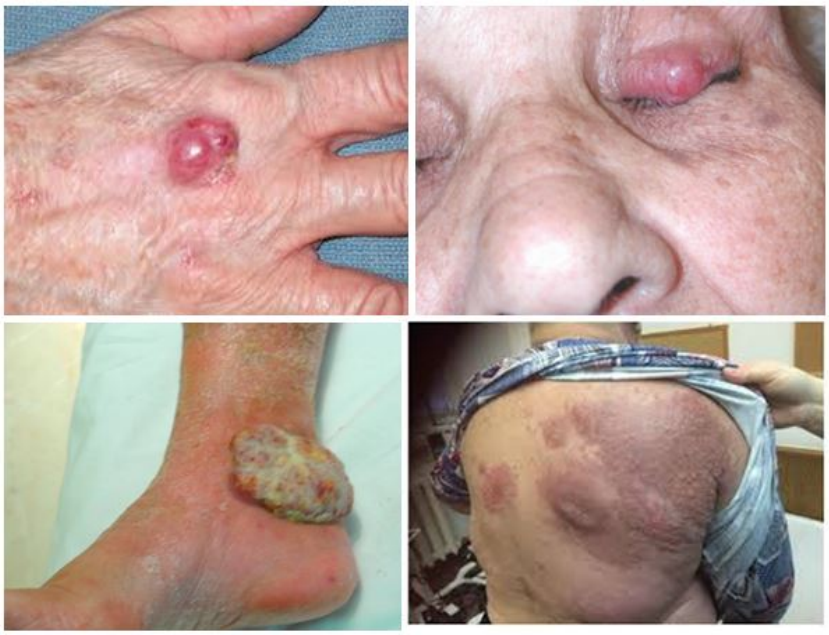
Merkel Cell carcinoma

It is an uncommon and deadly aggressive cancer. This type of cancer frequently manifests as a glossy pink, red, or bluish lump that grows swiftly. It develops in the Merkel cells, which are located well within the upper layer of the skin. Although dangerous, Merkel cell carcinoma (Fig.1.6) can often be successfully treated in many situations with early identification.

Figure 1.6 Merkel cell carcinoma



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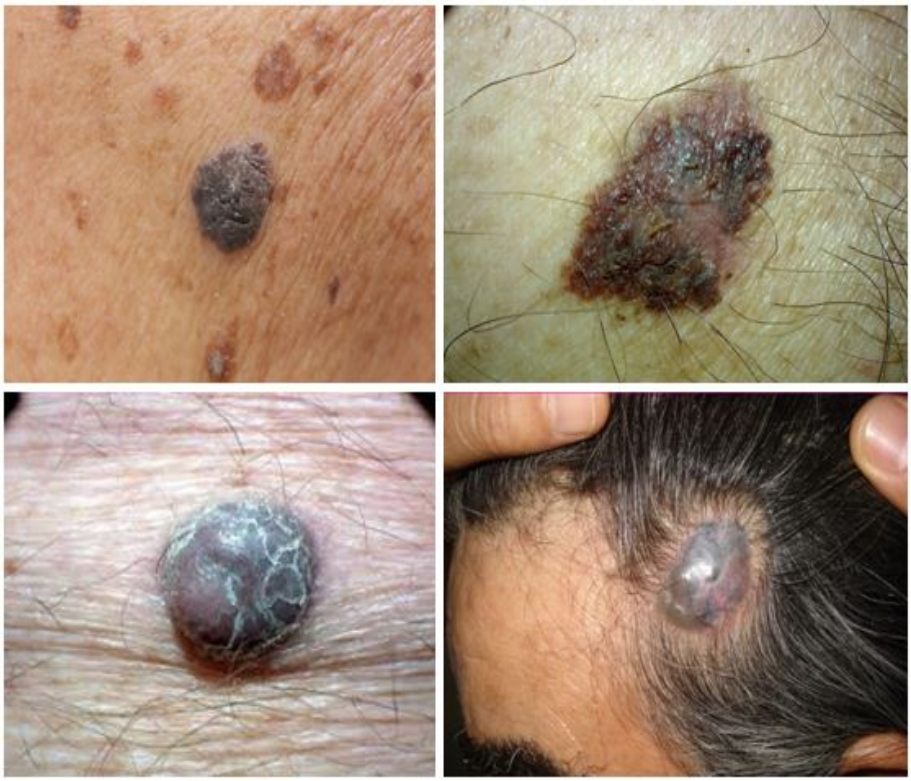
1.7.4 Melanoma

Melanoma (Fig.1.7) is a kind of cancer that develops in the melanocytes, the pigment-producing cells found in the epidermis of the skin. However, melanoma can also appear in parts of the body that aren't exposed to the sun due to damage produced by UV light from the sun and tanning beds. The symptoms and signs include the different sizes, shapes and colours of moles on the body; the colour ranges from tan to brown to black. It possesses an uneven border and is not a perfect circle and changes its colour or shape gradually. Though melanoma is an extremely dangerous kind of cancer, early identification helps many people survive longer despite the fact that melanoma skin cancer can manifest itself almost anywhere on the body; it is most frequently discovered on the face, back, arms and legs, which are exposed to the sun. The initial symptom that stands out could be an altered mole that already exists, skin that has recently developed. Researchers in the general medical field still don't fully comprehend the precise reasons for the cellular DNA mutations that result in the onset of melanoma skin cancer. But scientists have conclusively proven that being exposed to UV light from the sun and other sources, such as tanning beds, significantly raises the chance of developing melanoma..

Figure 1.7 Melanoma



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- https://encrypted-tbn0. gstatic. com/images?q=tbn:ANd9GgQ74s2UeeQBYL5yvJKldK2X14NaWMbt8U9IOw&usqp=CAU

However, not all melanomas are caused by exposure to UV light. Some melanomas, often known as hidden melanomas, appear in parts of the body that are never exposed to sunlight, including: between two fingers or toes (the lesion may resemble a subungual

hematoma, which is a collection of blood in the nail bed that may appear within a few days of a crushing injury; the main distinction between the two is that the nail discoloration from the former will gradually go away as the nail heals), on the sole of the foot or the palm of the hand, under the scalp's hair, within the digestive system, mouth, or nose, the genitalia inside the vagina, urinary tract or anus. Seborrheic keratosis is sometimes mistaken for melanoma. There is a significant distinction between seborrheic keratosis and melanoma, despite the tumours' similar appearance. In particular, seborrheickeratoses are benign growths that frequently appear as a result of ageing and are completely safe.

This is why a lot of scientists think hereditary and environmental variables work together to cause melanoma cancer. Other risk factors for melanoma skin cancer, besides UV light exposure include white skin, a history of sunburn, 50 or more moles (most adults have between 10 and 40 moles), a melanoma diagnosis in the family and a lowered immunological response. It's crucial to remember that people of all hues, including those with black and dark brown skin, can develop skin cancer. However, melanoma on black skin tends to occur less commonly than melanoma on white skin.

Skin (cutaneous) melanoma is the most prevalent form of melanoma cancer. Typically, the top layer of skin is where cutaneous melanoma first appears (epidermis). If left unattended, it may spread and infiltrate the dermis and hypodermis, two of the deeper layers of skin. The following are cutaneous melanoma subtypes; superficial

spreading melanoma, nodular melanoma, lentigomaligna melanoma, acrallegtiginous melanoma, desmoplastic melanoma and amelanotic melanoma. The other melanoma cancer types which are less frequent than cutaneous melanoma includes ocular melanoma (an eye cancer) and mucosal melanoma. A mucous membrane, such as the lining of the mouth, nose or digestive tract, can develop a malignancy called mucosal melanoma. Melanoma with an unknown primary cause (a tumour with no known cause that spreads throughout the body), paediatric melanoma (melanoma that develops in a child) are some other types. The degree of the tumour, the patient's general health and their personal preferences can all influence how their melanoma is treated. Surgery is frequently used to treat early-stage melanoma skin cancer. Occasionally, a biopsy might eliminate the entire melanoma, negating the need for further therapy.

1.8. Treatment procedures for colon and skin cancers

The various types of treatment procedures include

i) **Surgery:** The most frequent form of treatment for colorectal cancer is surgery, which can be either minimally invasive (such as the removal of a polyp during a colonoscopy) or, in extremely rare circumstances, total colon removal. Tumors, the area of the colon where the tumour was discovered, surrounding healthy tissue, and close-by lymph nodes are frequently removed during procedures for colorectal cancer. Excision of the malignant melanoma and any lymph nodes involved (as identified through a sentinel lymph node biopsy) is the major treatment protocol for melanoma.

ii) **Chemotherapy:** One of the most popular forms of cancer treatment is chemotherapy. These anticancer medications function by focusing on cancer cells that are actively developing either throughout the body or in a particular region of the body.

iii) **Radiation therapy:** It is a treatment that targets specific energy sources, such as X-rays and radioactive materials, to kill cancer cells, shrink tumours, and/or treat certain cancer-related symptoms. High-energy X-rays and other radiation therapies are used by medical professionals to harm cancer cells' DNA, either killing them or preventing them from proliferating or dividing. High-energy beams that target and destroy melanoma cells, such as X-rays or protons, are used in radiation therapy of skin cancer.

iv) **Targeted therapy:** A type of targeted cancer treatment, targeted therapy, is a type of chemotherapy. Targeted therapy directs medications to particular characteristics of cancer cells, in contrast to traditional or standard chemotherapy, which targets rapidly proliferating cells throughout the body, whether they are cancerous or not. The goal of conventional chemotherapy is to eradicate cancer cells that the body has already produced. Targeted therapies aim to obstruct or stop cancer cells from replicating and limit the growth of new cancer cells. Targeted therapies have a lower risk of killing normal cells than conventional chemotherapies because of how they operate differently. Therapy that specifically targets the melanoma's distinctive cellular features includes skin cancer treatment.

v) **Hormone therapy:** Hormone therapy is a type of cancer treatment that slows or prevents the growth of cancer cells that use hormones as a growth factor. It causes the tumour to shrink and slowdown in size. Other names for hormone therapy include hormonal therapy, hormone treatment, and endocrine therapy.

vi) **Immunotherapy:** Drugs that support the body's immune system in locating and destroying cancer are employed in this mode.

1.9 OBJECTIVES OF THE STUDY

Proteases are engaged in several of biological functions, including cell differentiation, motility, division, and death. They are crucial in intracellular protein degradation through a variety of mechanisms, including lysosomes. Research suggests that proteases may also be involved in the genesis and growth of tumours, which are mostly reliant on the availability of oxygen and nutrients. Hence PIs could be used successfully for treating cancer. Trypsin inhibitors are found in a wide variety of genera and species of plants. Research is now being conducted on the potential anti-carcinogenic properties of the inhibitors. According to certain studies, PIs can permanently limit the growth of cancerous cells. The mechanism is still unknown, though.

In the current study we focus on the isolation of trypsin inhibitors from some indigenous plant seeds. Our primary goal is the isolation, purification and characterization of the trypsin inhibitors from selected seeds. The subsequent aim is to investigate the

anticancer efficacy of the isolated trypsin inhibitors against skin and colon cancer cell lines. The specific objectives of the research are the following:

1. Collection and Screening of plant seeds of Malabar region for trypsin inhibitors (TIs)

Screen different plant seeds for the presence of trypsin inhibitory proteins and standardise the extraction protocol. Different extraction media will be used to extract the TIs as part of standardization and select the ideal one for the isolation of protein with highest trypsin inhibitor activity.

2. Isolation and Purification of effective trypsin inhibitors

Purification of trypsin inhibitor protein by ammonium sulphate fractionation, dialysis, ion exchange chromatography and size exclusion chromatography.

i. Ammonium sulphate Precipitation

The fractionation of TI protein will be done by ammonium sulphate precipitation as 0-30%, 30-60% and 60-90%. The fraction after Ammonium sulphate precipitation will be dialysed in a suitable dialysis bag against the same buffer. The fractions collected with highest TI activity will be further purified by Ion exchange chromatography.

Ion exchange chromatography using DEAE cellulose

The TI protein with the highest TI activity collected after ammonium sulphate precipitation will then be purified by DEAE cellulose ion exchange chromatography and the protein fraction with TI activity will be collected and pooled for the next step of purification. The specific inhibitory activity of the pooled fraction will also be calculated.

ii. Size exclusion chromatography

The pooled fractions with highest TI activity after ion exchange chromatography will be then subjected to gel filtration chromatography with Sephadex G75 as the resin. The collected fractions with highest TI activity will be used for further characterization and anticancer studies.

iii. Determination of Yield and fold of purification

Yield and fold of purification of Trypsin inhibitor protein will be calculated after each stages of purification.

3. Characterisation of trypsin inhibitor

The characterisation of TI protein is also one of the objectives which include molecular weight determination, molecular weight confirmation by MALDI-TOF analysis, determination of thermal and pH stabilities and determination of the type of inhibition.

i. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE analysis will be performed to determine the homogeneity and molecular weight of the purified enzyme.

ii. Activity staining by reverse zymography

Activity staining by reverse zymography will be conducted to determine the presence of a protein with trypsin inhibitory activity, which will then be confirmed using activity staining by reverse zymography.

iii. Molecular weight confirmation by MALDI-TOF/TOF analysis

The molecular weight of the purified TI protein will be confirmed by MALDI-TOF analysis.

iv. Determination of Thermal Stability of TI protein

Thermal stability of the purified TI protein will be determined by TI activity assay after keeping the samples at different temperatures (37°C, 40°C, 60°C, 80°C, and 100°C).

v. Determination of pH Stability of TI protein

The pH stability of the TI protein also will be determined by TI activity assay after keeping the samples at different pH conditions.

vi. Determination of Inhibitor constant from Dixon Plot

By analysing the Dixon plot, the nature of inhibition can be found. Hence the method will be used to know the type of inhibition, whether it is competitive, non-competitive or uncompetitive.

4. Determination of the potential of isolated trypsin inhibitor in cancer therapy using cell lines

The anticancer potential of purified TI protein will be analysed against colon cancer (HT29) and skin cancer (A431) cell lines. The normal cell lines (L929) also will be analysed to check the cytotoxicity of the purified protein. Soybean trypsin inhibitor (STI) will be used as a standard (for comparison) protein for the anticancer potential analysis.

i. Direct microscopic observation

The images after treatment with different concentrations of the purified TI protein can reveal if there are significant morphological changes occurs. Detectable changes in the morphology of the cells such as rounding or shrinking of cells, granulation and vacuolization in the cytoplasm of the cells will be considered as indicators of cytotoxicity.

ii. MTT Assay of cells lines (L929, A431, and HT29 cells) after treatment with the TIs

Cytotoxicity will be determined by the MTT assay in both the cancer cell lines and normal cell lines. IC_{50} values will be calculated,

which reflect the comparative effectiveness of the TIs in inhibiting the proliferation of skin and colon cancer cells.

iii. LDH leakage Assay of cells lines (L929, A431, and HT29 cells) after treatment with TIs

The anti-proliferative activity of the TIs will be further confirmed by measuring the LDH leakage from the cancer cells after treatment with various concentrations of TIs.

iv. Neutral red uptake assay

Neutral red uptake assay also will be performed to detect the anti-proliferative activity. Cytotoxicity tests with neutral red will be conducted based on how well living cells can absorb and bind neutral red.

v. Comet Assay for the Evaluation of DNA Damage after Treatment with TIs

The detection of DNA damage will be done and evaluated by Comet assay after treatment with IC_{50} concentrations of the purified TI proteins using standard protocol. The tail length and comet length will be measured.

vi. Annexin V-FITC Apoptosis of Skin Cancer Cells by Flow Cytometry after treatment with TIs

The Annexin V-FITC apoptosis of skin and colon cancer cell lines will be performed by flow cytometry analysis. The flow cytometry analysis after treatment with IC_{50} concentrations of the purified TIs can reveal live, apoptotic, and necrotic cell data.

Chapter 2

Review of Literature

2.1 Proteases and their Inhibitors

Proteases are a wide and varied collection of hydrolytic enzymes that are categorised according to their region of activity, the makeup of the enzyme active site and particular reaction processes. They are pervasive in biology and play a role in a variety of physiological and biochemical processes that affect how cells and organisms function. These processes include nutrient uptake; protein synthesis and breakdown, adaptation, regulation, growth, disease and death (OP Ward, University of Waterloo, Waterloo, ON, Canada).

Proteases are categorized into different types based on a variety of factors like the sort of reaction catalysed, the catalytic site's chemical composition, the link between evolution and structure, functional group that is present at the active site, by many scientists (Barett *et al*., 1994; Hartley, 1960; Menon *et al*., 1987; Argos, 1987; Rawlings *et al*., 1993).

Several secondary metabolites and phytochemical substances found in plants are widely investigated at the biochemical and molecular genetics levels and used extensively in the field of human health care. Richardson and Weder reported that the three primary plant groups of Fabaceae, Poaceae, and Solanaceae, have produced the most researched PIs (Richardson, 1991; Weder, 1981). Some studies have also documented PIs from families like Malvaceae, Rutaceae, Poaceae, and Moringaceae (Srikanth *et al*., 2016). Plant PIs (PPIs) are typically tiny proteins that have been found in plant aerial parts as well as storage tissues like tubers and seeds. They have primarily been

reported as occurring in storage tissues (De Leo *et al*., 2002). By direct testing or expression in transgenic agricultural plants, many PIs have been demonstrated to behave as defensive chemicals against pests, and a body of evidence for their function in plant defence has continued to grow (Krattiger, 1997). For viral and parasite infections, cancer, inflammatory, immunological and pulmonary disorders, numerous PIs have demonstrated therapeutic potential in preclinical studies in animals and early human clinical trials (Hugli, 1996; Fath *et al*., 1998). The *Bauhinia bauhinioides* kallikrein-inhibitor (BbKI), an 18-kDa protein, was identified by Oliva *et al*. (1999) from the seeds of *B. bauhinioides*. A 7.7 kDa buckwheat inhibitor (BWI)-1 protein that was isolated from common buckwheat seeds (Dunaevsky *et al*., 1997). Huang *et al*. (2007) reported the anti-proliferative action and the mechanism of a 22 kDa trypsin inhibitor (TI) protein from the storage roots of the sweet potato [*Ipomoea batatas* (L.)]. High amounts of PIs have been reported in numerous plants belonging to the Solanaceae family, despite the fact that they are present in plants from a variety of taxonomic groupings (Plate *et al*., 1993).

2.2 Role of Proteases in Cancer

Many proteases are involved in cancer progression and metastasis. Leukocytes and epithelial cells produce cysteine cathepsin proteases, which have recently come to be recognised as key mediators of the development of cancer (Turk *et al*., 2004). Trypsin, a well-known digestive serine protease that promotes invasion, proliferation and metastasis, has also been linked to a number of malignancies. The

prognosis and length of disease-free life are poor for colorectal cancers that express trypsin. Trypsin digestion of type I collagen may directly encourage the invasion of the basal membrane by cancer cells. Trypsin activates matrix metalloproteases (MMPs), which are known to promote invasion and metastasis and co-express with them (Nyberg *et al*., 2002). The use of PIs as anticancer medications is suggested by the role of proteases in cancer. According to Yamamoto *et al*. (2003) and Soreide *et al*. (2006), trypsin triggers proliferation, invasion and metastasis and is involved in the development of colorectal cancer. A poor prognosis and a shorter disease-free life are also associated with colorectal tumours that express trypsin (Rakash *et al*., 2012).

Trypsin from the serine protease group, which has been evaluated for many diseases, was the major target enzyme of many of naturally occurring PIs that were described from various plant species (Tamir *et al*., 1996; Majumdar, 2013). Troncoso *et al*., (2003) isolated a trypsin inhibitor PDTI from the seeds of *Peltophorum dubium* with a molecular mass of 20-22kDa. The mass, cysteine concentration, and number of reactive sites of the various serine protease families vary (Lingaraju and Gowda, 2008). A serine PI (trypsin inhibitor) was isolated from the seeds of *Sinapis alba* (L.) and it differed structurally from other families of plant-derived serine PIs and had more cysteine and glycine residues (Menegatti *et al*., 1992).

2.3 Protein Purification

Purwanto *et al*. testified that the process of purification may first separate the mixture's protein and non-protein components before

separating the desired protein from other proteins. The most time-consuming step in protein purification is often separating one protein from another. Prior to utilising a more complex process for protein purification, it has historically been conventional to perform fractionation, for instance, by using ammonium sulphate as a precipitating agent (Purwanto *et al*., 2016). Ammonium sulphate precipitation is a typical first step in bulk separation of separation techniques to isolate a typical first step in bulk separation techniques to isolate proteins (Wingfield, 2001). This is achieved by progressively adding ammonium sulphate and collecting the various protein precipitate fractions. After that, the ammonium sulphate can be removed using dialysis.

Ion-exchange chromatography is an established method that has been extensively utilised for the separation of peptides, proteins, nucleic acids and related biopolymers which are charged molecules in different molecular sizes and molecular nature (Okada, 1998; Bruch *et al*., 2009). This method is widely used for the characterization of therapeutic proteins. It is a potent method for the qualitative and quantitative assessment of charge heterogeneity. The ability to analyse a wide variety of compounds in the biotechnology, medical, agricultural, environmental and other industries has enhanced the appeal of ion exchange chromatography in recent years (Bhattacharyya *et al*., 2012). DEAE (diethylaminoethyl) is a typical functional group available for anion exchangers. For the best separation, the elution buffer's concentration can also be changed. Reducing agents or other buffer components necessary for protein stability should also be

included (Duong-Ly *et al*., 2014). Quaternary aminoethyl (QAE), diethylaminoethyl (DEAE) and quaternary anion are the most common functional groups available for anion exchangers (Q). Carboxymethyl (CM), sulfopropyl (SP) and methyl sulfonate are the functional groups in cation exchange beads (Duong-Ly *et al*., 2014).

By filtering molecules over a gel, size exclusion chromatography (SEC) separates molecules according to their size. Wheaton and Bauman recognised the earliest instances of size-based liquid chromatographic separations (Wheaton and Bauman, 1953). The biological activity of the macromolecule is preserved when proteins are separated by size-exclusion chromatography (SEC) in their natural environment (Lathe and Ruthevin 1955, 1956). Because of its speed and reliability, SEC has been widely used for routine and validated analyses (Yu *et al*., 2008). Singh *et al*., in 2017 reported that s ize exclusion chromatography using a Sephadex G-75 gel filtration column was performed to separate the preheated extract that contained SOSPI (serine PIs from squid ovary). A Bowman-Birk-like trypsin and chymotrypsin inhibitor of 17.8kDa from tepary bean (*Phaseolus acutifolius*) PI (TBPI) was purified using ion exchange, hydrophobic interaction and size exclusion chromatography (Maiman *et al*., 2019).

Dixon plot was used to determine the dissociation constant (K_I) value and mechanism of inhibition of PI from *Solanuma culeatissimum* Jacq. (SAPI).The SAPI exhibits competitive inhibition, as seen by the two lines corresponding to each substrate intersecting above the x-axis, a feature of competitive inhibition (Krishnan *et al*., 2015). Howard *et*

al. (2006) reported that trypsin was assayed with varying SSTI concentrations (at two substrate concentrations) under standard assay conditions, and the K_I value was calculated by Dixon's plot. The Dixon method of visualising enzyme inhibition data is used when the inhibitor and enzyme interaction is more complex than in cases of classical and non-competitive inhibition (Butterworth *et al*., 1973).

A comparable technique for detecting proteinase inhibitors is reverse zymography. Notably in the field of medicine, reverse zymography techniques are useful for isolation and characterization of natural PIs (Herron *et al*., 1986; Michcaud *et al*., 1996). When the proteins have been re-natured, the gel is treated with metalloproteinases, which degrade the substrate that has been integrated into the gel. When stained, inhibitors appear as black zones of inhibition against a clear background (Hawkes *et al*., 2010). Tissue inhibitors of metalloproteinases (TIMPs) are detected using reverse zymography in a variety of experimental materials. Zymography is a method of electrophoretic separation of proteases in a polyacrylamide gel-containing substrate under non-reducing conditions (Choudhary *et al*., 2022). The reverse zymography approach can be used to find inhibitors of a range of proteolytic enzymes that are both low- and high-molecular-weight in tiny amounts of natural materials (Le *et al*., 2004).

2.4 *Artocarpus hirsutus*

Extracts from aerial and underground components from *A. hirsutus*, in addition to fruits and seeds, have been traditionally used to

cure a variety of illnesses including diabetes, diarrhoea, dermatitis, malarial fever, asthma, tapeworm infection, anaemia and many others. Additionally, the seeds are typically fried as a snack. In earlier structures, notably in Kerala, timber of *A.hirsutus* was extensively used to build ceilings, door frames and furniture and to construct the fabled snake boats of Kerala. It is well known that *A.hirsutus* is a great source of bioactive secondary metabolites such as xanthones, triterpenoids, stilbenes and flavanoids. After the spiny outer skin is removed, its ripe fruit can be consumed as well and are typically fried as a snack. Its bark treats diabetes, anaemia, malaria, dermatitis, diarrhoea, acne, tapeworm infection and ulcers. When it comes to the availability of complex phytochemical components, antibacterial activity, anti-helminthic, anti-inflammatory and antiviral properties, *A.hirsutus* has made significant contributions to the study of phytochemistry (Monisha *et al*., 2017). Although the leaf, bark and fruit extracts of *A.hirsutus* were subjected to phytochemical screening, only a few research has been conducted to determine the health advantage of its seeds. Methanolic extract of the bark has anti-ulcer properties. It guards against stomach ulcers brought on by pylorus ligation in experimental mice. Rats with their pylorus tied had less gastric secretion, acidity and ulceration after receiving the test substance (Dibinlal *et al*., 2013). The anti-diabetic and antioxidant effects of *A.hirsutus* seed extract support its use as medication to treat diabetes mellitus and the oxidative damage (Sireesha *et al*., 2016). *A.hirsutus* bark infusion was used to treat minor skin fissures and pimples (Varma *et al*., 2008).

2.5 *Bauhinia acuminata*

Bauhinia acuminata is found in tropical Southeast Asia and is a flowering shrub species. Its leaves, bark, roots, flowers and seeds are used in traditional medicines. During a phytochemical analysis of the leaves of *Bauhinia acuminata*, researchers discovered starch, alkaloids, flavonoids, tannins, reducing sugars, amino acids and lignins (Sudipa Nag *et al*., 2013). Various pathogenic gram-positive and gram-negative bacteria were sensitive to the crude extract from seed kernels of *B.acuminata*, with *Bacillus subtilis* being the most sensitive and *Pseudomonas aeruginosa* being the least sensitive (Karaket *et al*., 2011). The substantial antioxidant and total phenolic content activities found in *B.acuminata* leaves indicate that various oxidation related illnesses are treated naturally with anticancer and anti-microbial preparations from the plant (Md. Reyad-ul-Ferdous *et al*., 2014). A study observed that sodium arsenite induced toxicity in adult albino rats could be reduced by powdered *B.acuminata* stem bark. Significantly less arsenic accumulated in tissues, hair and faeces after treatment with stem bark powder. The results demonstrated that induced arsenicosis could be ameliorated by the oral administration of *B.acuminata* stem bark powder (De A *et al*., 2016). Another study assessed *B.acuminata'*s ability to protect the liver from damage caused by carbon tetrachloride (CCl_4) in a single dose, along with olive oil caused hepatic damage. The reduction of SGOT, SGPT, ALP and bilirubin levels caused by CCl_4 as well as a confirmatory histological analysis of liver tissues indicated its hepatoprotective action (Jamadar *et al*., 2021). Various quantities of *B.acuminata* leaf extracts were

tested for their anthelmintic effectiveness against housefly worms in aqueous and ethanolic extracts. Tannins and saponins were found in both extracts after a phytochemical screening. Results indicated considerable dose-dependent anthelmintic action by paralysis and death indicators. In comparison to the water extract, the ethanol extract demonstrated more activity (Radha Prabhu *et al*., 2018).

2.6 *Garcinia gummi-gutta*

The Clusiaceae family includes more than 300 species of the genus Garcinia, which is indigenous to Asia and Africa and includes species like *Garcinia cambogia (Gummi-gutta)*, *Garcinia mangostana* and *Garcinia atroviridis* (Hemshekhar *et al*., 2011). According to studies, garcinol (40-200mg/kg) oral dosing prevented rats from developing stomach ulcers caused by indomethacin (Yamaguchi *et al*., 2000). Additionally, studies have demonstrated that the commercially available concentrated syrup, cold aqueous and hot (cooked) aqueous extracts of *G.gummi-gutta* are efficient at neutralising free radicals (Preuss *et al*., 2004). Commercially, the rinds of the *G.gummi-gutta* fruit are used to make concentrated syrups that, when properly diluted, can be used to make ready-to-drink chilled health beverages, particularly during the off-season. Additionally, the rinds are used by the Goan locals to make wine. In order to be utilised as an acidulant in traditional curries, dried rinds are ground into powder and sold (Nayak *et al*., 2010). The effectiveness of *G.gummi-gutta extracts* as weight-loss aids was examined in a systematic review and meta-analysis of randomised clinical trials (Onakpoya *et al*., 2011). By acting as a

selective serotonin reuptake inhibitor, hydroxycitric acid (HCA) from *G.gummi-gutta* raises serotonin levels and increases the possibility of neurotransmitter poisoning. Irritability and agitation were the main symptoms. The symptoms subsided after the supplement was stopped and all the changed metrics went back to normal (Lopez *et al*., 2014; Hendrickson *et al*., 2016).

2.6 *Syzygium cumini*

India and the East Indies are *Syzygium cumini*'s original home countries. Its habitat extends from Myanmar to Afghanistan and other regions like Thailand, the Philippines and Madagascar. It is found all over India up to an elevation of 1800 m. *S. cumini* is a member of the Myrtaceae family, which also includes tannins, flavonoids, essential oils, gallic acid, betulic acid, malic acid and oxalic acid. The fact that *Syzgium cumini* is so commonly used in traditional medicine shows how significant it is pharmacologically. 75 percent of the total fruit is made up of the edible pulp. Ca, Mg, P, Fe, Na, K, Cu, S, Cl, vitamin C, vitamin A, riboflavin, nicotinic acid, choline and folic acid were among the reported minerals and vitamins. The National Institute of Science Communication, Council of Scientific and Industrial Research reported in "Wealth of Indian", A Dictionary of Indian Raw Materials and Industrial Products, that the main sources of sweetness in ripe fruit of *Syzygium cumini*, which has no trace of sucrose, are glucose and fructose. The predominant acid, accounting for 0.59 percent of the fruit's weight, is maleic acid (Ivan, 2006). Additionally, there is a small amount of oxalic acid. The fruits' sourness is caused by tannins,

particularly gallic acid (Ivan, 2006; Evans, 2007). It is present in edible pulp, seed and bark. Tannins are also effective at preventing ulcers and protecting the digestive system (Evans, 2007; Kokate, 2008). One or two cyaniding diglycosides are responsible for the fruit's purple colour (Ivan, 2006; Margaret *et al*., 2015). Pentacyclic triterpenoid tannins are present in bark portion. Gallitanins, Essential oil, Terpenes, 1-limonene, Dipenten are present in the seed and leaves (Sah *et al*., 2011). Both biochemical and histological investigations show that the seed's methanolic extract has hepatoprotective effects against carbon tetrachloride-induced damage (Sisodia & Bhatnagar, 2009).

2.7 *Thevetia neriifolia*

Thevetia neriifolia (*Thevetia peruviana*) is a medicinal plant with a wide range of pharmacological properties; comparatively a few scientific researches have been done on it. The plant, also referred to as yellow oleander, is a member of the Apocynaceae family. The plant is indigenous to Central and South America, particularly Mexico, Brazil and the West Indies, but it is now widely grown as a decorative plant across the tropical regions, including India and Sri Lanka. This plant contains numerous kinds of secondary metabolites including alkaloids, flavonoids, steroids, terpenoids, tannins, cardiac glycosides and saponins. In the plant's leaves, flowers, fruits, seeds and bark numerous pharmacological properties have been discovered by a number of studies. The plant poisons from *T.neriifolia* was evaluated for potential applications in biological pest management. Yellow oleander paint

with antifungal, antibacterial, and antitermite characteristics was created using *Thevetia peruviana* seed oil (Kareru *et al*., 2010). The isolated chemical's anti-inflammatory properties were assessed using an *in vitro* approach, and the study's findings showed that the isolated compound displayed a biphasic feature (Thilagavathi *et al*., 2010). Rat spermatogenesis was decreased by *T. peruviana*, suggesting its potential as a herbal male contraceptive (Gupta *et al*., 2011). *T. peruviana* is a plant whose milky sap contains a substance called thevetin that is used as a heart stimulant but which, like all sections of the plant, is exceedingly deadly in its natural state, especially the seeds. The ethanol-extracted leavesof *T.neriifolia* was screened for their antidiarrhoeal, antimicrobial and cytotoxic effects (Hassan *et al*., 2011). Tachycardia, arrhythmia, paralysis, ataxia and disorientation are examples of poisoning symptoms that suggest significant cardiac, neuromotor and mental dysfunction. Aqueous kernel extract caused speedier death in rats administered by injection within 10 h than aqueous leaf or stem bark extracts, which caused death to occur after 260h in the rats (Oji and Okafor, 2000). *Escherichia coli*, *Staphylo cocccus aureus*, *Bacillus subtilis* and *Candida albicans* were all inhibited by the paint made from *Thevetia peruviana* (Pers.) Schum.oil extract" in a concentration-dependent manner (Kareru *et al*., 2010). *Thevetia neriifolia* bark was tested for *in vivo* antidiabetic action in streptozotocin-induced diabetic rats and it demonstrated considerable effectiveness in a concentration-dependent manner (Gogoi and Bhuyan, 2017). Changes in the total, free amino acids, DNA and RNA protease activity and acid and alkaline phosphate levels were observed

in the muscle, liver, gonadal tissue of experimental fish after exposure to sub-lethal dosage of this plant's acetone-leaf and bark extracts in laboratory settings (Singh *et al*., 2010).

2.8 PIs and Cancer

A set of disorders known as cancers are linked to unnatural cell proliferation. Uncontrolled and unrestrained cell multiplication is referred to as cancer. Clinically, it manifests as a growth. It is one of the most terrible diseases in the world and if cancer incidence and mortality grow by 1% annually, there might be 26.4 million additional cancer patients, 1.7 million cancer deaths annually, and 80 million individuals with cancer within five years of diagnosis by 2030 (Schomall *et al*., 2009).According to Jeremy *et al*., (2021) cancer is the second leading disease, and multiple strategies are needed to prevent, control, and treat it.

A malignant tumour has the ability to spread to distant areas, harm nearby structures, and ultimately lead to death. Not all cancers result in death; some tumours are curable with the right diagnosis and care. According to Fields *et al*. (2012), naturally occurring plant-based BBI is regarded as a therapeutically significant possibility for the treatment of a number of disorders, particularly in the area of cancer prevention. A putative chemopreventive for human cancer, the Bowman-Birk inhibitor (BBI) is a serine PI developed from soybeans (Armstrong *et al*., 2000). Investigations on chymostatin, a highly specific inhibitor of chymotrypsin and a soybean extract containing the Bowman-Birk inhibitor (BBI) to suppress dimethyl hydrazine-induced

colon carcinogenesis when added to the diet of mice (Billings *et al*., 1990) were reported. BBI concentrate has undergone significant research in both in vitro and animal carcinogenesis model systems. In numerous settings, a BBI has been demonstrated to suppress animal carcinogenesis without producing negative side effects (Kennedy, 1993).

The growth of tumours is inhibited by many plant PIs, in addition to their ability to stop the spread of cancer cells. As a result, PIs are emerging as potentially effective therapeutic strategies for cancer treatment (De Clerck *et al*., 1994). Studies on various types of cancer are going on worldwide. PIs have undergone significant testing in recent years as therapeutic agents, primarily to treat various forms of human cancer (Srikanth *et al*., 2016). The main aim of the present study is to isolate suitable trypsin inhibitors from selected, easily available plant seeds and to detect their activity against the growth of skin and colon cancer cell lines.

Chapter 3

Screening of plant seeds for trypsin inhibitory activity, extraction and isolation of TIs from selected seeds

Proteases and PIs are widely distributed in many plants, predominantly in the seeds. Various plant species represent the sources of many biochemical compounds which have numerous pharmacological activities. The compounds could be obtained in pure forms to develop a product of interest. The isolation of such compound in pure forms takes various steps before it reaches commercially to the society.

Trypsin is a serine protease enzyme that hydrolyses proteins with substrate specificity based on positively charged lysine and arginine side chains. Trypsin inhibitors are serpins present in many plant and animal species. They can act as suicide inhibitors. Suicide inhibition or suicide inactivation is an irreversible type of enzyme inhibition in which the mechanism-based inhibition happens when an enzyme binds an analogue of a substrate and creates an irreversible complex with it by a covalent bond during a typical catalytic reaction. Previous research by various scientists showed that over expression of trypsin promotes the growth of different cancer cells *in vitro*. Hence the discovery of a highly active trypsin inhibitor of plant origin may be a helpful way to develop a cost-effective drug for cancer treatment.

3.1 OBJECTIVES

The aim of this part of the study is to screen different plant seeds for the presence of trypsin inhibitory proteins and to isolate TIs from those plant seeds that prove to be potent sources. Selection of suitable solvent for extraction which gives maximum recovery of TI activity and total protein is our subsequent aim.

3.2 MATERIALS AND METHODS

The materials and methodology for the extraction, screening, and isolation of the TI proteins were done according to the protocols described in detail as follows.

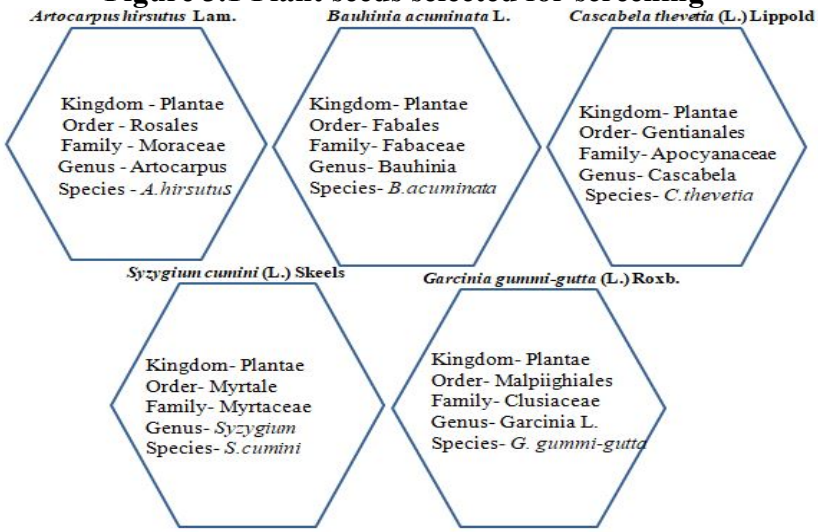
3.2.1 Materials

BAPNA (Nα-Benzoyl-D, L-arginine p-nitroanilide hydrochloride) and Trypsin was purchased from Sigma Aldrich, USA. All other chemicals including n-Hexane, tris-(hydroxymethyl) aminoethane, DMSO (Dimethyl Sulfoxide) and BSA was procured from SRL, India. Whatmann No. 1 filter paper was purchased from Himedia, India.

3.2.2 Collection of Plant Seeds

Seeds of five different plants were selected for the study. The selection was made on the observation that cattle usually reject to feed on plant parts rich in trypsin inhibitors. The seeds selected for this study come under this category of cattle deterrents; hence may contain trypsin inhibitors. The seeds (details are given in Fig.3.1) were collected from different villages of Calicut and Malappuram Districts, Kerala. Seeds of *Bauhinia acuminata* and *Cascabela thevetia* were collected from Thamarassery, Calicut, that of *Garcinia gummi-gutta* and *Artocarpus hirsutus* from Engapuzha, Calicut District and the seeds of *Syzygium cumini* from the campus of the University of Calicut, Malappuram District.

Figure 3.1 Plant seeds selected for screening



The samples were identified by Dr. A. K. Pradeep Kumar, Assistant Professor, Department of Botany, University of Calicut. All the Specimens were identified and deposited at the Calicut University Herbarium (CALI) with the following accession numbers (Table 3. 1). In terms of holdings among Indian University herbaria, the CALI, founded in 1968, is at the forefront.

Table 3.1 Accession numbers of the selected plants in the Herbarium CALI

Sl. No.	Plant name	Accession Number
1	*Artocarpus hirsutus* Lam.	6989
2	*Cascabela thevetia*	6990
3	*Bauhinia acuminata* (L).	6991
4	*Syzigium cumini* (L.) Skeels	6992
5	*Garcinia gummi-gutta* (L.)Roxb.	6993

All the five plant species selected are seasonal and locally available. All the seeds were collected in bulk, washed, dried under shade and stored with proper labelling until use. Their images are given in Fig. 3.2.

Figure 3.2 Plants and their seeds selected for screening

3.2.3 Sample Preparation

Matured seeds were collected in bulk and washed properly with running tap water and then with distilled water. The washed seeds were kept under shade until dry. Dried seeds were powdered using a mixer grinder to a particle size of 20 meshes. The seed powder was defatted using n-hexane at the ratio 1:5 (w/v) for 10 min and filtered through a Whatmann No. 1 filter paper. This procedure was repeated three to five times until total removal of residual oil in the powdered sample. The defatted sample was air-dried until dry and free of hexane odour. Later the seed flour was de-pigmented using acetone at the ratio 1:3 (w/v), washed and allowed to dry in air. It was then kept in a sealed container and stored at -20°C until use.

3.2.4 Extraction of Trypsin Inhibitor

The extraction procedure followed was as reported by Maggo *et al*., (1999) with slight modifications. The solvent used for extraction was 50mM Sodium phosphate buffer at pH 7.6, which has been reported as one of the most effective solvents for TI extraction (Marconi *et al*., 1993; Hajela *et al*., 1999; Kansal *et al*., 2008; Deepika *et al*., 2010). The extraction was done by continuously stirring the powered sample for 4h using a magnetic stirrer under ice cold condition in the ratio of 1:5 (w/v) in the solvent. The crude extracts of all the five seeds were then subjected to centrifugation in a cooling centrifuge at 10000xg for 30 min. The supernatant collected was stored at -20°C until used.

3.2.5 Trypsin Inhibitory Activity Assay

Trypsin inhibitory activity was assayed according to the procedure described by M.L Kakade *et al*., (1974).

a. Reagents

i. Tris buffer (0. 05M, pH 8. 2)

ii. 40mg of BAPNA (Nα-Benzoyl-D, L-arginine p-nitroanilide hydrochloride) dissolved in minimum volume of DMSO (Dimethyl Sulfoxide) was diluted to 100mL with Tris buffer. The BAPNA solution was prepared fresh and kept at 37°C.

iii. 4mg trypsin was dissolved in 0.001M HCl and made up to 200mL with distilled water.

b. Procedure

To the extracted samples pre-incubated with trypsin, BAPNA (pre-incubated at 37°C for 10 min) was added to start the reaction. After incubating at 37°C for 10 min, 0.25mL 30% acetic acid (v/v) was added to terminate the reaction. The reaction mixture was centrifuged at 10000xg for 3min. Activity of trypsin was measured by the absorbance at 410nm due to p-nitroaniline released. One trypsin unit (TU) is arbitrarily defined as an increase of 0.01 absorbance units at 410nm per 10mL of the reaction mixture under the conditions used herein. Trypsin inhibitory activity is expressed in terms of trypsin units inhibited (TIU).

3.2.6 Confirmation of proteinacious nature by Trichloroacetic Acid (TCA) Precipitation

The supernatant collected after extracting the sample with 50mM Sodium phosphate buffer (pH 7.6) was subjected to TCA precipitation. The proteinaceous nature of the TIs was confirmed by precipitating the samples with TCA and estimating the TI activity of the precipitate and the supernatant individually.

a. Reagent

Trichloroacetic acid (TCA) 100% [w/v]

b. Procedure

1 volume of TCA stock was added to 4 volumes of protein sample (250µL of TCA to 1mL of the sample) in a tube with a 1.5mL maximum volume.The tube was incubated at 4°C for 20 min. The tubes were centrifuged at 10000xg for 15 min. The protein pellets and supernatant were collected separately. Pellets were washed with 200µL cold acetone, washed two to three times and dried until the removal of acetone. The dried pellets were dissolved in 50mM Sodium phosphate buffer (pH 7.6) and the TI activity of both the supernatant and dried pellets (dissolved in 50mM Sodium phosphate buffer with pH 7.6) was determined separately. TI activity of both the supernatant and dried pellets (dissolved in 50mM Sodium phosphate buffer with pH 7.6) was determined separately.

3.2.7 Selection of ideal solvent for TI extraction

Since the seeds of *A.hirsutus* and *G.gummi-gutta* showed significant TI activity, further studies were conducted with them only.

For the extraction and best recovery of trypsin inhibitor, different extraction solvents (Distilled water, 0.01M NaOH, 0.02M NaOH, 0. 15M NaCl, 0.30M NaCl and 50mM Sodium phosphate buffer) were used as a part of standardization. The TI activity of the samples in each buffer was examined.

Defatted seed powder was weighed and taken in a beaker with the solvents (Distilled water, 0.01M NaOH, 0.02M NaOH, 0.15M NaCl, 0.30M NaCl and 50mM Sodium phosphate buffer) in the ratio of 1:5 (w/v) and stirred continuously for 4h under cold condition. It was then centrifuged for 30 min at 10000xg in a cold centrifuge. The supernatant was collected and used for further studies; the sediment portion was discarded. The TI activity of the supernatant was estimated by the afore-mentioned protocol. The total protein content was determined by the protocol of Lowry *et al*., (1951).

Among the above mentioned solvents, Sodium phosphate buffer (50mM) with pH 7.6 was identified as the best one for the extraction of TI protein from the seed samples. Hence further extraction of the samples was done with this buffer.

3.2.8 Estimation of Protein content

Protein content was determined using the method described by Lowry *et al.* (1951), and the specific activity of TIs was calculated. In an alkaline solution, the -CO-NH-bond (peptide) in the polypeptide chain interacts with copper sulphate to produce a blue-coloured complex. Additionally, the phosphomolybdate and phosphotungstate

components of the Folin-Ciocalteau reagent are reduced as a result of tyrosine and tryptophan residues in protein, producing bluish products that help to increase the sensitivity of this approach.

a. Reagents

- Reagent A: Sodium carbonate (2%) in 0.1N Sodium hydroxide.
- Reagent B: 0. 5% Copper sulphate ($CuSO_4.5H_2O$) in 1% potassium sodium tartarate. Freshly prepared by mixing stock solutions.
- Alkaline copper solution (Reagent C): 50mL of reagent A and 1mL of reagent B were combined.
- Folin's reagent (Reagent D): Folin-Ciocalteau reagent was diluted with an equal volume of 0.1N NaOH.
- Standard: 50mg of BSA was dissolved in 50 mL of distilled water in a volumetric flask. 10mL of this stock standard was taken and diluted to 50mL in another flask to create the working standard solution. 200µg of protein are present in one mL of this solution.

b. Procedure

0.2 to 1mL (0.2, 0.4, 0.6, 0.8, and 1mL) of working standard solution were taken in a series of labelled test tubes. 1mL of the sample alone was taken into another test tube. The volume in all the tubes was made up to 1mL with distilled water. A tube with 1mL of distilled water served as the blank. 5mL of reagent C was added to all the test tubes, including the test tubes labelled "blank" and "unknown". By vortexing or shaking the tubes and allowing them to stand for 10

min, the contents of the tubes were mixed. Following that, 0.5mL of reagent D was immediately added and mixed thoroughly. All the tubes were kept at room temperature in the dark for 30 min. After 30 min, the absorbance was recorded at 660nm against the blank. A standard curve was made by taking the concentration of protein along the x-axis and the absorbance at 660nm along the y-axis. The concentration of protein in the sample was determined from the standard curve.

3.2.9 Trypsin Inhibitor activity assay of germinated seeds

Among all the five distinct species of plant seeds selected, the seeds of *A.hirsutus* and *G.gummi-gutta* showed significant TI activity. It has been reported that large amounts of stored proteins accumulated in the seeds of higher plants during seed development and maturation are released in the germination period to give energy and building blocks for germination and seedling growth (Bewley *et al*., 1994; Wang *et al*., 2007). Moreover, various research papers proved that there is a reduction in the anti-nutrient levels in the germinated seeds. (Mubarak, 2005; Kumari *et al*., 2014).Trypsin inhibitors are classified as anti-nutrients, since they decrease the digestibility of proteins. Hence, the germinated seeds of the selected plants were also checked for TI activity.

The collected seeds were soaked in warm water for 24h to enhance the seed germination. The seeds were then sowed in a pot containing a mixture of soil and cocopeat in 1:1 ratio. The seeds of *A.hirsutus* started germinating after 10-12 days, and those of *G.gummi-gutta* took a period of about 80-85 days for germination. The seeds of *A.hirsutus* with a one-month germination period and *G.gummi-gutta*

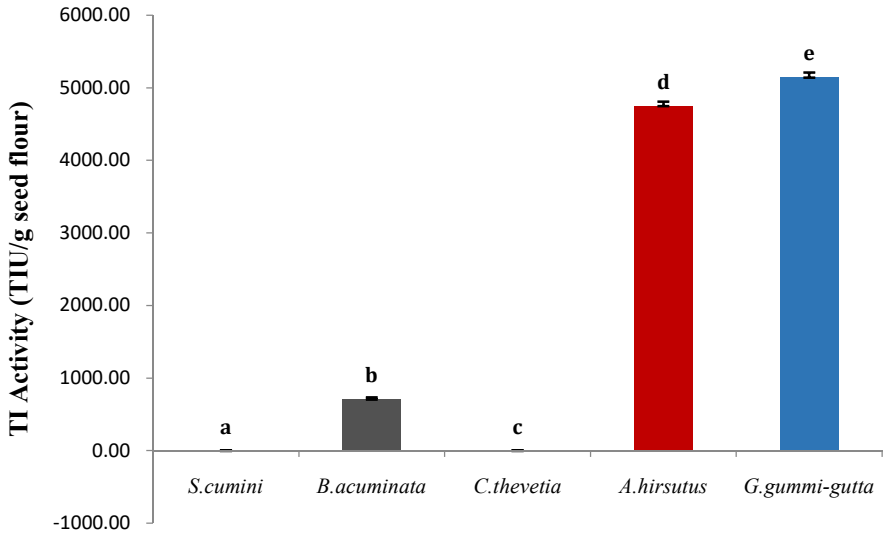
with a three-month germination period were used for the assay of TI activity. The procedure followed was the same as mentioned earlier.

3.3 RESULTS

3.3.1 Trypsin Inhibitor (TI) activity of Seeds

Five different plant seeds were screened for the presence of trypsin inhibitor protein. The plant seed were fine powdered and extracted using 50mM Sodium phosphate buffer with pH 7.6 under ice cold condition.The supernatant collected were used for TI assay by the protocol followed by Kakade *et al*., 1974. The results are given in Fig.3.3.

Figure 3.3 TI activity of seeds



Values are Mean ± Standard Deviations (n=6). Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. F value=$2.23x10^3$.

Among the five different plant seeds selected for screening, *S.cumini* and *C.thevetia* did not possess any trypsin inhibitory activity. Higher and comparable TI activity was found with the seeds of *A.hirsutus* (4748.70±61.99TIU) and *G.gummi-gutta* (5142.59± 67.36TIU). Hence they were selected for further studies and will be denoted as AhTI and GgTI hereafter.

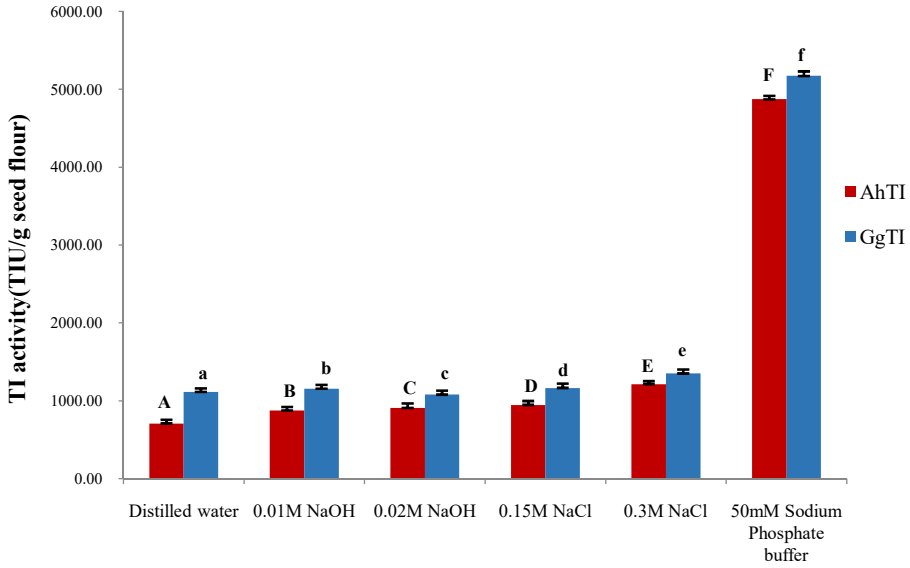
3.3.2 Confirmation of proteinacious nature of TIs by TCA precipitation

Only the precipitates obtained in the TCA precipitation experiment showed TI activity, for both seed samples; the supernatants remain inactive in this respect. Hence it can be confirmed that the TI extracted out using 50mM Sodium Phosphate Buffer (pH 7.6) is of proteinacious nature, for both the seeds.

3.3.3 Effect of different Extractants on Trypsin Inhibitor activity

Different solvents were used for the extraction of trypsin inhibitor to select the ideal one for further extraction. Distilled water, 0.01M NaOH, 0.02M NaOH, 0.015M NaCl, 0.3M NaCl and 50mM sodium phosphate buffer pH 7.6 were used for the extraction of TI proteins. The results are represented in Fig. 3.4.

Figure 3.4 Effect of extractants on TI activity



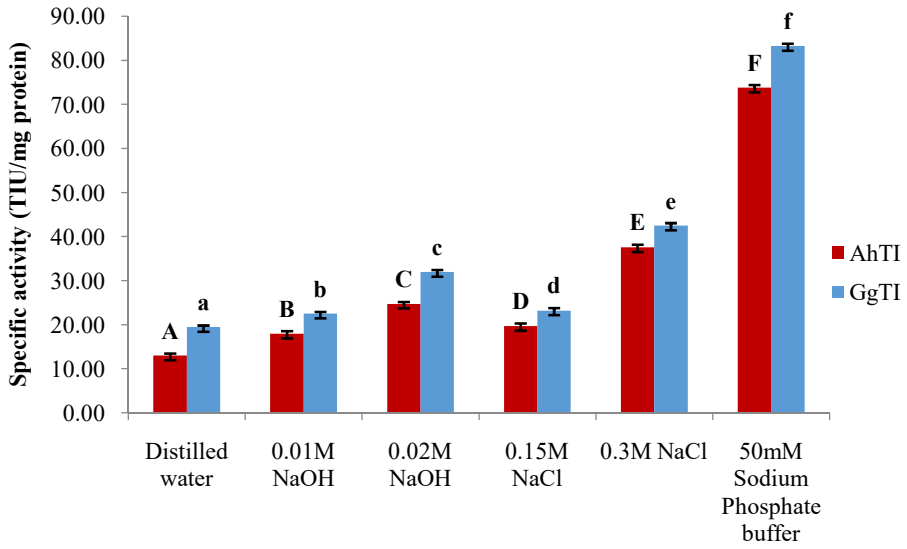
Values are Mean ± Standard Deviations (n=6). Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. F value for AhTI=$7.23x10^3$ and GgTI=$6.14x10^3$.

Among the extraction media used 50mM Sodium phosphate buffer with pH 7.6 showed the highest recovery of seed protein with TI activity. Distilled water showed the lowest. AhTI showed an activity of 4873.20±43.99TIU and that of GgTI was 5172.17±58.30TIU in 50mM Sodium phosphate buffer with pH 7.6.

3.3.4 Effect of extractants on specific activity of TI protein

The specific activity of the TI protein in different extraction solvents was also determined. The results are represented in Fig. 3.6.

Figure 3.5 Effect of extraction media on Specific activity



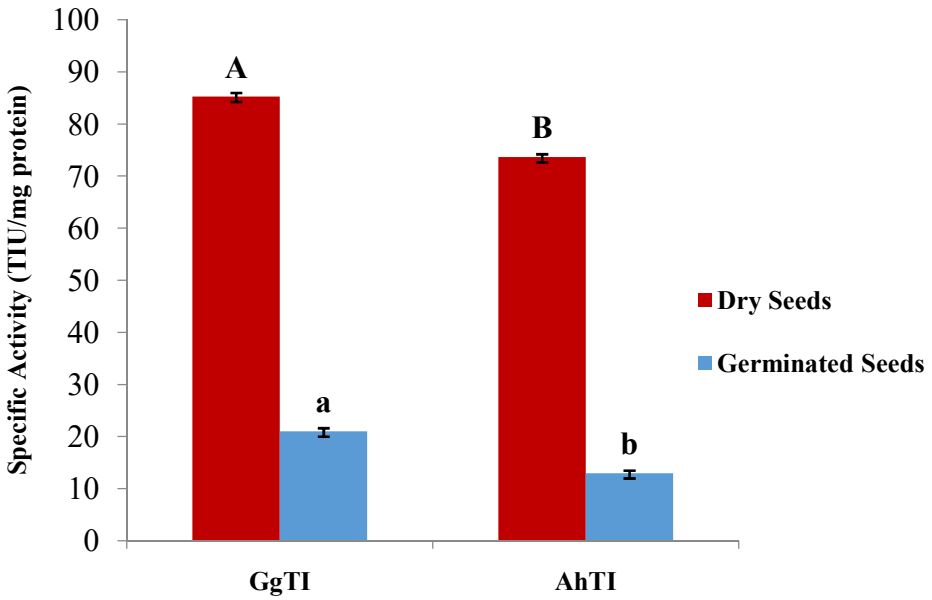
Values are Mean ± Standard Deviations (n=6). Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. F value for AhTI=$3.7x10^3$ and GgTI=$4.2x10^3$.

0.3 M NaCl and 50 mM Sodium Phosphate Buffer extracts showed comparably higher TI activities than the others, for both the seeds. TI protein from *A.hirsutus* showed a specific activity of 73.78±0.652TIU/mg protein and that from *G.gummi-gutta* showed 83.23±0.569TIU/mg protein, when extracted with 50mM Sodium phosphate buffer. TI activity while using 0.3M NaCl was 37.54±0.654TIU/mg protein in the case of *A.hirsutus* and 42.46±0.621TIU/mg protein in the case of *G.gummi-gutta*.

3.3.5 Trypsin Inhibitory Activity of germinated seeds

The TI activity of germinated seeds of both *A.hirsutus* and *G.gummi-gutta* was determined after germination. The seeds of *A.hirsutus* after 30 days of germination were used for the TI activity assay, and those of *G.gummi-gutta seeds* were used after 3 months of germination. The results are given in Fig.3.6.

Figure 3.6 Trypsin Inhibitor Activity of dry and germinated seeds



Values are Mean ± Standard Deviations (n=6). Error bars indicates Standard Deviations. $p < 0.05$ considered statistically significant. F value for AhTI=$1.x10^3$and GgTI=$6x10^3$.

Both the germinated seed samples showed lesser TI activity than the corresponding non- germinated ones. The TI activity of germinated seeds of *A.hirsutus* was 12.96±0.875TIU/mg protein, whereas that of dry seeds is 73.67±0.522TIU/mg protein. *G.gummi-*

gutta showed an activity of 85.28±0.637TIU/mg protein in the dry seeds but the germinated seeds showed an activity of 21.005±0.729TIU/mg protein.Both the plant seeds showed a reduced TI activity after germination. Hence for further studies, only the non-germinated (dry) seeds were used, in both the cases.

3.4 DISCUSSION

The substrate used for the trypsin inhibitor assay was BAPNA (N-α-benzoyl DL- arginine-p-nitro anilide). Five different plant seeds were randomly selected and used for the isolation of trypsin inhibitor protein. Seeds of *Artocarpus hirsutus*, *Bauhinia acuminata*, *Garcinia gummi-gutta*, *Syzygium cumini* and *Cascabela thevetia* were selected for the study. The plant seeds except *Cascabela thevetia* and *Syzygium cumini* showed TI activity and the seeds from *A.hirsutus and G.gummi-gutta* showed the highest TI activity. Although reports are available on different plant seeds with TI activity (Giri *et al*., 2003; Kansal *et al*., 2008), there have been no reports on *A.hirsutus* and *G.gummi-gutta* seeds having PIs. Among the TIs from plant seeds reported so far, most are proteins (Ribeiro *et al*., 2015; Serquiz *et al*., 2016; Cristina *et al*., 2019). Our results also show that the seeds of *A.hirsutus* and *G.gummi-gutta* contain proteinaceous TIs.

Different extraction solvents such as distilled water, 0.01M NaOH, 0.02M NaOH, 0.15M NaCl, 0.30M NaCl and 50mM Sodium phosphate buffer were used for the standardization of the extraction of TI proteins. Among these solvents sodium phosphate buffer with pH 7.6 was identified as the ideal extractant for the isolation of TI protein

from both the plant seeds. Hence, 50 mM phosphate buffer (pH 7.6) was used in all further studies. Different media were used by various researchers for the extraction of PIs (Maggo *et al*., 1999; Hajela *et al*., 1999). In many studies alkaline solvents (Jyothi *et al*., 1995; Peace *et al*., 1992), aqueous salt solutions with changing pH (Tan *et al*., 2006; Kakade *et al*., 1974) or distilled water (Klomklao *et al*., 2011; Karray *et al*., 2020) were used to extract PIs. The inhibitor from buckwheat was quite resistant to acid, while exposure to alkali decreased its inhibitory power (Ikeda *et al*., 1978). In a study by Kansal *et al*., (2008), it was reported that the seed flour extracted with 50 mM phosphate buffer (pH 7.6) showed maximum inhibitory activity.

The extraction medium's pH and ionic strength have an impact on how successfully winged bean (*Psophocarpus tetragonolobus*) seed trypsin inhibitors can be extracted (Tan *et al*., 1982). The highest specific inhibitory activity for Navy bean (*Phaseolus vulgaris*) and red kidney bean (*Phaseolus vulgaris* L) was given by 0.02 NaOH, among the several extractants used (Kusuma *et al*., 2009). Several buffers were used to extract a trypsin inhibitor from eggplant exocarps. The extract with the highest specific activity was 0. 1 M acetate buffer at pH 5.5, (Kanamori *et al*., 1975).In another study, buckwheat seeds (*Fagopyrum esculentum Moench*) were found to contain a protein-like trypsin inhibitor that could be recovered from the aqueous extract using a combination of gel filtration, salting out and heat treatment (Ikeda *et al*., 1978).

The results show that the TI activity was reduced in germinated seeds of *A.hirsutus* and *G.gummi-gutta* compared to the respective dry seeds. The trypsin inhibitory activity of the dry Indian bean is very strong, but it gradually declines by 51% over the course of 12 h of soaking, falling to 17% by the end of the 32h germination period (Ramakrishna *et al*., 2006). It has been reported that during germination, the amount of trypsin inhibitors in the chickpea cotyledon decreased (Neves and Laurenco, 2011). In comparison to dry seeds, trypsin inhibitor activity in horsegram and mung bean seeds decreased to 16% and 40%, respectively, after 72h of germination (Subbulakshmi *et al*., 1976). According to Mohamed *et al*. (2011), after soaking for 24 h, soybean, mung bean and kidney bean seeds lost 15.9, 27.5 and 39.5% respectively, of their trypsin inhibitor activity when compared to dry seeds. Another research reported that the TI content in sprouting *Cicerarietinum* seeds remained stable during the course of the germination period; however, in this instance, significant changes in the total protein profile were not noticed and in the TI isoform studies, the composition of the *Viciafaba* and *Cicer arietinum* species changed during germination (Muzquiz, *et al*., 2004). After three days of germination, the trypsin inhibitor activity of the seed flour of *Mucana* bean seeds was reduced (Mungendi *et al*., 2010). With longer germination time, *Dolichosbi florus* seeds' ability to block trypsin activity diminished. Milky phases in growing seeds showed the least amount of trypsin inhibitory activity, which gradually rose after 21 days of flowering until the maturation period (Nath *et al*., 2014). It is noteworthy that the trypsin inhibitor in buckwheat grain essentially

disappears after germination because germination treatment can successfully remove the inhibitor from the grain (Ikeda *et al*., 1978). When compared to non-germinated seeds, the protein content in the germination period is higher, indicating that the anti-nutritional components of the proteins were either hydrolyzed or separated from them. The types of anti-nutritional agents that affect protein digestion include trypsin inhibitors and phytate. Hydrolysis removes the phytate-related inhibition during germination and suppresses the activity of trypsin inhibitors (Ikenna *et al*., 2020). Hence it can be inferred that in the case of *A.hirsutus* and *G.gummi-gutta* seeds, the decrease in TI activity might be due to the same reason.

Chapter 4

Purification and characterization of TI from *Artocarpus hirsutus* Lam. (AhTI)

Five types of plant seeds were collected from different localities in the Malappuram and Calicut districts of Kerala. Among these the seeds of *A.hirsutus* and *G.gummi-gutta* showed significant trypsin inhibitory activity. Thus, both the seeds were selected for the isolation of TI proteins. The purified forms of these TI proteins were used for further research.

4.1 OBJECTIVES

This chapter describes the purification and characterization of trypsin inhibitor proteins extracted from the seeds of *Artocarpus hirsutus* Lam. The purification techniques include ammonium sulphate precipitation, dialysis, ion exchange chromatography and size exclusion chromatography. The confirmation of TI activity of the purified protein was done by activity staining by Reverse zymography. Another goal of the study was to determine the molecular weight of the isolated protein by Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and confirmation of the molecular weight by Matrix-Assisted Laser Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry (MS). The study then focuses to determine the stability of purified TI protein under different pH and temperatures. Understanding the type of inhibition and determination of K_I value from the Dixon plot are also our aim.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Trypsin, BAPNA (N-α-benzoyl DL- arginine-p-nitro anilide) and Protein molecular weight Marker were purchased from Sigma

Aldrich, USA. Acrylamide, bis acrylamide, TEMED, Methylene green, Bromophenol blue, Coomassie Brilliant Blue G-250, Coomassie Brilliant Blue R-250, BSA (Bovine Serum Albumin, fraction V), β-Mercapto ethanol (2-ME), cyanogen bromide (CNBr), β- alanine and Ammonium sulphate were from Sisco Research Laboratories Limited, Mumbai. *Diethyl amino ethyl cellulose* (DEAE), Sephadex G75 and Dialysis membrane were purchased from Himedia, India. All other general chemicals used were of high-purity and available commercially.

4.2.2 Ammonium Sulphate Precipitation

An aggregate of hydrophobic components is formed when hydrophobic groups on the proteins exposed to the environment during the ammonium sulphate precipitation process attract additional hydrophobic groups. Normally, the protein precipitate in this situation can be seen with the naked eye. This approach has the benefit of being inexpensive to use, even at very high volumes.

The supernatant collected from the centrifuged seed extract was subjected to Ammonium sulphate precipitation. The precipitation calculations were done with the help of an online ammonium sulphate calculator ("https://www.encorbio.com/protocols/AM-SO4.htm") by Encor Biotechnology Inc. (2018). The benefit of using Ammonium Sulphate is that it is highly soluble and stabilizes protein structures. It has a low density, is easily available and reasonably cheap. So it is used as an ideal precipitant. The Ammonium Sulphate fractionation was done at 0 to 30%, 30 to 60% and 60 to 90%.

a. Reagents

- Solid Ammonium sulphate
- 50 mM phosphate buffer (pH 7.6)

b. Procedure

Solid ammonium sulphate was slowly added to the crude extract (supernatant) with gentle stirring initially from 0 to 30% saturation. The crude extract was kept under ice cold condition. The ammonium sulphate was entirely dissolved, and the solution was kept overnight at 4°C while the precipitation process continued. The proteins precipitated were then recovered by centrifugation at 10000xg for 15 min. The supernatant was set aside so that the needed amount of ammonium sulphate could be added for the next saturation level. The preceding stages were continued until the ammonium sulphate saturation level reached 90%. The pellets in each step were dissolved in a small volume of 50mM sodium phosphate buffer with pH 7.6, and kept at 4°C for further purification analysis.After ammonium sulphate separation, the stored pellet was dialyzed against 50 mM phosphate buffer (pH 7.6) to remove ammonium sulphate from the precipitate and trypsin inhibitory activity, protein content and specific activity were determined.

4.2.3 Dialysis

a. Reagents

- 0.2% H_2SO_4 (v/v)
- 0.3% Sodium sulfide (w/v)
- Sodium phosphate buffer (pH 7.6)

b. Procedure

Pretreatment of the dialysis membrane is required to remove the normally associated additives like glycerine and sulphur compounds, to prevent protein absorption into the membranes as well as to clear the pores. The dialysis tube (MWCO 7kDa) was washed under running tap water for a period of 3-4h. After rinsing the tube, washed it in a 0.3% (w/v) solution of sodium sulfide for 1min at 80°C. The tubes were washed immediately with hot water (60°C) for 2min. The tubes were then acidified with 0.2% (v/v) H_2SO_4. It was again rinsed with hot water (60°C).

After ammonium sulphate precipitation, the re-suspended protein pellets in 50mM Sodium phosphate buffer was transferred to the pre-treated dialysis bag and dialysed against 50mM Sodium phosphate buffer at 4°C for 24h with continuous stirring. The buffer was replaced two to three times during this period. After dialysis the sample was freeze dried using INLABCO INFD3P lyophilizer and stored at -20°C until use. TI activity, protein content and specific activity were determined using the lyophilised sample.

4.2.4 Ion exchange chromatography

Active fractions from dialysis after ammonium sulphate fractionation were pooled, lyophilised in 1mL aliquot, re-suspended in sample buffer and subjected to ion exchange chromatography.

a. Reagents

- 50mM Sodium Phosphate buffer (pH 7.6)

- 1M NaCl
- NaCl (0.1M, 0.2M, 0.3M, 0.4M and 0.5M prepared in 50mM sodium phosphate buffer with pH 7.6)
- DEAE Cellulose

b. Procedure

i. Activation of DEAE cellulose

10g of DEAE Cellulose (SRL, India) was soaked in 50mM Sodium Phosphate buffer (pH 7.6) and allowed the resin to settle down. The fine particles were removed by decanting. It was then re-suspended in 1M NaCI and kept overnight. Decanted the sodium chloride solution and washed several times with distilled water in a sintered glass funnel using vacuum filtration, until the pH of the washings became neutral. Equilibrated the resin within the buffer by repeated washing.

ii. Purification with DEAE-Cellulose Column

The activated DEAE Cellulose was carefully packed in XKI6/26 column (30cm height) (Amersham Pharmacia) without any air bubble and the column was equilibrated with Phosphate Buffer (50mM, pH 7.6) overnight. 5mg of the sample was dissolved in 15mL of 50mM Sodium Phosphate buffer (pH 7.6) and loaded to the pre-equilibrated DEAE Cellulose columns. After the complete entry of the sample in to the column it was connected with the reservoir containing 50mM Sodium Phosphate buffer (pH 7.6). The flow rate was adjusted to 2mL/min. The unbound proteins were washed out with the buffer

until the absorbance reached zero at 280nm. The sample were eluted with a flow rate of 2mL/min with stepwise gradients of sodium chloride ranging from 0.1M, 0.2M, 0.3M, 0.4M and 0.5M prepared in 50mM sodium phosphate buffer with pH 7.6. 2mL fractions were collected and OD of each fraction was read at 280nm. The TI activity of each fraction having an A_{280} of 0.5 and above was assayed, and only those showing significant TI activity were pooled. The pooled fraction was dialysed against 50mM Sodium Phosphate buffer (pH 7.6). The trypsin inhibitory activity, total protein and specific activity were calculated. The yield and fold of purification were calculated. The dialysed sample in 50mM Sodium Phosphate buffer (pH 7.6) was subjected to gel filtration chromatography.

4.2.5 Gel filtration chromatography

After the purification by ion exchange chromatography the sample was subjected to size exclusion chromatography using the resin Sephadex G75.

a. Reagents

- 50mM Sodium Phosphate Buffer (7.6)
- Sephadex G-75

b. Procedure

5g of Sephadex G-75 was suspended in distilled water and allowed to hydrate for 3h in a boiling water bath, decanted the fine particles. The hydrated gel suspension was degassed under vacuum to remove the air bubbles. Gel suspension was poured in to the column

without air bubbles and allowed to settle under gravity. The column was allowed to reach the room temperature. Added 50mM sodium phosphate buffer with pH 7.6 through the column continuously and allowed it to drain out completely. This was done to equilibrate the column.

0. 5mL of the sample (the pooled fraction obtained from the ion exchange chromatography) was applied to the column. After the complete entry of the sample to the column, the proteins were eluted using 50mM sodium phosphate buffer. The flow rate was adjusted to 1mL/minute. The protein content was estimated by measuring the absorbance at 280nm. The trypsin inhibitory activity, total protein and Specific activity of the peak fraction were calculated as per the respective protocols described in Chapter III. The yield and fold of purification also were calculated.

4.2.6 Estimation of Protein content

Protein content was determined using the method described by Lowry *et al.* (1951), and the specific activity of inhibitors from the five seed samples was calculated. The principle and procedure for the estimation of protein was described in detail in the chapter III (3.2.8).

4.2.7 Yield and fold of Purification

Yield and fold of purification of Trypsin inhibitor protein was calculated after each stages of purification. Yield of total protein, Specific inhibitor activity, Yield of activity, Fold of

purification, Total inhibitor activity was calculated from the below mentioned formulae:

$$\textbf{Yield of Activity} = \frac{\text{Total activity of the purified fraction}}{\text{Total activity of the crude extract}} \text{x100}$$

$$\textbf{Fold of purification} = \frac{\text{Specific activity of the purified fraction}}{\text{Specific activity of the crude extract}}$$

4.2.8 SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis)

SDS-polyacrylamide gel electrophoresis was done the protocol described by Laemelli (1970).

a. Reagents

40% Acrylamide (Stock)

- Acrylamide 116.8g
- N, N'-Methylene bisacrylamide 3.2g
- DDI (Distilled De-Ionised water) 300mL
- (It was Filtered and stored in a dark bottle at 4°C until each use)

30% Ammonium Persulfate

- Ammonium Persulfate 1.5g
- DDI water 5mL

Resloving Gel (RG) Buffer-1.5 M Tris-HCl, pH 8.8

- Tris-free base 90.75g
- Conc. HCl 8mL

- DDI water 300mL

  Adjusted the pH to 8.8 with conc. HCl, and made final volume to 500 mL with DDI water.

Stacking Gel (SG) Buffer -1.0 M Tris-HCl, pH 6.8

- Tris-free base 60.54g
- Conc. HCl 36mL
- DDI water 300mL

  Adjusted the pH to 6.8 with conc. HCl, and bring final volume to 500mLwith DDI water

4x SDS-PAGE Sample Buffer (pH 6.8)

- Tris HCl, (1M) - 5mL
- Glycerol- 8mL
- 20% SDS - 8mL
- ß-Mercaptoethanol- 4mL
- 0.5mg/mL Bromophenol Blue-20mg
- DDI water -15mL

10x SDS-PAGE Running Buffer (pH-8.3)

- 30.3g Tris base
- 144.0g Glycine
- 10.0g SDS

Dissolved well in DDI and made the volume up to 1000mL.

Coomassie Brilliant Blue Stain Solution

- Coomassie brilliant blue R250 - 100mg
- Methanol - 40mL
- Glacial acetic acid - 10mL
- Distilled water - 50mL

Destaining solution

- Methanol - 40mL
- Glacial acetic acid - 10mL
- Distilled water - 50mL

Resolving Gel (12%)

- 1. 5 M Tris-HCl, (pH 8. 8) - 2.6mL
- 40% Acrylamide Stock - 2.4mL
- 20 % SDS - 20µL
- 10% Ammonium Persulfate - 20µL
- TEMED - 8µL
- DDI water - 2.8mL

Stacking Gel (4 %)

- M Tris-HCl, (pH 6.8) - 500µL
- 40% Acrylamide - 500µL
- 20 % SDS - 100µL
- 30% Ammonium Persulfate - 16µL

- TEMED - 8µL
- DDI water - 3.9mL

b. Procedure

i. Casting the Gel

The glass plates and spacers were assembled in the gel casting apparatus according to the BioRad instruction manual. The components of the resolving gel were mixed as described by the protocol followed by Laemmlli (1970). The resolving gel mixture was poured into the gel plates up to 2cm down from the top of the shorter plate. To stop meniscus formation in the resolving gel, DDI water was applied on top of the gel. The resolving gel was allowed to stand for 30 min at room temperature. The DDI water was drained off from the surface of the resolving gel. Drained it completely after a repeated DDI water rinse and used a Kimwipe to wipe away any leftover DDI water. The components for stacking gel were mixed well. Gel plates were filled by pouring the stacking gel solution into them (on top of the flowing gel). The comb was placed on top of the spacers. The gel was allowed to stand at room temperature for 1h.

ii. Preparation of Sample

The samples were thoroughly mixed in 4X SDS-PAGE sample buffers (3:1) and boiled for 5 min in a water bath, immediately spun to remove any debris, and then allowed to cool to room temperature.

iii. Running SDS PAGE

The cast gel was placed in the PAGE apparatus after removing the combs. Freshly prepared 1x running buffer (300mL) was added to both chambers of the apparatus. Then the prepared samples (30µL) and molecular weight marker (15µL) were loaded into the wells of the gel. The gel was run at 100V until the dye front migrated into the running gel and increased to 150V until the dye front reached the bottom of the gel.

- **Staining and De-staining of Gel**

The glass plates and spacers as well as the run gel were removed from the apparatus. The gel was placed in a tray and added around 20mL of staining solution, stained for at least 30 min, and gently shaken. The remaining stain was then poured off. Approximately 5mL of the de-stain solution was added and gently shaken for 1 min and poured off. Destaining was done with 30mL of de-stain solution for more than 2h with gentle shaking until the gel is clearly destained. Poured off the de-stain solution and discarded it. DDI water (30mL) was added, gently shaken and rinsed for 5 min. The gel was dried by placing a piece of Whatman filter paper below the gel.

4.2.9 Activity staining by Reverse Zymography

Activity staining by reverse zymography was performed using the protocol followed by Felicioli *et al*., (1997).

a. Reagents

- 2.5% (v/v) Triton X-100
- M Tris-HCl with pH 7.4
- 50mM Tris-HCl (pH 7.6)
- Trypsin
- 10% methanol
- 10% acetic acid
- 200mM NaCl
- 1% gelatine
- Isopropanol
- Coomassie Brilliant Blue

b. Procedure

i. Preparation of Gelatin-SDS-PAGE gel for Reverse Zymography

The gel cassette was assembled after thoroughly cleaning and drying the glass plates, combs, and spacers (0.75-1mm). 1% gelatin in distilled water was heated until dissolved, then kept warm to prevent gelling.

The separating gel (12%) was prepared by adding the gelatin solution to the equivalent volume of water until it reached a final concentration of 0.1%. To prevent the gelatin from improper gelling, the mixture must be well combined and swiftly transported to the casting chamber. Prior to polymerization, a thin layer of isopropanol was applied to the gel to level it. The Isopropanol layer was removed and dried off well with filter paper after the gel had polymerized. The comb was inserted and the stacking solution was prepared and poured into the casting chamber.

ii. **Preparation of Sample**

An equal volume of 2x protein sample loading buffer was added to TI sample. Electrophoresis was conducted at 100 V for about 3-4h at 4°C according to the procedure followed by Laemmlli (1970). SDS must be removed from the gel before the gel is treated with the protease since it can affect the activity of the PI being analyzed. Thus, the SDS was removed from the gel by; the gel was rinsed twice with 30mL of 2.5% (v/v) Triton X-100 solution by agitation for each 10min. Again the gel was rinsed twice for 10 min each with 30mL of a solution of 2.5% (v/v) Triton X-100 + 50 mM Tris-HCl with pH 7.4. It was rinsed for 10 min with 30mL of 50 mM Tris-HCl, pH 7.4. The gel was incubated for protein digestion for 2h at 37°C in a buffer solution containing Trypsin, 50mL of 50 mM Tris-HCl (pH 7.6) and 200mM NaCl. The gel was placed in a 10% methanol and 10% acetic acid solution for fixation with gentle shaking for 30min, and then discarded the solution.

iii. Staining for detection of Trypsin inhibitor

After incubation, the gel was stained with Coomassie Brilliant Blue with the addition of approximately 100mL of stain solution and kept in a shaker overnight. The gel was rinsed with distilled water after discarding the stain until the background became clear. The clear gel part revealed the gelatin copolymer's effective degradation with the enzyme trypsin. The stained band indicated the presence of trypsin inhibitor where the gelatin was protected from degradation by the activity of the inhibitor sample.

4.2.10 Determination of pH stability

a. Reagents

- Tris-HCl
- Sodium phosphate buffer (pH 6. 0 and 7.6)
- Tris-HCl buffer (8.0)
- Sodium acetate (pH 4.0)
- Sodium phosphate buffer (pH 6.0 and 7.6)
- Glycine-sodium hydroxide buffer (pH 10)

b. Procedure

After incubating the purified AhTI in various pH buffers, each for 4 h at 4°C, the pH stability of the pure TI was tested throughout a range of pH (4, 6, 7, 8, and 10) and the activity of TI was assessed.

300µL of the appropriate buffers Sodium acetate (pH 4.0), Sodium phosphate buffer (pH 6.0 and 7.6), Tris-HCl buffer (8.0) and Glycine-sodium hydroxide buffer (pH 10) were carefully combined with 30µL of purified TI. After incubation the TI activity of each samples treated with different pH buffers were calculated according to previously mentioned protocol.

4.2.11 Determination of thermal stability

Thermal stability of the TI was determined by incubating the TI sample at various temperatures ranging from 37°C-100°C for 20 min each. After that, the samples were taken back and allowed to cool. Then the samples were centrifuged at 8000xg for 10 min and the supernatant were used for Trypsin inhibitory activity assay which was done by the previously described procedure.

4.2.12 Determination of Inhibitor constant from Dixon Plot

Inhibitory activity of TI was determined at two different substrate concentrations (1mM and 0.5mM) by varying inhibitor concentrations (1µg/mL, 2µg/mL, 3µg/mL, 4µg/mL and 5µg/mL) and the inhibitor constant (K_I) was determined from Dixon plot (Dixon *et al*., 1979).

4.2.13 Molecular weight Confirmation by MALDI TOF/TOF

a. Reagents

- 0.1% TFA

- 0.1% TFA in water
- Sinapic acid (matrix) saturated in TA50 (50:50 v/v acetonitrile: 0.1% TFA in water)

b. Procedure

The matrix/analyte mixture was spotted on the polished steel MALDI target plate (BRUKER DALTONICS) and allowed to air dry. One part of sinapic acid (matrix) saturated in TA50 (50:50 v/v acetonitrile: 0.1% TFA in water) was combined with one part of sample solution (Dried Droplet method). After that, the dried samples were examined with an Ultraflextreme MALDI TOF/TOF (BRUKER DALTONICS) instrument that was operated in positive ion mode and was managed by Compass for flex software [flex control (version 3.3) and flex analysis (version 3.2)]. In MS, each spectrum had an average of 3500 laser pulses with a mass window of 5000-30000 Dalton. Ion source 1 was set to 25KV, Ion source 2 was set to 22.25KV and the detector gain for MS mode was set to 8.5 xs. The mass spectrometer's times of flight analyser (TOF) were calibrated using a Bruker protein calibration standard I solution with a mass range of 5000-20000 Da.

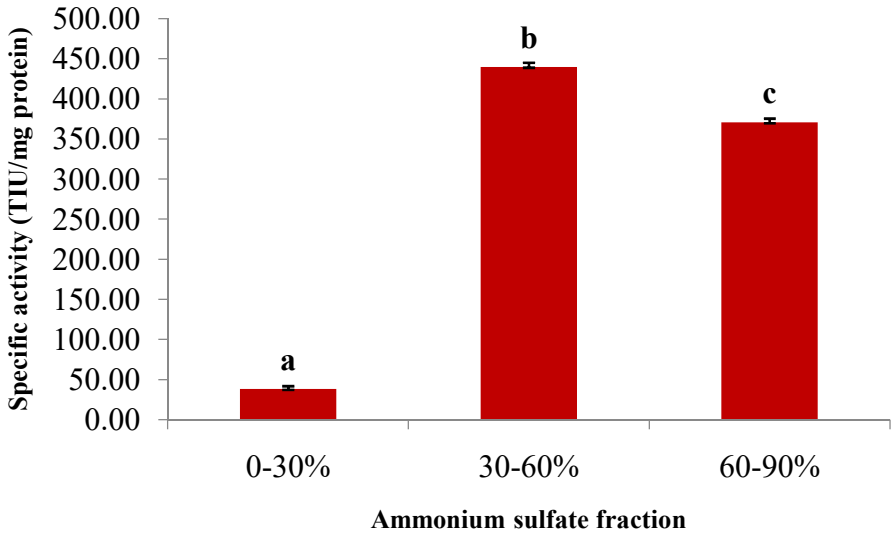
4.3 RESULTS

4.3.1 TI activity of Ammonium sulphate fractions

Ammonium sulphate precipitation was done as 0-30, 30-60, and 60-90% fractions at 4°C. After ammonium sulphate precipitation, the protein precipitate of each fraction was re-suspended in 50mM

Sodium phosphate buffer (pH 7.6) and was dialysed against same buffer at 4°C. The precipitation calculations were done with the help of an online ammonium sulphate calculator ("https://www.encorbio.com/protocols/AM-SO4.htm") by Encor Biotechnology Inc. (2018). The results are given in Fig.4.1.

Figure 4.1 Specific activity after Ammonium sulphate precipitation and dialysis



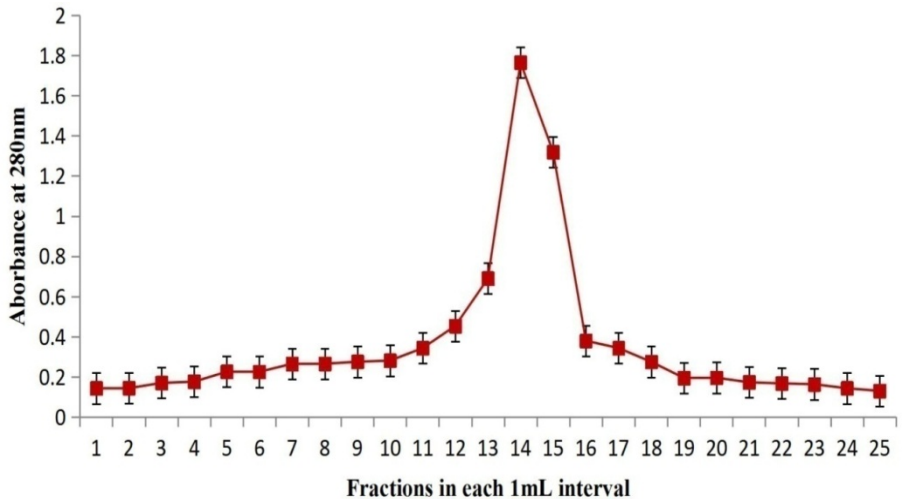
Fraction 30-60% showed highest TI activity of 439.69±5.11TIU/mg protein; Values are Mean± Standard Deviations. n=6; $p<0.05$ considered statistically significant. F value =$1.4x10^3$.

All the precipitated proteins of ammonium sulphate fractions of *A.hirsutus* showed Trypsin Inhibitor activity. 0-30% fraction has a trypsin inhibitor activity of 38.16±3.52 and that of 30-60% is 439.69±5.11 and 60-90% fraction is 370.55±4.65TIU/mg protein.The highest TI activity was found in the 30-60% fraction (439.69±5.11TIU) and it was used for further purification.

4.3.2 Purification by Ion exchange chromatography

The fraction (30-60%) with highest TI activity isolated after ammonium sulphate precipitation and dialysis was used for further purification by ion exchange chromatography. It was lyophilised in 1mL aliquot, re-suspended in sample buffer and ion exchange chromatography was performed with the anion exchange resin DEAE cellulose. The results are given in Fig.4.2.

Figure 4.2 Purification by ion exchange chromatography

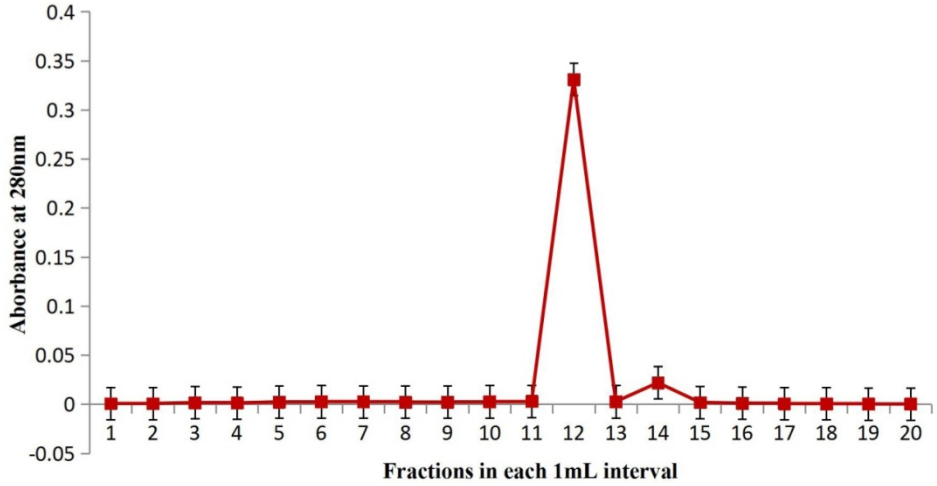


Fractions 11-19 were the fractions with significant protein content. Among these fractions only 14th and 15th showed TI activity. These were pooled, and the specific inhibitory activity of the pooled fraction is 458.67±5.049TIU/mg protein.

4.3.3 Purification by Gel filtration chromatography

The fractions with highest TI activity from ion exchange chromatography were subjected to size exclusion chromatography with the resin Sephadex G75. Fig.4.3 represents the results.

Figure 4.3 Purification by gel filtration chromatography



Fraction 12 and 14 possessed protein content. Fraction 12 is the only one with trypsin inhibitory activity. The Specific inhibitory activity of fraction 12 is 2048±27.3 TIU/mg protein.

4.3.4 Yield and Fold of Purification

Yield and fold of purification was determined after ech step of purification. The results are shown in Table 4.1.

Table 4.1 Yield and fold of Purification

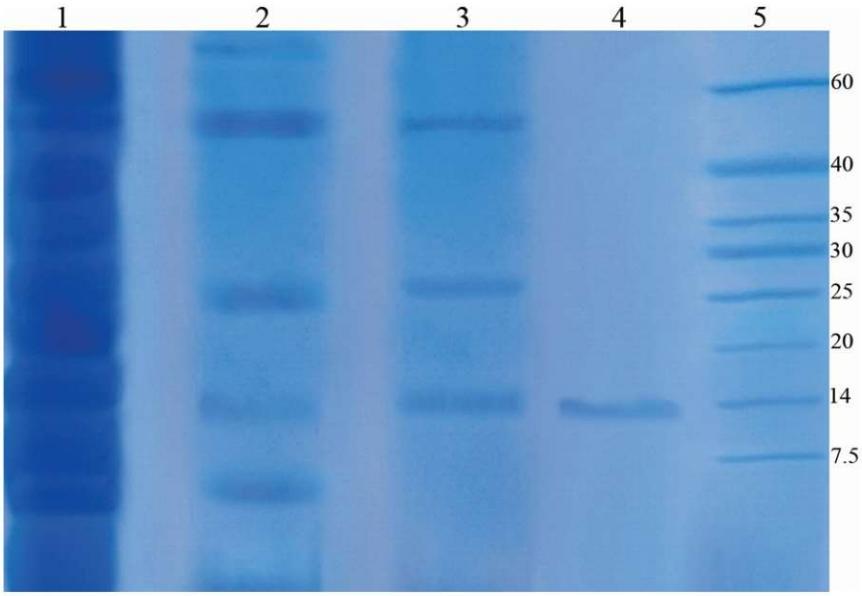
Sample	Total protein (mg/mL)	Trypsin inhibitor activity (TIU/mL)	Specific inhibitor activity (TIU/mL)	Yield of activity %	Fold of purification
Crude extract	0.67±0.020	49.04±0.021	73.78±0.653	100	1
Ammonium sulphate fraction (30-60%)	0.40±0.084	37.2±0.200	439.69±5.11	75.85	5.96
DEAE Cellulose	0.06±0.004	27.52±0.151	458.67±5.049	56.12	6.22
Sephadex G 75	0.01±0.002	20.48±0.015	2048±27.3	41.76	27.76

Values are Mean±Standard Deviations. n=3; $p<0.05$ considered statistically significant. F value for Crude extract=$1.1x10^3$, Ammonium sulphate fraction (30-90%) =$1.3x10^3$, DEAE Cellulose=$1.1x10^3$, Sephadex G75=$1.6x10^3$.

4.3.5 Molecular weight determination by SDS-PAGE

SDS-polyacrylamide gel electrophoresis was done following the protocol described by Laemelli (1970). A 12% gel was prepared for resolving and 4% gel for stacking. The results are given in Fig.4.4.

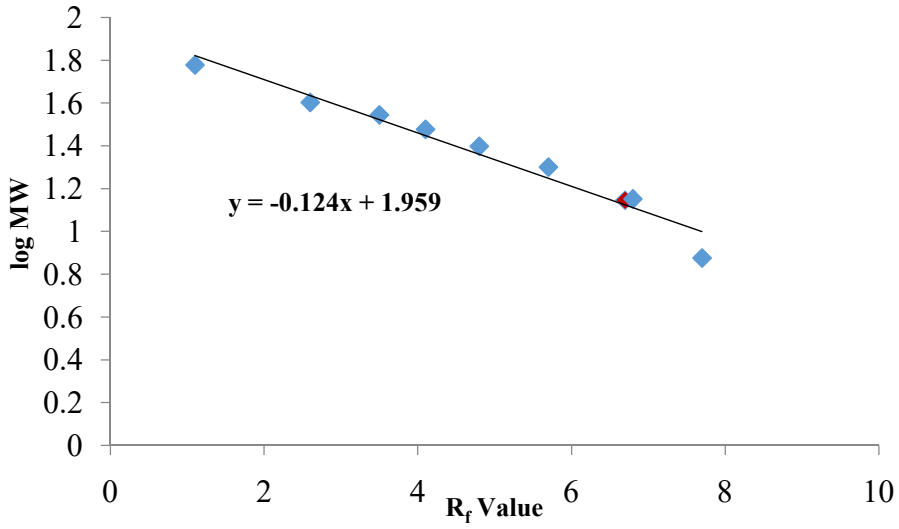
Figure 4.4 SDS PAGE of fractions obtained after different steps of purification



SDS PAGE was done in 12% gel. Lane 1: crude extract, Lane 2: 30-60% Ammonium sulphate precipitated dialysed fraction, Lane 3: DEAE Ion exchange fraction, Lane 4: Sephadex G-75 Size exclusion fraction and Lane 5: Protein molecular weight marker (7.5-60kDa).

The molecular weight of AhTI was determined from the SDS PAGE. It was determined using a standard curve plotted by log MW versus R_f (Fig.4.5).

Figure 4.5 Molecular weight determination from standard curve



Molecular weight determination of the AhTI protein from a standard curve of the log MW versus R_f value.

The molecular weight obtained from the graph was 14.18kDa. This value is very close to the molecular weight determined from MALDI analysis (14.28kDa). (Results of MALDI analysis is given in section 4.3.7).

4.3.6 Activity staining by Reverse Zymography

The trypsin inhibitor protein eluted from gel filtration chromatography was subjected to activity staining by reverse zymography as per the protocol described by Felicioli *et al*., (1997). The results are given in Fig.4.6.

Figure 4.6 Activity staining by reverse zymography

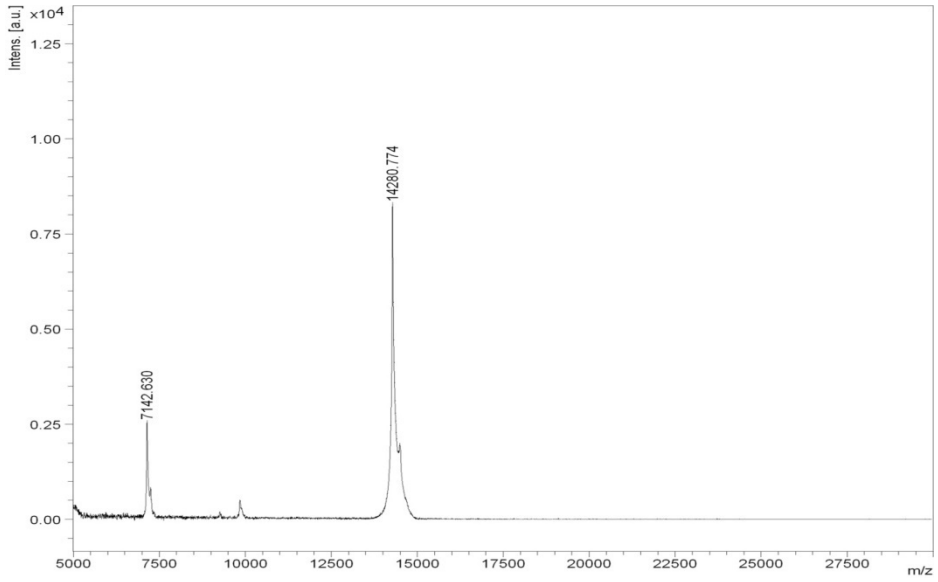
Reverse zymography: Presence of AhTI as a single band against a clear back ground.

The presence of a single protein with trypsin inhibitory activity is confirmed by the result of activity staining by reverse zymography.

4.3.7 MALDI TOF/TOF Spectrum

The molecular weight of AhTI obtained from SDS-PAGE analysis was confirmed by MALDI-TOF/TOF analysis. The purified fraction of TI protein after gel filtration chromatography was subjected to MALDI analysis (Fig.4.7).

Figure 4.7 MALDI TOF/TOF Spectrum

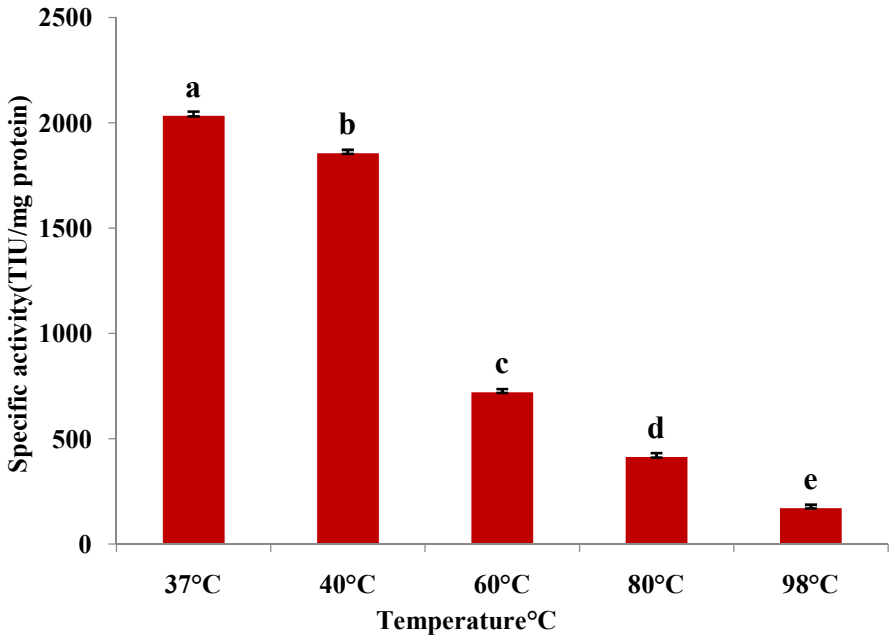


Molecular weight confirmation by MALDI-TOF analysis indicated that AhTI has a molecular weight of 14.28KDa.

4.3.8 Determination of Thermal Stability

The thermal stability of the purified TI protein was determined by incubating the purified fractions after gel filtration chromatography at different temperatures (37^{o} to 100^{o}C) for 20min. The results are given in Fig.4.8.

Figure 4.8 Thermal Stability



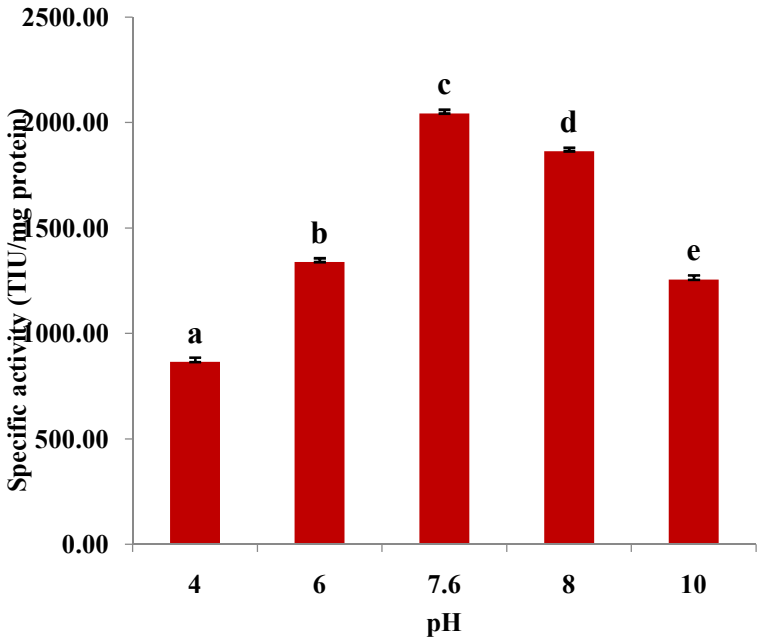
Specific activity of AhTI after treatment at different temperatures for 20min/h; n=6; Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. F value= $1.6x10^3$.

The result of thermal Stability of AhTI at different temperatures reveals that the AhTI retains activty up to 98°C and the highest activity is seen at 37°C.

4.3.9 Determination of pH Stability

The pH stability of TI protein was determined by incubating the purified AhTI in various buffers (Sodium acetate (pH 4.0), Sodium phosphate buffer (pH 6.0 and 7.6), Tris-HCl buffer (pH 8.0) and Glycine-sodium hydroxide buffer (pH 10) for 4h at 4°C. The results are given in Fig.4.9.

Figure 4.9 pH Stability



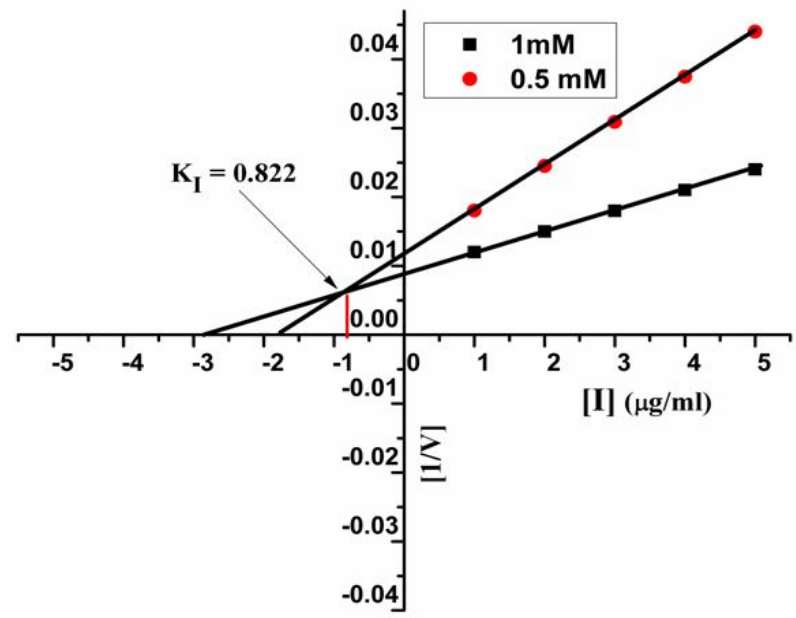
Specific activity of AhTI after treatment with buffers of different pH for 4h; n=6; Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p < 0.05$ considered statistically significant. F value=1.8×10^3.

At different pH the AhTI showed highest activity at pH 7.6 (2043.39±17.61TIU/mg protein).

4.3.10 Determination of Inhibitor constant from Dixon Plot

Trypsin Inhibitory activity of AhTI was determined at two different substrate concentrations and the inhibitor constant (K_I) was determined from the Dixon plot. The results are given in Fig. 4.10. For plotting the Dixon plot we used the software Origin Pro8. At the point where $[I] = K_I$, competitive inhibition produces straight lines that converge on the abscissa.

Figure 4.10 Dixon Plot



Trypsin Inhibitory activity of AhTI was determined at two different substrate concentrations and the inhibitor constant (K_I) was determined from the Dixon plot.

By analysing the Dixon plot, the nature of inhibition is found to be of competetive type and AhTI has a K_I value of 0.82µg/mL.

4.4 DISCUSSION

Because salting out separates proteins that readily combine from those that are very soluble, it is an appropriate first step in the purification of small soluble proteins (Englard and Seifter, 1990). Most proteins need at least a tiny concentration of salt to stay folded and stable, and very few proteins are soluble exclusively in water. Under conditions of extremely low salt, proteins with positively and negatively charged areas frequently self-aggregate (Scopes, 1993). The anions and cations in salt, however, balance charges on the protein surface and stop aggregation. Even more salt concentration will cause the protein's surface to become so charged that the protein molecules will re-aggregate (Arda *et al*., 1955).

For the purification of AhTI we have used the Ammonium sulphate precipitation method in the beginning stage to remove the non protein part. Different fractions (0-30%, 30-60% and 60-90%) were precipitated according to the protocol previously described. Amongst all the fractions, 30-60% fractions of the AhTI showed highest TI activity (439.69±5.11TIU [Fig.4.1]). Hence it was selected for further purification analysis. Similar results were reported by Mello *et al*., 2001 who purified second trypsin inhibitor (DMTI-II) from the seeds of *Dimorphandra mollis* a by ammonium sulphate precipitation (30-60%), ion-exchange and gel filtration chromatography.

After the ammonium sulphate precipitation, the 30-60% fraction was subjected to ion exchange chromatography using DEAE as the resin. The result of ion exchange chromatography shows that the

fractions 11-19 have significant protein content (Fig.4.2). Among these fractions, only the 14^{th} and 15^{th} showed TI activity and these were pooled, and the specific inhibitory activity of the pooled fraction is 458.67±5.049TIU/mL/mg protein.Using an isolation protocol that included ion exchange chromatography on DEAE-cellulose a 17.5-kDa trypsin inhibitor was purified by Li *et al*., 2017 from *Phaseolus vulgaris* cv. "gold bean." There are three categories of trypsin inhibitors from plant seeds: squash type (Telang *et al*., 2003) bowman-birk type (Kumar *et al*., 2002) and Kunitz type (Machado *et al*., 2013). The molecular masses of the three kinds are, respectively, nearly 20 kDa, 8 kDa, and 3 kDa. A low-yielding process of acid precipitation, cation exchange and anion exchange chromatography was used by (Chaudhary *et al*., 2008) to purify trypsin inhibitor from *Putranji varoxburghii* seeds. By utilising ammonium sulphate fractionation, followed by ion exchange, affinity, and gel filtration chromatography, Prasad *et al*., (2010) isolated Bowman-Birk proteinase and a proteinase inhibitor (BgPI) from blackgram, *Vigna mungo* seed.

After the ion exchange chromatography, the pooled fraction with highest TI activity was subjected to size exclusion chromatography. The result (Fig.4.3) shows that fraction 12 and 14 possessed protein content and the fraction number 12 is the only one with trypsin inhibitory activity. The Specific inhibitory activity of fraction 12 was 2048±27.3TIU/mL/mg protein. Several reports show the involvement of similar techniques for the isolation of TIs from plant and animal sources. Chen *et al*., (1973) isolated a trypsin inhibitor from sweet potatoes. The trypsin inhibitor from sweet corn

was extracted by lyophilization, chromatography using Sephadex G75 and CM-cellulose, extraction in diluted salt solution, and ammonium sulphate fractionation (Chen *et al*., 1973). *Enterolobium contortisiliquum* seeds were used to isolate a trypsin inhibitor using a variety of chromatographic techniques, including gel filtration with Sephadex G-75 resin (Batista *et al*., 1996). Using acid treatment and column chromatographies with Sephadex G-50, DEAE-Sephadex and Sephadex G-75, an inhibitor known as a cytosol thiol PI was isolated from rat liver cytosol (Hirado *et al*., 1981).

The SDS-PAGE analysis was used to determine AhTI's molecular weight. A standard curve drawn by log MW vs. R_f was used to calculate it (Fig.4.6). The graph yielded a molecular weight of 14.18kDa. The molecular weight measured by MALDI analysis (14. 28 kDa) and this value are fairly similar. The SDS-PAGE examination of a trypsin inhibitor isolated from *Murraya koenigii* after HPLC, gel filtration and anion exchange column (DEAE) revealed molecular weight around 27kDa (Shee *et al*., 2007). A novel inhibitor purified from potato tubers of was characterised by MALFI-TOF analysis (Cotabarren *et al*., 2018).

The fraction collected after the gel filtration chromatography was subjected to activity staining by reverse zymography as per the protocol described by Felicioli *et al*., The result (Fig.4.6) shows the presence of a single protein with trypsin inhibitory activity, which was confirmed using activity staining by reverse zymography. Alves *et al*., (2010) reported similar observations. From a crude protein mixture of

cotyledons of *Phaseolus vulgaris* seeds they identified nine novel TIs using a reverse zymography approach.

Thermal stability of AhTI was determined by incubating the purified fractions after gel filtration chromatography at different temperatures (37°C to 100°C for 20 min). AhTI showed stability up to 100°C but it was reduced when the temperature increased from 37°C. It showed the highest activity at 37°C and 40°C. Most of the TIs of plant origin showed similar range of temperature stability. Between 25 and 50°C, the gold bean trypsin inhibitor exhibited outstanding thermal stability, which diminished after the temperature reached 50°C (Li *et al*., 2017). Heat treatment alters the hydrophobic polymerization, precipitation and structure of TIs, which has an impact on the inhibitory activity (Wang *et al*., 2001). The dominanace of aromatic amino acid molecules, which are frequently associated with energy transfer and disulfide bonds, as well as reversible denaturation via transitory intermediate may further, contribute to the inhibitor's tolerance toward temperature and pH (Krishnan *et al*., 2015).

The pH stability of TI protein was detected by incubating the purified AhTI in various buffers for 4h at 4°C. The results showed that the optimal pH for AhTI is 7.6 (Fig.4.9).Similar studies reported that *Murraya koenigii* trypsin inhibitor is totally soluble at and above pH 7.5, with the maximum inhibition seen at pH 8.0 (Shee *et al*., 2007).

The type of inhibition by AhTI is found to be of competetive type (Fig.4.10) and AhTI has a K_I value of 0.82µg/mL. A measure of an inhibitor's strength is the inhibitor constant, K_I which is the

concentration needed to generate half the maximum inhibition. Dixon plot data were used used by several investigators to calculate the K_I value of TIs. *S. aculeatissimum* PI (SAPI) dissociation constant (K_I) value and mechanism of inhibition was determined by Krishnan *et al*., (2015). According to the findings, the SAPI exhibits competitive inhibition, which is a characteristic of competitive inhibition where two lines that correspond to different substrates overlap above the x-axis.

In this part of the study, a trypsin inhibitor protein from the seeds of *A.hirsutus* was purified and characterised by the applying relevant methods.

Chapter 5

Purification and characterization of TI from *Garcinia gummi-gutta* (L.)Roxb. (GgTI)

Since the seeds of *A.hirsutus* and *G.gummi-gutta* showed significant trypsin inhibitory activity among the five seeds selected for screening, both these seeds were used for the isolation, purification and characterisation of TI proteins.

5.1 OBJECTIVES

The purification and characterization of the trypsin inhibitor protein extracted from the seeds of *Garcinia gummi-gutta* (L.) Roxb. are the major objectives of this part of the study. The parameters focused on and the techniques used are the same as that described in the previous chapter, for the purification and characterization of AhTI.

5.2 MATERIALS AND METHODS

The protocols followed for the purification and isolation of GgTI Ammonium Sulphate precipitation, Dialysis, Ion exchange chromatography using DEAE cellulose, Gel filtration chromatography using Sephadex G-75, Calculation of yield and fold of purification, SDS-PAGE, Activity staining by Reverse Zymography, Determination of pH stability and thermal stability, Determination of Inhibitor constant from Dixon Plot, Molecular weight confirmation by MALDI TOF/TOFare as described in detail in Chapter IV (4. 2).

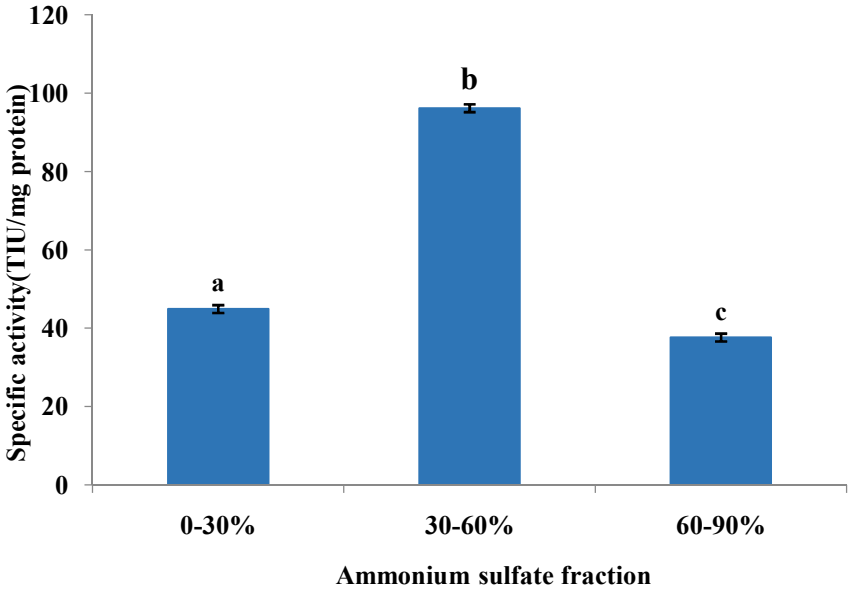
5.3 RESULTS

5.3.1 Ammonium Sulphate Precipitation

At 4°C, ammonium sulphate precipitation was carried out in fractions of 0-30, 30-60, and 60-90%. The protein precipitate from each fraction was then resuspended in 50mM sodium phosphate buffer

(pH 7.6) and dialyzed at 4°C against the same buffer after ammonium sulphate precipitation. Encor Biotechnology Inc. (2018) provided an online ammonium sulphate calculator that was used to calculate the precipitation (https://www. encorbio. com/protocols/AM-SO4. htm). The outcomes are presented in Fig.5.1.

Figure 5.1 Specific activity after Ammonium sulphate precipitation and dialysis

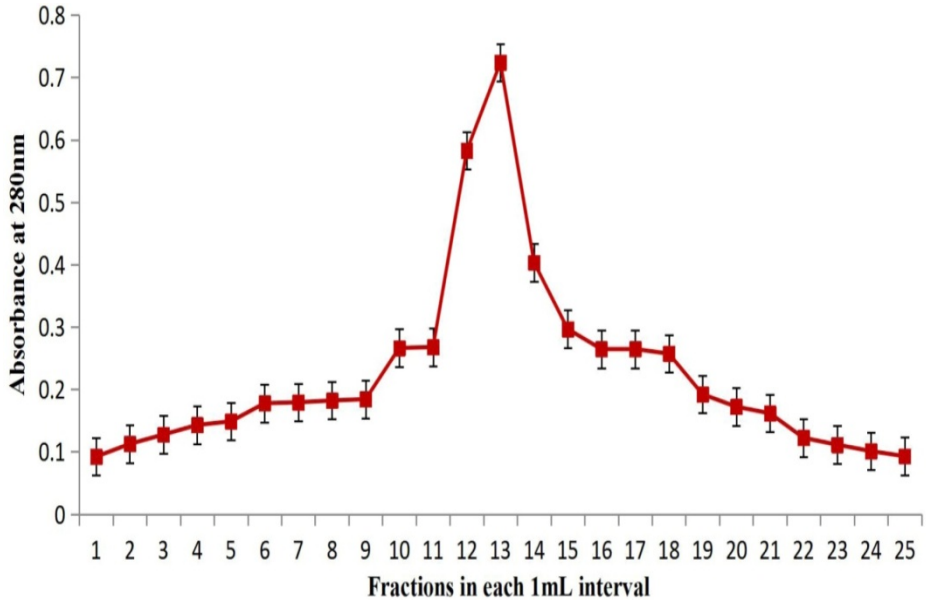


Fraction 30-60% showed highest TI activity of 96.1±0.43 (TIU/mg protein); Values are Mean± Standard Deviations. n=6; $p<0.05$ considered statistically significant. F value = 3.4.

5.3.2 Purification Ion exchange chromatography

Fig.5.2 represents the chromatogram obtained for GgTI. The ammonium sulphate fraction with highest activity was further purified by ion exchange chromatography. The active fraction was lyophilised in 1mL aliquot, re-suspended in sample buffer and chromatographed using DEAE cellulose as the anion exchange resin.

Figure 5.2 Purification by ion exchange chromatography

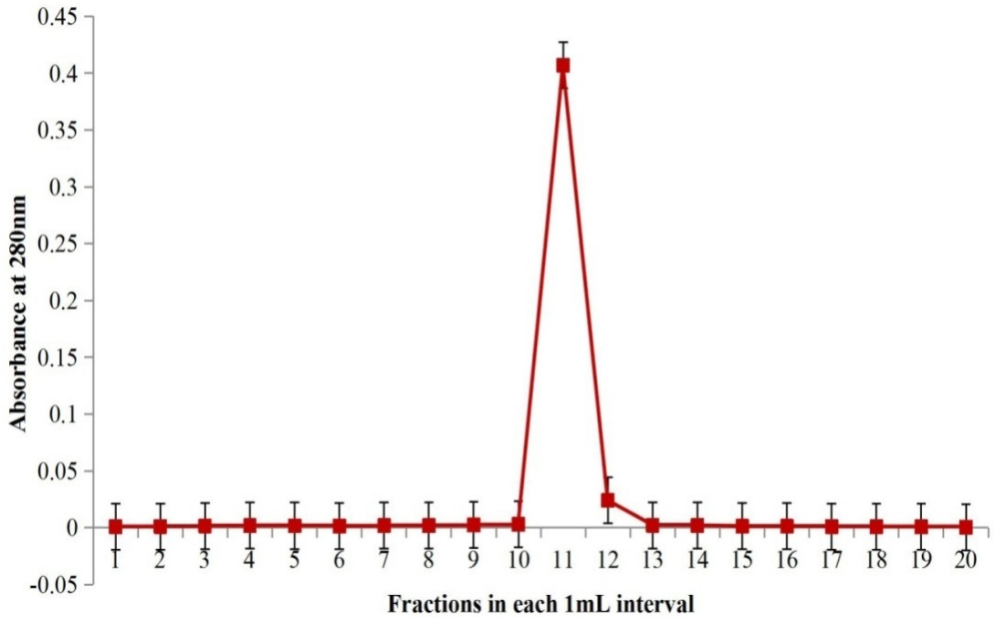


Fractions 9-20 were the fractions having measurable protein content. The fractions 13, 14 and 15 showed TI activity among these. They were pooled and the Specific inhibitory activity of the pooled fraction is 259.26±2.66 TIU/mg protein.

5.3.3 Purification by Gel filtration chromatography

The fractions of GgTI after ion exchange chromatography with highest activity were used for further purification by size exclusion chromatography. The resin used was Sephadex G75. Fig.5.3 shows the results.

Figure 5.3 Purification by gel filtration chromatography



Fraction 12 is the only one with trypsin inhibitory activity. The specific inhibitory activity of fraction 12 is 281.74± 0.13 TIU/mg protein.

5.3.4 Yield and fold of Purification

Yield and fold of purification was determined after ech step of purification. The results are shown in Table 5.1.

Table 5.1 Yield and fold of Purification

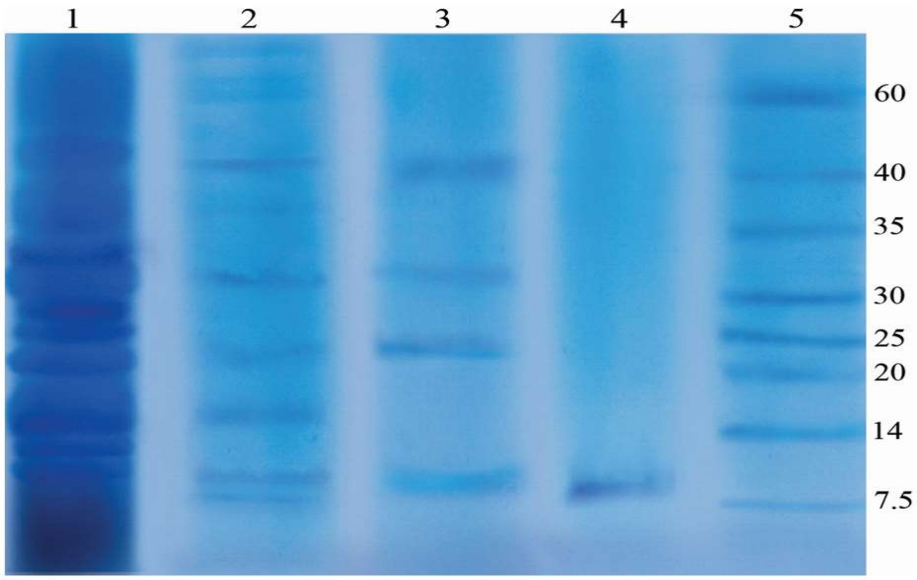
Sample	Total protein (mg/mL)	Trypsin inhibitor activity (TIU/mL)	Specific inhibitor activity (TIU/mL)	Yield of activity %	Fold of purification
Crude extract	0.62± 0.015	51.72± 0.023	83.42± 0.026	100	1
Ammonium sulphate fraction (30-60%)	0. 2± 0.010	19.22± 0.012	96.1± 0.436	37.1	1.15
DEAE Cellulose	0.054±0.003	14± 0.115	259.26± 0.151	27.1	3.11
Sephadex G 75	0.023± 0.002	6.48±0.097	281.74± 0.131	12.5	3.38

Values are Mean± Standard Deviations. n=3; $p<0.05$ considered statistically significant. Fvalue for Crude extract=$1.7x10^3$, Ammonium sulphate fraction (30-90%) =$1.4x10^3$, DEAE Cellulose=$1.3x10^3$, Sephadex G75=$1.5x10^3$

5.3.5 Molecular weight determination by SDS-PAGE

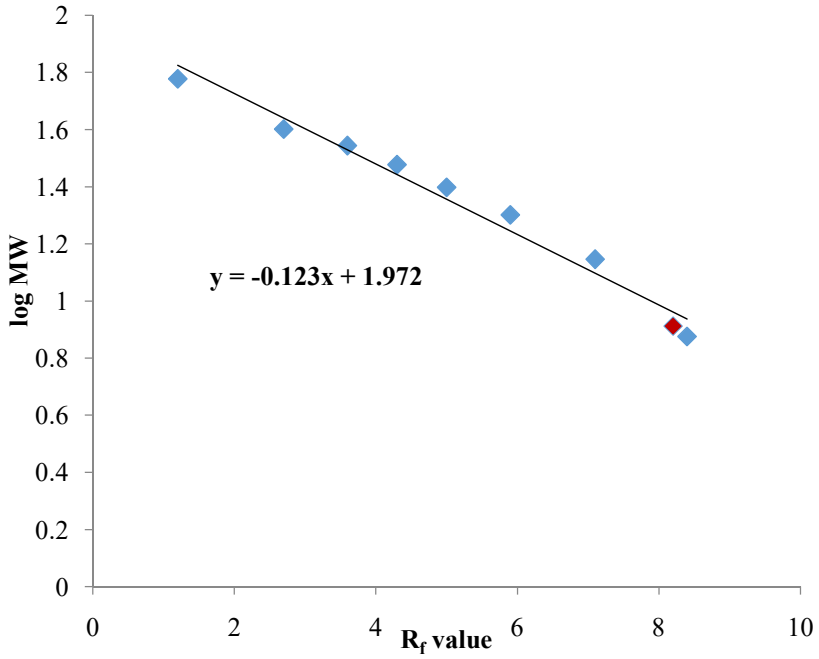
The molecular weight of the GgTI protein was determined by SDS-polyacrylamide gel electrophoresis followed by the protocol described by Laemelli (1970). A 12% resolving gel and 4% stacking gel were prepared for the same. The Fig.5.4 and Fig.5.5 indicate the results.

Figure 5.4 SDS PAGE of fractions obtained after different steps of purification



SDS PAGE was done in 12% gel. Lane 1: crude extract, Lane 2: 30-60% Ammonium sulphate precipitated dialysed fraction, Lane 3: DEAE Ion exchange fraction, Lane 4: Sephadex G-75 Size exclusion fraction and Lane 5: Protein molecular weight marker (7.5-60kDa).

Figure 5.5 Molecular weight determination from standard curve



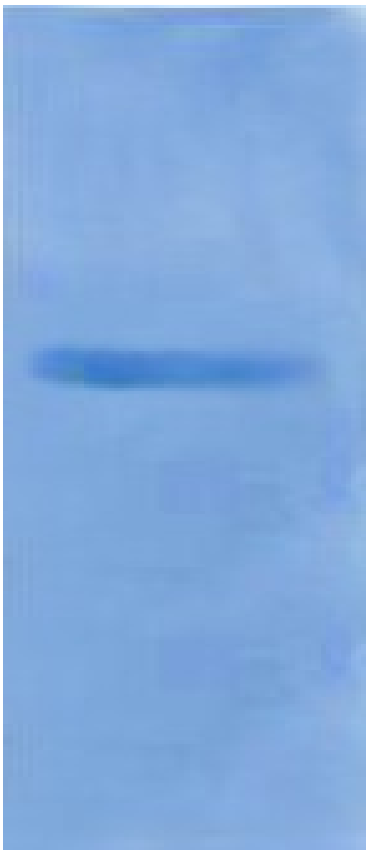
The molecular weight was calculated from a standard curve plotted by log MW (of standard protein markers) versus R_f.

Molecular weight of GgTI determined from the standard curve plotted by log MW (of standard protein markers) versus R_f is 8.18kDa.

5.3.6 Activity staining by Reverse Zymography

The trypsin inhibitor protein eluted from gelfiltration chromatography was subjected to activity staining by reverse zymography. The result is represented in Fig.5.6.

Figure 5.6 Activity staining by reverse zymography

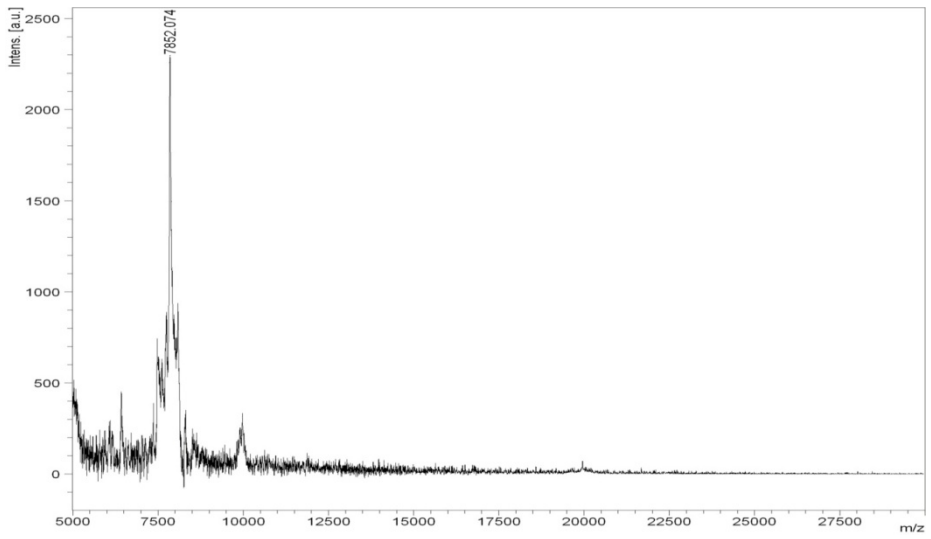


The result showed the presence of a single protein with trypsin inhibitory activity.

5.3.7 MALDI-TOF/TOF analysis

The molecular weight of the GgTI protein determined from SDS PAGE was alsoconfirmed by the result of MALDI-TOF analysis (Fig.5.7).

Figure 5.7 MALDI-TOF/TOF Spectrum



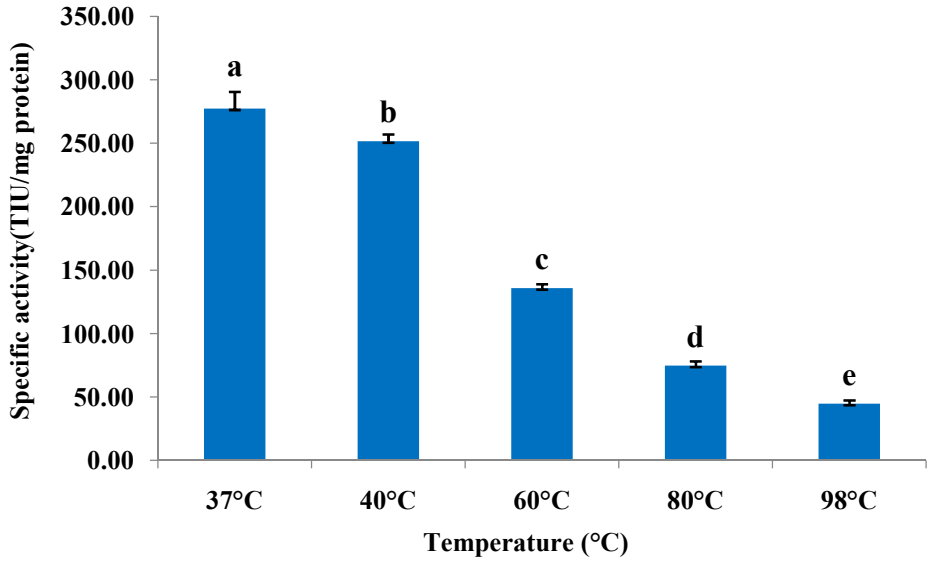
Molecular weight confirmation by MALDI-TOF analysis indicated that GgTI has a molecular weight of 7.85kDa.

The Fig.5.5 shows the result of molecular weight of purified TI protein in SDS PAGE after gel filtration chromatography and the molecular weight confirmed from the graph (Fig.5.5) is 8.18kDa and that from MALDI analysis (Fig.5.6) is 7.85kDa.

5.3.8 Determination of thermal Stability

The stability of GgTI protein under different temperature was analysed. The TI protein was incubated at different temperatures (37°C, 40°C, 60°C, 80°C and 98°C). The results were shown in the (Fig.5.8).

Figure 5.8 Thermal stability



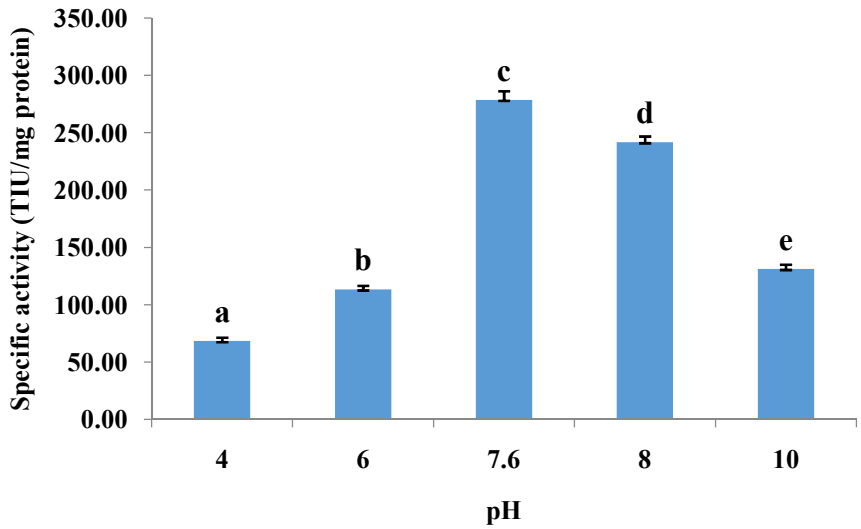
Specific activity of GgTI after treatment at different temperatures for 20min/h; n=6; Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p < 0.05$ considered statistically significant. F value=235.4.

The result of thermal Stability of GgTI at different temperatures reveals that the GgTI retains activity up to 98°C and the highest activity is seen at 37°C.

5. 3.9 Determination of pH Stability

TI activity was determined after incubating the purified GgTI in various pH buffers (Sodium acetate (pH 4.0), Sodium phosphate buffer (pH 6.0 and 7.6), Tris-HCl buffer (8.0), and Glycine-sodium hydroxide buffer (pH 10) for 4h at 4°C. The optimal pH is found to be 7.6 (Fig.5.9).

Figure 5.9 pH Stability



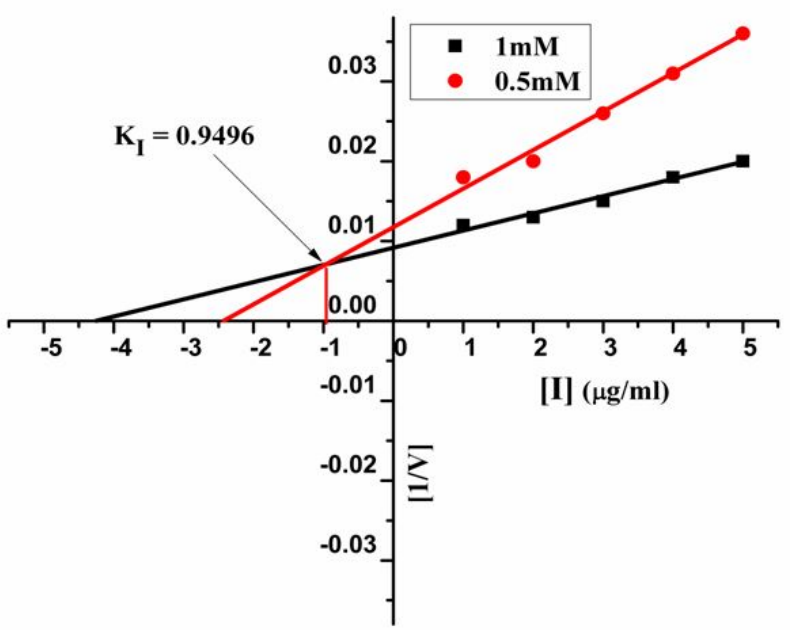
Specific activity of GhTI after treatment with buffers of different pH for 4h; n=6; Error bars indicates Standard Deviations, (not visible when smaller than symbol size). $p < 0.05$ considered statistically significant. F value=365.67.

At different pH the GgTI showed highest activity at pH 7.6 (278.65±6.0 TIU/mg protein). The TI activity at pH 4.0 is 68.40±1.50 and at pH 10 it is 131.28±1.21TIU/mg protein.

5.3.10 Determination of Inhibitor Constant from Dixon Plot

Inhibitor constantof GgTI was determined at two different substrate concentrations (1mM and 0.5mM) by varying inhibitor concentrations (1µg/mL, 2µg/mL, 3µg/mL, 4µg/mL and 5µg/mL) and the inhibitor constant (K_I) was determined from Dixon plot (Fig.5.10).

Figure 5.10 Dixon Plot



Trypsin Inhibitory activity of AhTI was determined at two different substrate concentrations and the inhibitor constant (K_I) was determined from the Dixon plot. The K_I value is 0.95µg/mL.

By analysing the Dixon plot (Fig.5.10), the nature of inhibition isfound to be of competetive type and GgTI has aK_I value of 0. 95µg/mL. Dixon plot was plotted using the software Origin Pro8.

5.4. DISCUSSION

The initial stage of purification have done with ammonium sulfate fractionation. Salting out is frequently used for the separation and enrichment of proteins. The result of ammonium sulphate precipitation **(**Fig.5.1) showed that all fractions showed TI activity. Among all the fractions the 30-60% fraction showed highest TI activity. The Specific activity of GgTI after Ammonium sulphate precipitation and dialysis was 49.64±4.88 (TIU/mg protein) and it was selected for further purification and studies. The hydration membrane of proteins can be destroyed by salt ions in extremely concentrated salt solution, which decrease the solubility of the protein and separate it from other contaminants like sugars (Duong *et al*., 2014).

After ammonium sulphate fractionation, the fraction with the highest TI activity was subjected to ion exchange chromatography with DEAE as anion exchanger resin. The fractions 13, 14, and 15 exhibited TI activity. The specific inhibitory activity of the pooled fraction is 259.26±2.66 TIU/mg protein. It was used for further purification. By using ion-exchange chromatography, seven proteinase inhibitors were extracted from winged bean seeds (Shibata *et al*., 1986). The*Phaseolus vulgaris* bean extract was split into three fractions of roughly comparable size after ion exchange chromatography on DEAE-cellulose (Li *et al*., 2017).

The fraction collected from ion exchange chromatography was assayed to check the TI activity. The fraction with highest TI activity was then subjected to size exclusion chromatography using sephadex-

G-75 as the resin. The result of gel filtration chromatography (Fig.5.3) showed that the fraction 12 was the only one with trypsin inhibitory activity. The Specific inhibitory activity of Fraction 12 was 281.74±0.131TIU/mL/mg protein.Following that, we performed poly acrylamide gel electrophoresis to determine the molecular weight of the TI protein. On the polyacrylamide gel electrophoresis with sodium dodecyl sulphate (SDS), the purified inhibitor produced a single protein band. The result (Fig.5.4 and 5.5) showed a single band with a molecular weight of 8.18kDa which was determined from a standard curve by the log MW versus R_f value. The molecular weight was again confirmed as 7.85 KDa by MALDI-TOF/TOF analysis (Fig.5.7).This fraction was then subjected to activity staining by reverse zymography for the confirmation of the presence of TI protein. The result of activity staining (Fig.5.6) showed the presence of a single protein as single band against a clear back ground. Reverse zymography is a sensitive method to find proteinase inhibitors. Proteins are renaturated, after which the gel is treated with metalloproteinases, which break down the substrate inserted into the gel. After staining, inhibitors are visible as black inhibition zones against a transparent backdrop (Hawkes *et al*., 2010). SDS-polyacrylamide gel electrophoresis and gel filtration on Sephadex G-75 revealed the inhibitor's molecular weight to be 12.4kDa (Hirado*et al*., 1981). Mung bean yielded a single trypsin inhibitor peak (Wilson *et al*., 1983) by gel filtration on Sephadex G-75 (5 x 95cm in 50mm Tris-Cl, pH 8.0).

The result of thermal studies revealed that (Fig.5.8) the TI activity decreases with increasing temperature, however it retains its

activity even at high temperatures. The purified TI was incubated with different temperatures from 37°C to 100°C. The highest TI activity was found at 37°C. It has been reported that metallo carboxypeptidase inhibitors are heat-stable molecules (Obregon *et al*., 2012). Thermal treatment of the crude extract of potato varieties (Lufrano*et al*., 2015) was carried out at 60, 70, 85 and 100°C for 60 min.

The purified TI fractions after gel filtration chromatography was treated with different pH buffers for 4h at 4°C. The highest TI activity found was at pH 7.6 (Fig.5.9).

The kinetic studies also were done by determining the Trypsin Inhibitory activity of GgTI at two different substrate concentrations and the inhibitor constant (K_I) was determined from the Dixon plot. By analysing the Dixon plot, the nature of inhibition was found to be of competetive type and the K_I value determined of 0.95µg/mL. According to kinetic studies, the *Murraya koenigii* trypsin inhibitor is a competitive inhibitor (Shee *et al*., 2007).

Chapter 6

Studies on the effect of AhTI and GgTI on skin cancer (A431) cell line

Any of the several illnesses characterised by the growth of aberrant cells that divide out of control and have the capacity to invade and destroy healthy bodily tissue are referred to as cancers. The propensity of cancer to spread throughout the body is common. In the millions of cells that make up the human body, cancer can develop practically anywhere. They result from the growth of abnormal cells that can invade or disseminate to different areas of the body. Skin cancer accounts for at least 40% of all cancer cases worldwide, making it the most prevalent type of cancer. Sometimes other histologies including squamous, adenosquamous, carcinoid and lymphoid tumors are found. Squamous cell tumours are extremely rare, making it challenging to research their development and therapeutic response. Merkel cell carcinoma (MCC), melanoma, squamous cell carcinoma, and basal cell carcinoma are the four main kinds of skin cancer. Exposure to UV light from the Sun is to blame for more than 90% of instances. Apoptosis is a highly controlled process of cell death that happens naturally during development. Apoptosis that is improperly controlled is connected to conditions like cancer and Alzheimer's disease. Inducing apoptosis is a very helpful method for treating such a condition.

TI proteins from *A.hirsutus* and *G.gummi-gutta seeds were* purified by ammonium sulphate fractionation, dialysis, ion exchange chromatography, and size exclusion chromatography. The molecular weight of the proteins was determined by SDS-PAGE which was confirmed by MALDI-TOF/TOF analysis. The TI activity was confirmed by activity staining by reverse zymography. The thermal

and pH stability of the TI protein were determined and are discussed in the previous chapters. This chapter includes an analysis on the effectiveness of isolated, purified TIs against skin cancer (A431) cell lines.

6.1 OBJECTIVES

The main focus of this part of the study is to investigate the anticancer potential of purified trypsin inhibitor proteins against skin cancer (A431) cell lines. The specific objectives of the study is to check the cytotoxicity of the purified TI proteins (AhTI and GgTI) against skin cancer cell lines by MTT assay, LDH leakage assay, and neutral red uptake assay. The next aim is to find out the DNA damage of the cells after the treatment with isolated TI proteins and to detect the apoptosis-inducing capacity of TI proteins by flow cytometry analysis. Soybean Trypsin Inhibitor (STI) was used for comparison in this part, since it is the well-studied TI from plant sources.

6.2 MATERIALS AND METHODS

6.2.1 Materials

Dulbecco's Modified Eagle *Medium* (DMEM), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) and Dimethylsulfoxide (DMSO) were procured from Sigma-Aldrich, USA. Trypsin, Fetal bovine serum (FBS), Sodium bicarbonate, L-glutamine and an antibiotic solution containing Streptomycin, Penicillin, and Amphoteracin were from Merck, Germany. All other

basic chemicals including BSA were purchased from SRL, Inda and Himedia, India.

Cell lines Used

1. Skin Cancer (A431): Human Skin cancer cells.
2. Normal (L929): Cells derived from normal subcutaneous areolar adipose tissue of mouse.

All the cell lines were purchased from The National Centre for Cell Sciences (NCCS), Pune, India and were cultured in DMEM (Dulbecco's modified Eagles media)- (Sigma-Aldrich, USA).

6.2.2 Anti-proliferative activity detection

The anti-proliferative activity of the TIs on skin cancer cells was detected by different cytotoxicity assays. For the determination of cytotoxicity and cell viability of the cells after treatment with the TIs (AhTI, GgTI and STI) MTT assay, LDH leakage assay and Neutral red uptake assay were performed. STI is used for comparison since it is a well studied TI isolated from plant source whose antiproliferative activity has already been reported. Detection of apoptosis in Skin Cancer (A431) cell lines after AhTI and GgTItreatment was done by Annexin V FITC Flow Cytometry analysis**.** Comet assay also was done for the evaluation DNA damage.

Each cell line was grown in a 25cm^2 tissue culture flask with DMEM supplemented with 10% FBS, Sodium bicarbonate (Merck, Germany), L-glutamine and an antibiotic solution comprising

Streptomycin (100µg/mL), Penicillin (100U/mL) and Amphoteracin B (2.5µg/mL). Cell lines were grown in a humidified 5% CO_2 incubator (NBS Eppendorf, Germany) at 37°C. By directly viewing the cells under an inverted phase contrast microscope and using MTT assay method afterward, the viability of the cells was assessed.

i. Seeding of cells in 96-well plate

96-well tissue culture plates were seeded with a 100µL cell suspension ($5x10^3$cells/well) that had been trypsinized and suspended in 10% growth media. The plates were then incubated at 37°C in a humidified 5% CO_2 incubator.

ii. Preparation of compound stock

After the cells were attached, the growth medium was replaced with 100µL of fresh medium. Each freshly prepared compound (AhTI and GgTI) was dissolved in 5% DMEM at different concentrations. A cyclomixer was used to mix the sample after it had been added to DMEM. To ensure sterility, the sample solution was filtered via 0.22µm Millipore syringe filter.The solutions were made in such a way that the final concentrations were 100, 50, 25, 12.5 and 6.25µg/mL, when 100µL of each concentration was added in triplicate to the appropriate wells. The mixture was then incubated at 37°C in an environment that was humidified with 5% CO_2. Control cells that hadn't been received any treatment were also kept.

6.2.2.1 Direct Microscopic observation

All of the plates were examined under an inverted phase contrast tissue culture microscope (Olympus CKX41 with an Optika Pro5 CCD camera) after the treatment for 24 h and microscopic observations were captured as photographs. Any observable morphological changes of the cells, such as shrinking or rounding of cells, granulation and vacuolization in the cytoplasm of the cells are all counted as signs of cytotoxicity.

6.2.2.2 MTT Assay

a. Reagents

- Phosphate buffered saline
- DMSO
- MTT solution(5mg/mL in PBS)

b. Procedure

After being thoroughly dissolved in 3mL of phosphate buffered saline, 15mg of MTT (Sigma, M-5605), was reconstituted and sterilised through filtering. After the initial 24h period of incubation, the sample content in the wells was removed and 30µL of reconstituted MTT solution was added to each test and cell control well. Then the plate was gently shaken and incubated at 37°C in an environment with humidified 5% CO_2 for 4h. Following the incubation period, the supernatant was taken out and 100µL of MTT solubilization solution

(DMSO, Sigma-Aldrich, USA) was added. The wells were then gently agitated by pipetting up and down in order to solubilize the formazan crystals. At a wavelength of 540 nm, the absorbance values were calculated using a microplate reader (Talarico *et al*., 2004).

The following formula was used to calculate the percentage of growth inhibition:

$$\% \text{ of viability} = \frac{\text{Mean OD Samples} \times 100}{\text{Mean OD of control group}}$$

6.2.2.3 Lactate dehydrogenase assay (LDH leakage assay)

a. Reagents

- Potassium phosphate buffer (pH 7.4)
- Sodium pyruvate solution (2.063 mM)
- 6 mM NADH solution

b. Procedure

The activity of lactate dehydrogenase, a quantitative marker enzyme for the entire cell, reveals the extent of the cell's glycolytic potential. LDH release (leakage) measurement is a crucial and commonly used diagnostic for severe irreversible cell damage. The LDH leakage assay was carried out according to the method followed by Andry and Erivch's (2006). How efficiently a cell can utilise glucose depends on the activity of lactate dehydrogenase, a quantitative marker enzyme for the complete cell. Measuring LDH

release is a critical and popular diagnosis for severe irreversible cell damage (leakage).

A test for LDH release was performed using cell-free supernatant taken from cell culture plates that had been exposed to different concentrations of AhTI and GgTI (6.25, 12.5, 25, 50, and 100µg/mL). A cuvette was used to combine this with 2.7mL of potassium phosphate buffer (pH 7.4), 0.1mL of 6 mM NADH solution and 0.1mL of sodium pyruvate solution (2.063 mM).The decrease in optical density was noticed at 340 nm in a spectrophotometer set to a temperature of 25°C. By replacing the sample with enzyme dilution buffer, the blank solution was prepared. The formula shown below was used to calculate lactate dehydrogenase activity.

$$\text{Activity of LDH}\left(\text{U/ mL}\right) = \frac{\left[\left(\text{Abs} - \text{Ab0}\right) \text{ x } 1\left(\text{mL}\right) \text{ x df}\right]}{\left[6.2 \text{ x } 0.1\left(\text{mL}\right)\right]} \left(\text{Where df is the dilution factor}\right)$$

6.2.2.4 Neutral Red Uptake Assay

a. Reagents

- Neutral red
- 1% acetic acid
- 50% ethanol

b. Procedure

The number of live cells in a culture can be quantitatively estimated using the neutral red uptake assay. It is one of the most employed cytotoxicity assays, with a wide range of biological and

environmental uses. It is predicated on how efficiently functioning cells can bind and integrate the supravital dye neutral red in lysosomes. The process is more affordable and accurate than other cytotoxicity testing methods (tetrazolium salts, enzyme leakage or protein content). The experiment can be finished in less than 3h after the cells have been treated.

The Neutral Red Uptake Assay was carried out using the method followed by Borenfreund *et al.* (1984). Cytotoxicity tests with neutral red were conducted based on how well living cells could absorb and bind neutral red. A positively charged dye known as neutral red rapidly diffuses across the cytoplasm and is stored in the lysosomes' acidic environment. Neutral red is only absorbed and bound by living cells; in dead cells, this ability is diminished or damaged. As a result, the number of living cells in the cell culture was strongly correlated with the amount of accumulated neutral red. KH_2PO_4 was added to the neutral red solution to bring the pH down to 6.35 (1M). Culture plates with the skin cancer cells were treated with different concentrations of AhTI and GgTI (6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL and 100µg/mL). Added 10µLof neutral red solution and the plates were incubated for 3h at 37°C in a CO_2 incubator. After that, cells were fixed with 200µL of the fixing solution (50% ethanol and 1% acetic acid) and cleaned with PBS. Following the 1min fixation period, the dye was extracted by thoroughly mixing 200µLof acidified ethanol solution. After that the plates were kept at room temperature

for 20 min. Using a microplate reader, the absorbance at 540nm was measured and the percentage viability was determined.

$$\% \text{ of cell viability} = \frac{\text{mean absorbance of sample}}{\text{mean absorbance of control}} \times 100$$

6.2.3 Comet Assay for the evaluation of DNA damage

a. Reagents

- 0.75% NMA (Normal Melting point Agarose)
- 1% SDS
- 12.5% NaCl
- 100 mM Na_2EDTA
- 10 mMTris Base (pH 10)
- 1% Na_2EDTA
- 300 mMNaOH
- 10% DMSO
- Ethidium bromide
- 1% Triton X 100

b. Procedure

The skin cancer (A431) cell lines were cultured in accordance with the earlier specified standard protocol. The IC_{50} concentrations of

both the samples were added (10.875µg/mL of AhTI and 15.625µg/mL of GgTI) to the cells ($1x10^4$) and incubated for 24h. After incubation the cells were trypsinized and used for the comet assay after being washed with fresh medium. A pre-coating of 1mL of 0.75% NMA (Normal Melting point Agarose, Invitrogen, USA) was applied to fully frosted microscope slides before they were placed in storage at 4°C. Before usage, this layer was removed and 120µL of 0.75% NMA was pipetted onto the slides. The slides were then covered with cover slips. 5-30µL of cell suspension were combined with 10µL of low melting point agarose (Invitrogen, Novex) and pipetted over the top of the agarose layer. 10µL of low-melting-point agarose (Novex, Invitrogen) were combined with cell suspensions ($1x10^4$/5-30µL) and pipetted over the top of the first layer of agarose. Finally, 80µL of NMA was employed as a protection layer. The slides were incubated at 4°C for 10 min after each stage to allow the agarose to set.

Slides were immersed in a cold lysing solution that contained 1% SDS, 12.5% NaCl, 100mM Na_2EDTA, 10mMTris Base (pH 10), and 1% Na_2EDTA. Immediately before usage, 10% DMSO and 1% Triton X 100 were also added to the solution. Slides were immersed in electrophoresis buffer (300mM NaOH and Na_2EDTA, pH 13) for 20 min following lysis to allow DNA to unwind. The same buffer was used for electrophoresis, and an electrical supply was used to provide an electric current of 0.8V/cm (300mA) for 20 min (Power case, Life Technologies). Slides were then dried and stained with 50µL of ethidium bromide (20µg/ mL) after being washed three times for 5 min

each in neutralisation buffer (0.4µLTris, pH 7.5). Using an Opitka Pro5 CCD camera and an Olympus CKX41 Inverted Epifluorescent Microscope, the slides were imaged. Tritek comet scoring software was used to score comets, and the results were statistically correlated.

6.2.4 Annexin V-FITC Flow Cytometry

a. Reagents

- Muse^{TM}Annexin V &7-AAD (7-amino-actinomycin D) Reagent

b. Procedure

Necrosis or unintentional cell death,is distinguished from apoptosis by distinctive morphological and biochemical alterations, such as nuclear chromatin compaction and fragmentation, cytoplasmic shrinkage and loss of membrane integrity. Flowcytometry can identify the Annexin V-labeled fluorescein, which binds selectively to the phosphatidyl serine (PS) on the outer leaflet apoptotic cell membrane. Nuclear dyes like PI, DAPI, and 7-AAD effectively block DNA binding and have a high DNA binding constant. During flow cytometric analysis, it is helpful for differentiating dead cells. Nuclear dyes can penetrate cells to stain DNA due to late apoptosis or loss of membrane integrity in necrotic cells. When combined with Annexin V, nuclear dyes can be used to discriminate between cells in different apoptotic phases.

The Muse^{TM}Annexin V flowcytometry analysis was done by the protocol followed by Schmidt *et al*., 1992. The A431 cell line was

cultured with the earlier specified routine protocols, and it was then given treatments at the IC_{50} concentrations (10.875µg/mL of AhTI and 15.625µg/mL of GgTI), which were allowed to act for 24h. After incubation the cells were trypsinized and 100µL of the cell suspension was divided among different tubes. Each tube was filled with 100µL of the Muse^{TM}Annexin V &7-AAD (7-amino-actinomycin D) Reagent. The tubes were completely mixed by pipetting up and down or vortexing at a medium speed for 3 to 5 sec, and then they were incubated in dark for 20min at room temperature. A flow cytometer was used to study the cells. The Muse FCS 3.0 software was used to evaluate cells for apoptosis while gating them against untreated control cells.

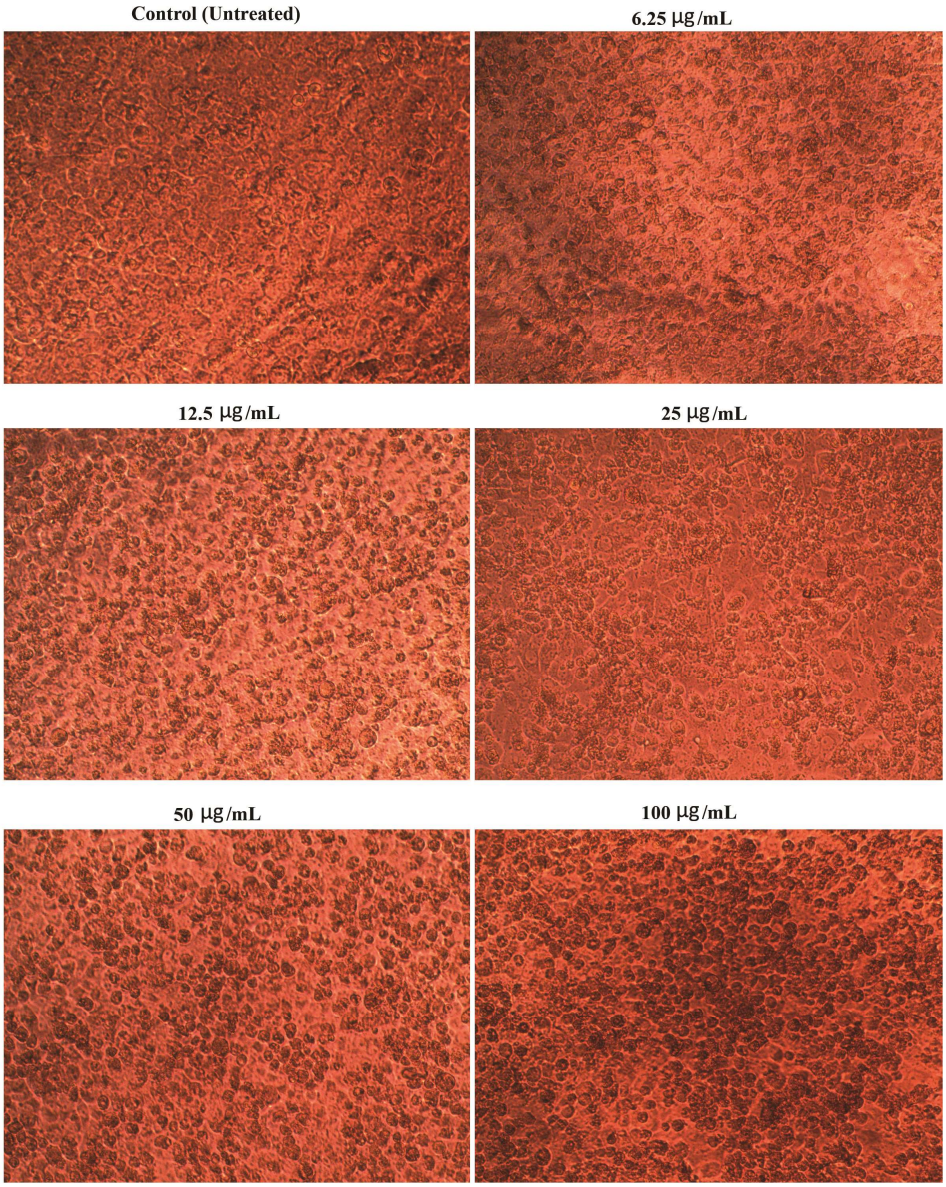
- non-apoptotic cells: Annexin V (-) and 7-AAD (-)
- early apoptotic cells: Annexin V (+) and 7-AAD (-)
- late stage apoptotic and dead cells: Annexin V (+) and 7-AAD (+)
- mostly nuclear debris: Annexin V (-) and 7-AAD (+)

6.3 RESULTS

6.3.1 Effect of AhTI, GgTI and STI on cell morphology

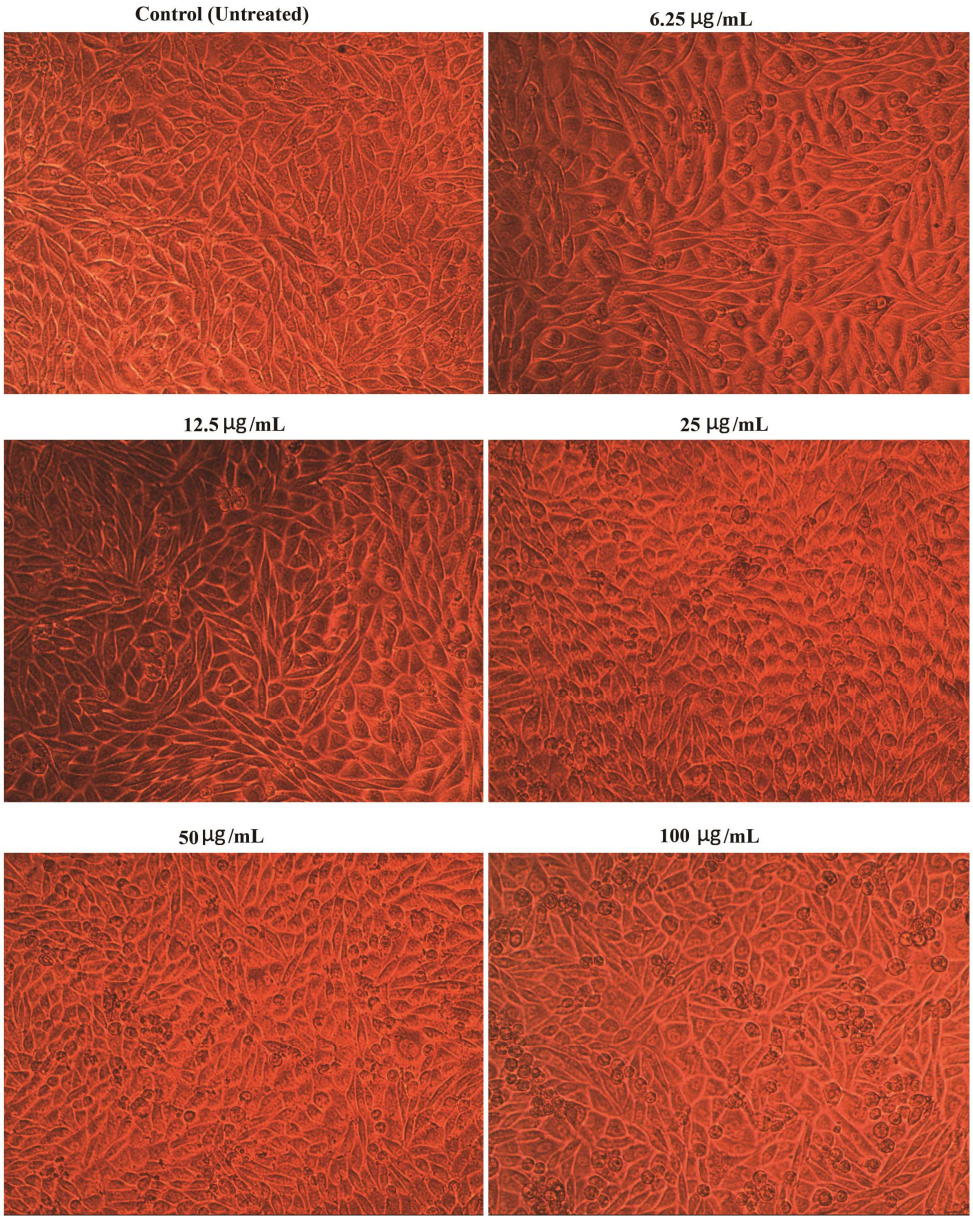
Changes in cell morphology were observed after 24h of incubation with different concentrations of the TIs by direct observation of cells through an inverted phase contrast microscope (Olympus CKX41 with an Optika Pro5 CCD camera). Normal cells (L929) were used for comparison. Figures 6.1 to 6.6 represent the results.

Figure 6.1 Skin cancer cells (A431) treated with different concentrations of AhTI



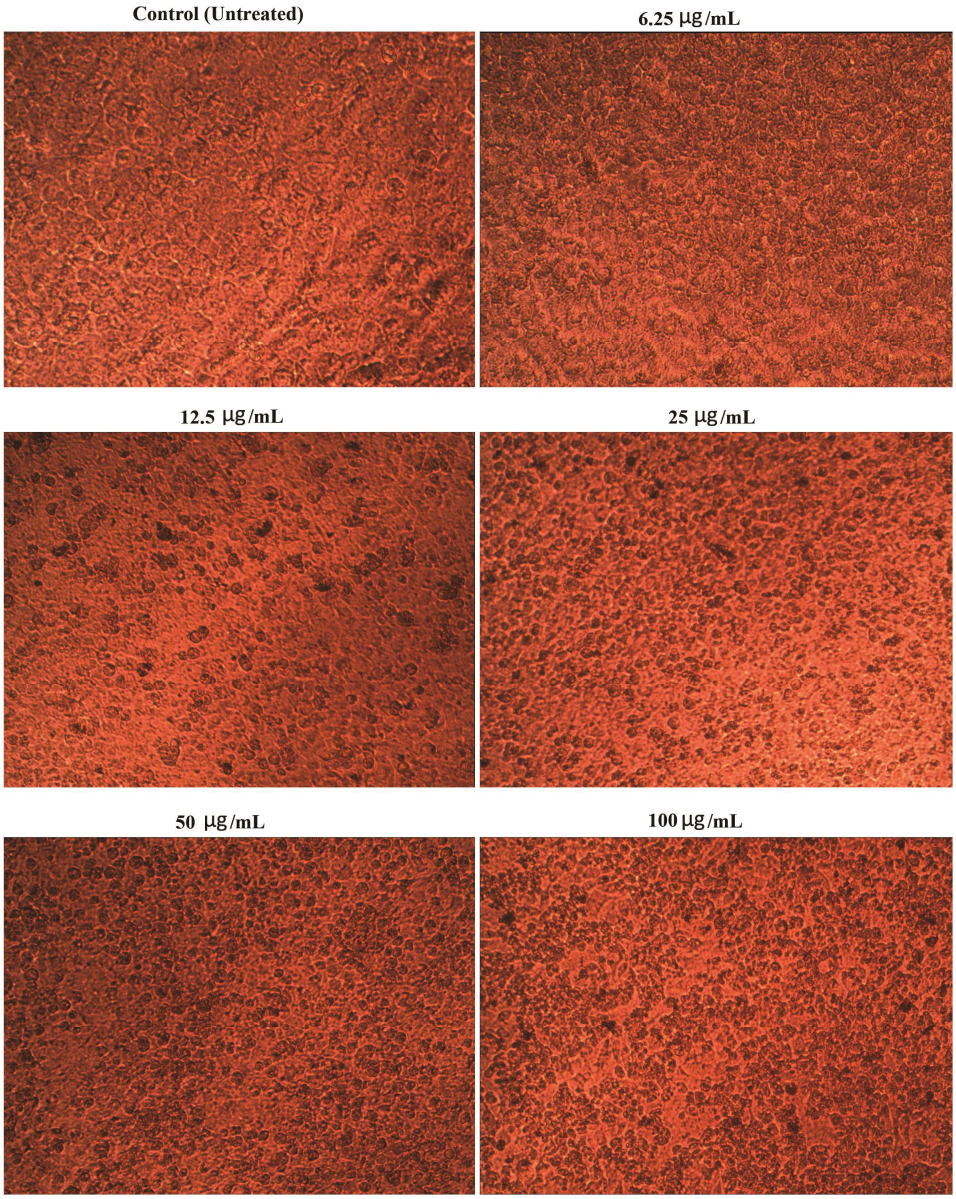
Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 6.2 Normal cells (L929) cells treated with different concentrations of AhTI



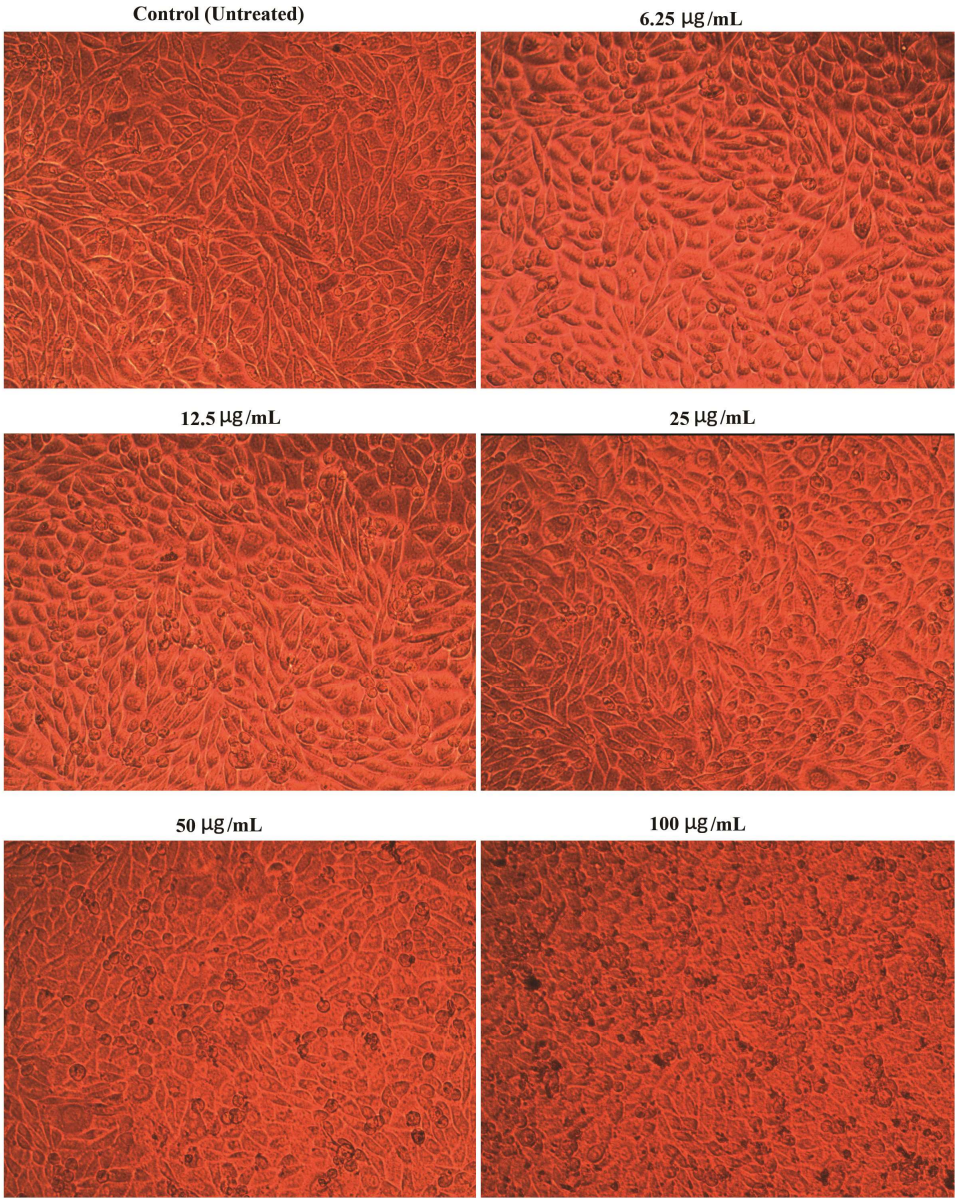
Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 6.3 Skin cancer cells (A431) treated with different concentrations of GgTI



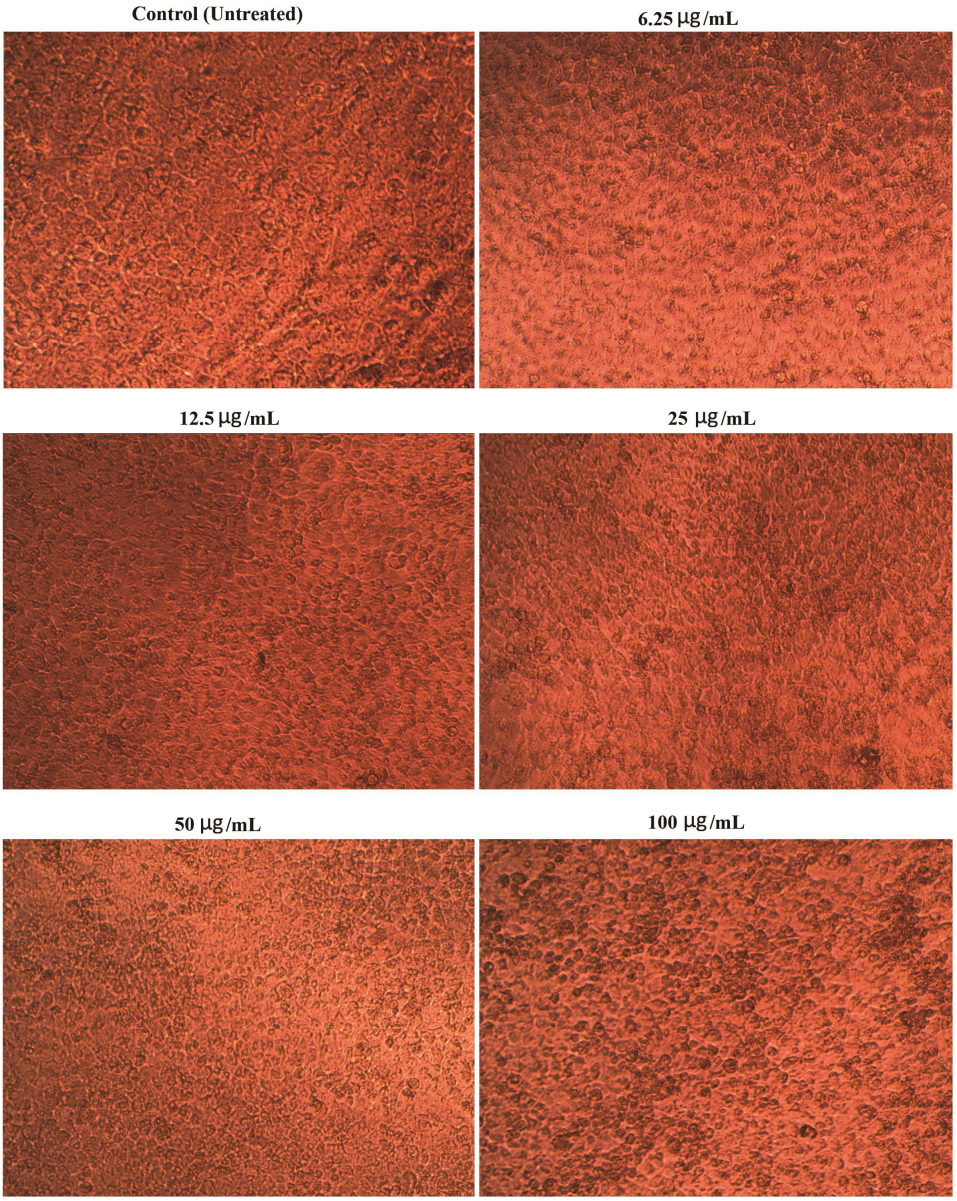
Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10xMagnification).

Figure 6.4 Normal cells (L929) treated with different concentrations of GgTI



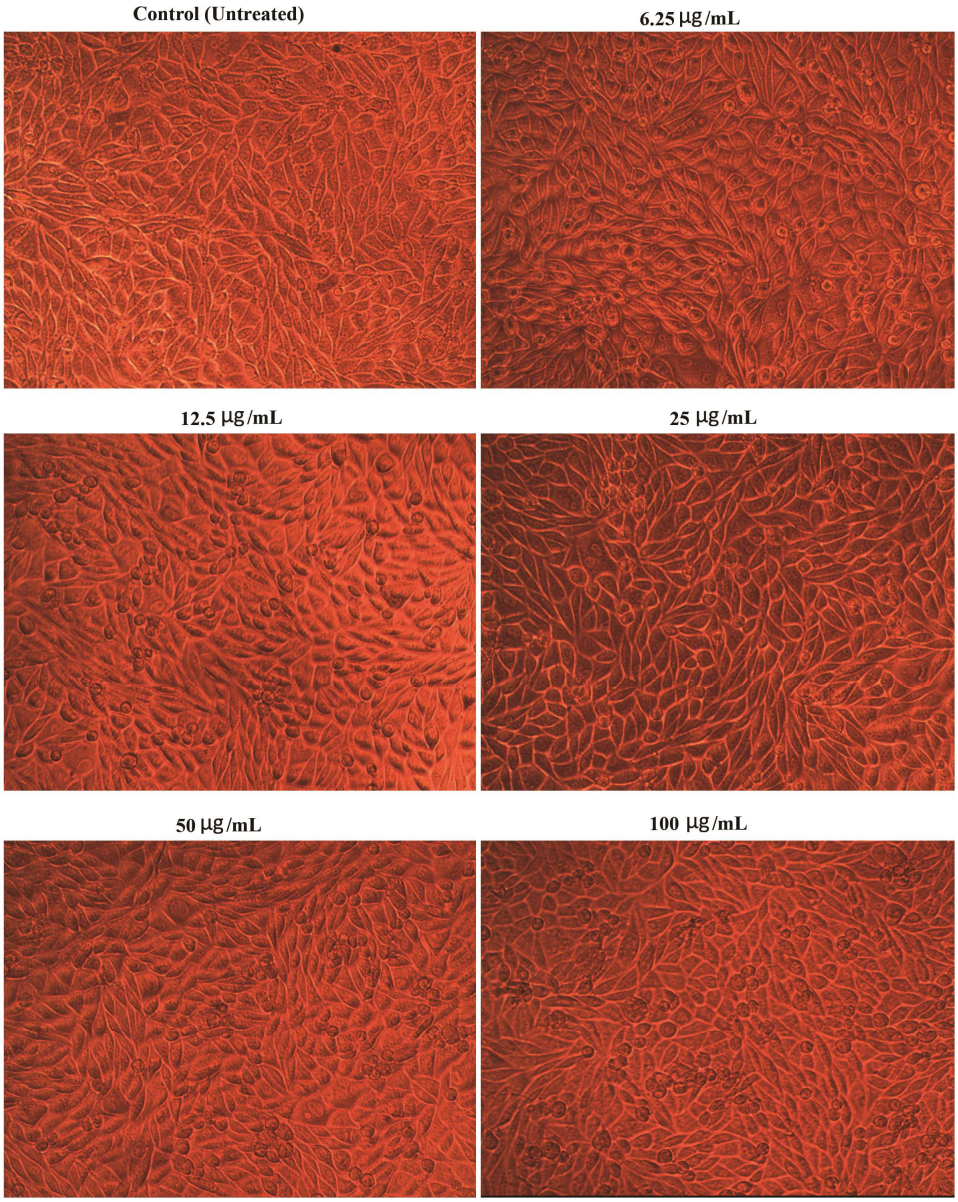
Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 6.5 Skin cancer cells (A431) treated with different concentrations of STI



Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 6.6 Normal cells (L929) treated with different concentrations of STI



Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

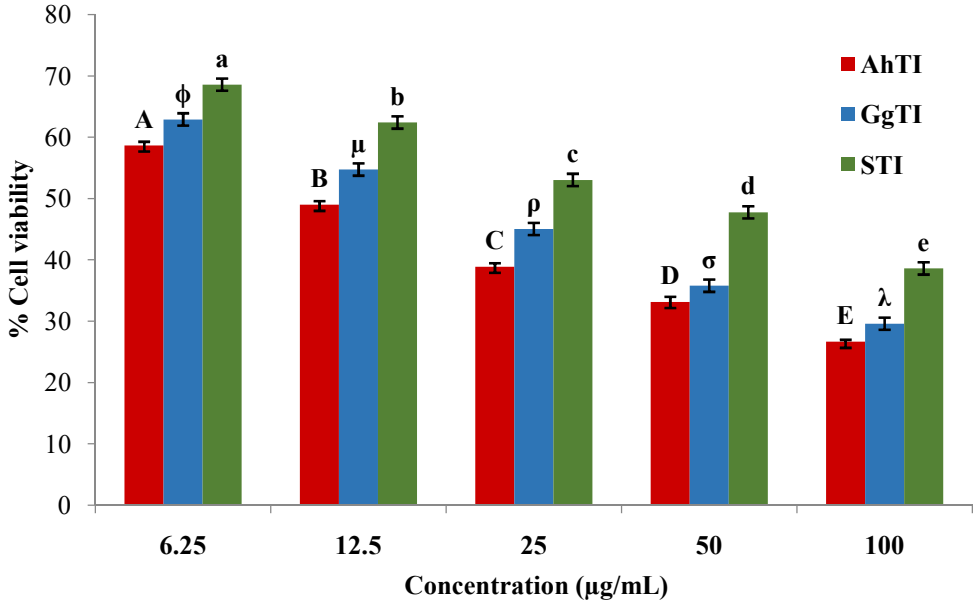
All the plates were examined under an inverted phase contrast microscope after 24h of incubation and images were recorded. The images of skin cancer cells after 24h of treatment with all the TIs at different concentrations reveal that the AhTI, GgTI and STI treated cells showed significant distortion in cell morphology like cell shrinkage, blebbing and granulation. The extent of morphological changes increases with the concentration of TIs. The normal cells (L929) treated with any of the TIs (24h) show any significant deviation from normal morphology. This proves AhTI, GgTI and STI to be non-toxic to normal cells.

6.3.2 MTT assay

i. Skin Cancer Cell lines (A431)

Analysis of anti-proliferative effect of TIs on skin cancer cells (A431) by MTT assay was done by the protocol described by Talarico *et al*., (2004). The skin cancer cell line was treated with the purified AhTI and GgTI for an incubation period of 24h. The effect of Soybean trypsin Inhibitor (STI) purchased from Sigma Aldrich was also studied for comparison.

Figure 6.7 MTT assay of skin cancer cells (A431) after 24h treatment



Percentage viability of cells treated with different concentrations of AhTI, GgTI and STI withA431 cells; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Data from three independent studies were given as Mean±Standard Deviation in six wells per treatments. $p<0.05$ considered statistically significant. Different letters/symbols indicate the significant differences between the groups. F value for AhTI=$1.4x10^3$; GgTI=881.5 and STI=969.11.

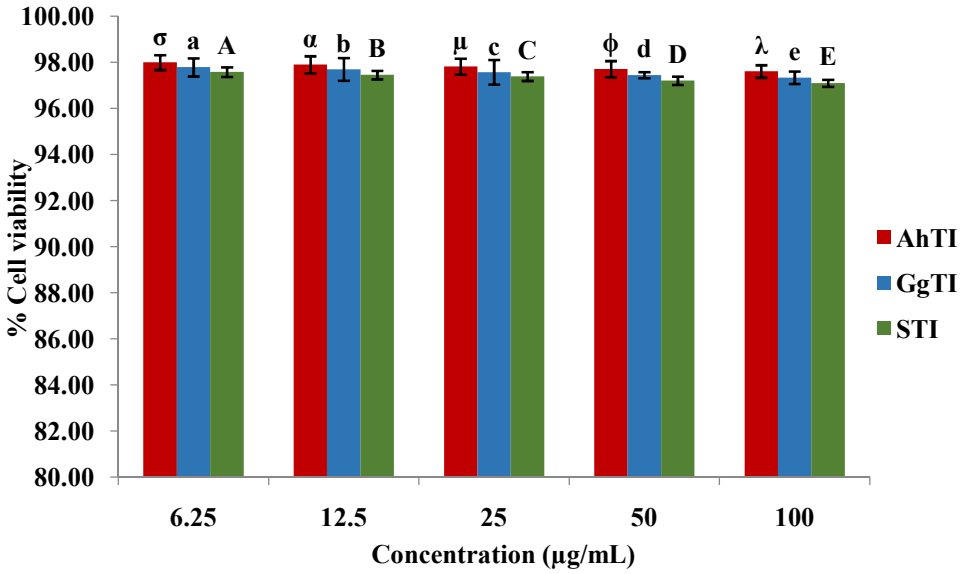
The result of MTT assay (Fig.6.7) shows that there is a decrease in viability of the cells after treatment with the AhTI, GgTI and STI in a concentration dependent manner. The maximum effect was shown by AhTI at 100µg/mL concentration (26.65±0.31%). Both the TIs under study are found to be more effective compared to the soybean TI, which is among the most studied and conventionally used TIs. IC_{50} of STI was calculated as 28.125µg/mL. But for AhTI and GgTI it was 10.875µg/mL and 15.625µg/mL respectively). This shows

that both these TIs are more effective than STI in terms of antiproliferative activity on skin cancer cell line.

ii. Normal Cell lines (L929)

Normal cells derived from the subcutaneous areolar adipose tissue of mouse also were treated with AhTI, GgTI and STI and the cells were assayed after 24h. The protocol followed was same as done in the MTT assay of the skin cancer cell line.

Figure 6.8 MTT assay of normal cells (L929) after 24h treatment



Percentage viability of cells treated with different concentrations of AhTI, GgTI and STI. Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Data from three independent studies was given as Mean±Standard Deviation in six wells per treatment. $p<0.05$ considered statistically significant. F value for AhTI=0.661; GgTI=0.736 and STI=3.3.

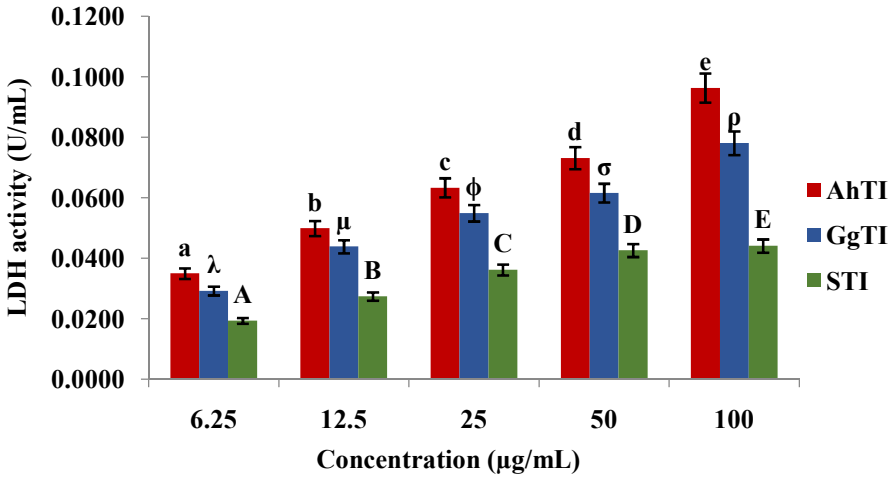
Fig.6.8 reveals that the normal cells (L929) showed no significant cell death even at higher concentrations of AhTI, GgTI and

STI after 24h treatment. About 97-98% of cells were viable after treatment with different concentrations (6.25µg/mL-100µg/mL) of AhTI, GgTI and STI. Since the TIs do not affect the morphology and viability of normal cells, further studies were performed only with cancer cells.

6.3.3 LDH Leakage assay

LDH leakage assay was carried out by the protocol reported by Andry and Erivch (2006). AhTI, GgTI and STI were used to treat the skin cancer cells to analyse the release of LDH enzyme which is a marker enzyme to detect the plasma membrane damage of the cells.

Figure 6.9 LDH Leakage assay of skin cancer cells (A431) after 24h treatment



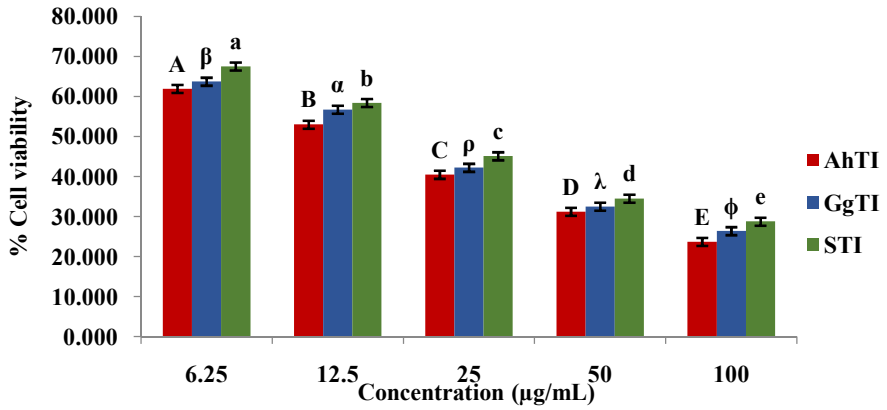
LDH Activity in cell free supernatant collected from tissue culture plates exposed to different concentrations of AhTI, GgTI and STI with A431 cells; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). F value for AhTI=813.45; GgTI=256.16 and STI=542.6.

The result of LDH leakage assay (Fig.6.9) shows that the AhTI treated cells exhibit a significant release of LDH enzyme than that of GgTI treated cells. STI leads to lesser LDH leakage when compared to AhTI and GgTI. Increased leakage was found in the cells treated with higher concentrations of TIs. Minimum concentration of AhTI, GgTI and STI treated was 6.25µg/mL. The maximum LDH release found was 0.0963±0.0008U/mL in the case of AhTI at 100µg/mL, while it was 0.078±0.0012U/mL, 0.044±0.0005U/mL in the case of GgTI and STI treated cells at 100µg/mL concentration.

6.3.4 Neutral red uptake assay

The number of live cells in a cultue can be quantitatively estimated using the neutral red uptake assay. The results are presented in Fig.6.10.

Figure 6.10 Neutral red uptake assay of skin cancer cells (A431) after 24h treatment



Cells treated with different concentrations of AhTI, GgTI and STI with A431 cells; Values Mean±Standard deviation (n=6), Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Fvalue for AhTI=2.39x103; GgTI=2.38x103 and STI=2.64 x103.

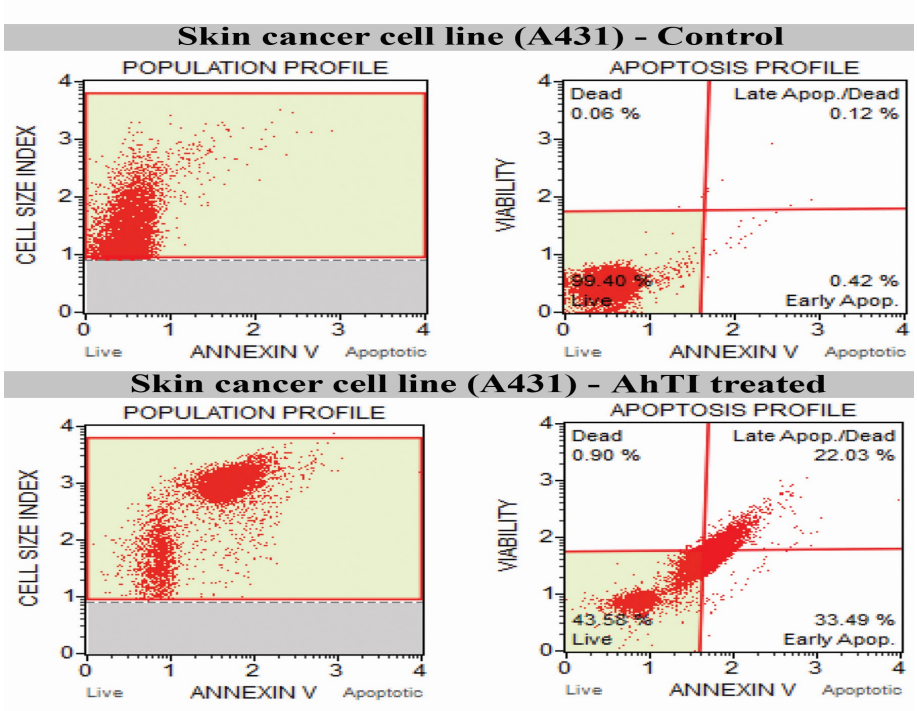
The result of Neutral red uptake assay (Fig.6.10) indicates that there is a decrease in cell viability of the skin cancer cells after treatment (24h) with AhTI, GgTI and STI in a concentration dependent manner. AhTI and GgTI are found to be more effective than STI. AhTI showed a cell viability of 61.93% at the minimum concentration (6.25µg/mL) and the viability of cells at maximum concentration (100µg/mL) was 23.70% after 24h treatment.

6.3.5 Apoptosis Profile of Skin Cancer cell lines by Annexin V-FITC Flowcytometry analysis

TheAnnexin V-FITC is a common method used for detecting apoptotic cells. The skin cancer cells were treated (24h) with both the samples AhTI and GgTI (at IC_{50} concentrations of 10.875µg/mL and 15.625µg/mL respectively). To evaluate and measure the Annexin V binding to apoptotic cells, Koopman *et al*. established the flow cytometric analysis in 1994, utilising Annexin V conjugated to FITC which is followed here.

The skin cancer cells treated with IC_{50} concentration of AhTI (10.875µg/mL), GgTI (15.625µg/mL) for 24h and non-treated control cells were subjected to Annexin V-FITC flowcytometry analysis to detect the number of apoptotic cells. Fig.6.11 and Table 6.1show the results of AhTI treated skin cancer cells while Fig.6.12 and Table 2 represents the result of GgTI treated skin cancer cells.

Figure 6.11 Annexin V-FITC -Flowcytometry profile of Skin cancer cells after AhTI treatment



Lower Left (LL): Live cells, Lower Right (LR): Early Apoptotic, Upper Right (UR): Late apoptotic/Dead, Upper Left (UL): Debris

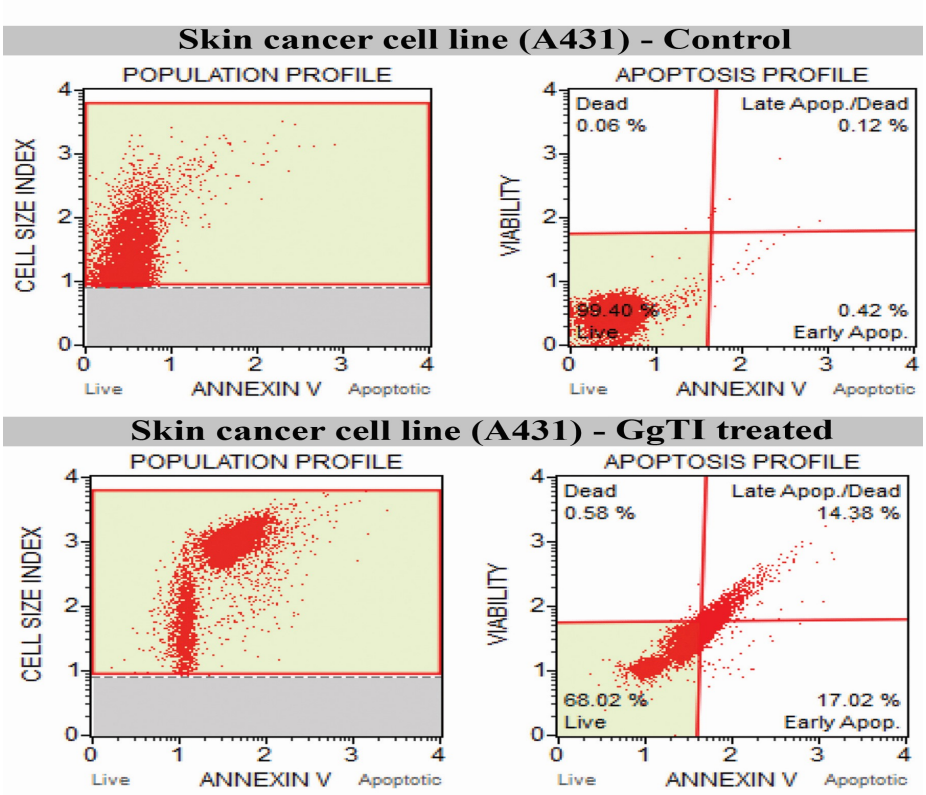
Table 6.1 Cells gated in Flowcytometry profile.

Skin Cancer Cells (A431) - Control			Skin Cancer Cells (A431) - AhTI Treated		
	Cell Conc. (Cells / mL)	% Gated		Cell Conc. (Cells / mL)	% Gated
Live (LL) :	4.05E+06	99.40 %	Live (LL) :	2.01E+06	43.58 %
Early Apoptotic (LR) :	1.71E+04	0.42 %	Early Apoptotic (LR) :	1.54E+06	33.49 %
Late Apop./ Dead (UR) :	4.88E+03	0.12 %	Late Apop./ Dead (UR) :	1.01E+06	22.03 %
Debris (UL) :	2.44E+03	0.06 %	Debris (UL) :	4.15E+04	0.90 %
Total Apoptotic :	2.20E+04	0.54 %	Total Apoptotic :	2.56E+06	55.52 %

Cells gated in Flowcytometry profile: % Gated of Live, Early Apoptotic, Late apoptotic or dead, debris and Total apoptotic cells are shown. Untreated Control and AhTI treated Skin cancer cells.

Annexin V-FITC - flow cytometry analysis reveals that 99.40% of cells in the non-treated control and only 43.58% of cells in the AhTI-treated skin cancer cells were alive after 24h.33.49% early apoptotic, 22.03% late apoptotic/dead cells were detected in the AhTI treated group. The total apoptotic cells detected was 55.52%.

Figure 6.12 The Annexin V-FITC-Flowcytometry profile of Skin cancer cells after GgTI treatment



Lower Left (LL): Live cells, Lower Right (LR): Early Apoptotic, Upper Right (UR): Late apoptotic/Dead, Upper Left (UL): Debris

Table 6.2 Cells gated in Flowcytometry profile

Skin Cancer Cells (A431) - Control			Skin Cancer Cells (A431) - GgTI Treated		
	Cell Conc. (Cells / mL)	% Gated		Cell Conc. (Cells / mL)	% Gated
Live (LL) :	4.05E+06	99.40 %	Live (LL) :	2.19E+06	68.02 %
Early Apoptotic (LR) :	1.71E+04	0.42 %	Early Apoptotic (LR) :	5.48E+05	17.02 %
Late Apop./ Dead (UR) :	4.88E+03	0.12 %	Late Apop./ Dead (UR) :	4.63E+05	14.38 %
Debris (UL) :	2.44E+03	0.06 %	Debris (UL) :	1.87E+04	0.58 %
Total Apoptotic :	2.20E+04	0.54 %	Total Apoptotic :	1.01E+06	31.40 %

Cells gated in Flowcytometry profile: % Gated of Live, Early Apoptotic, Late apoptotic or dead, debris and Total apoptotic cells are shown. Untreated Control and GgTI treated Skin cancer cells.

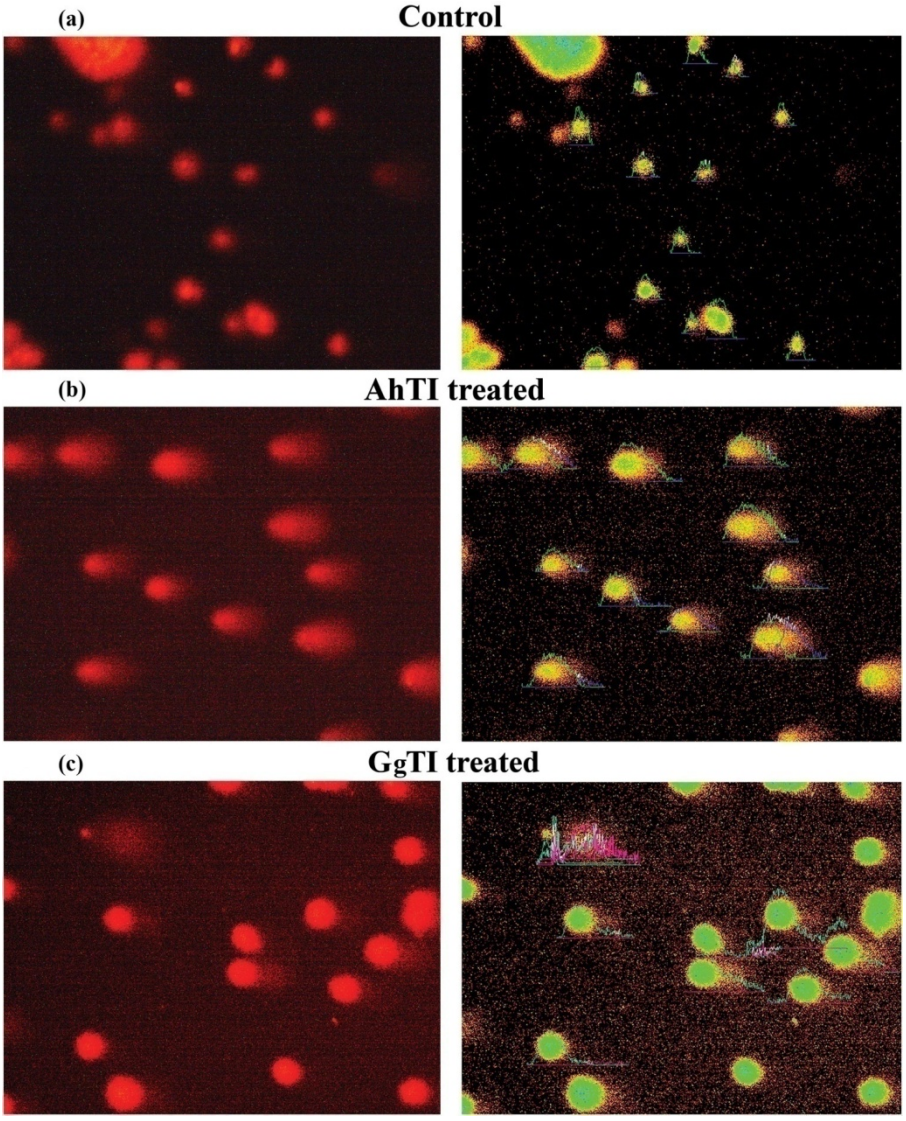
In the Annexin V-FITC flow cytometry examination, only 68.02% of the cells in the GgTI-treated skin cancer cells were still alive after 24h (Fig.6.11; Table 6.1), in contrast to 99.40% of the cells in the untreated control cells.It was shown that 17.02% of the apoptotic cells were early and 14.38% were late or dead. There were 31.40% of cells that had undergone cell death.

6.3.6 Comet Assay for the evaluation of DNA damage

Comet assay has been shown to be a very useful method for assessing DNA damage in cells. The comet test is a quick and accurate way to find individual cell DNA strand breaks. In the last few years, its use has considerably expanded (Daryl *et al*., 1995).

Comet assay was performed to check the DNA damage that occurred to skin cancer cells treated with the IC_{50} concentrations of AhTI and GgTI (10.875µg/mL and 15.625µg/mL respectively) and the non-treated control cells for 24h. The comet and tail lengths were measured and calculated with the TriTek software.

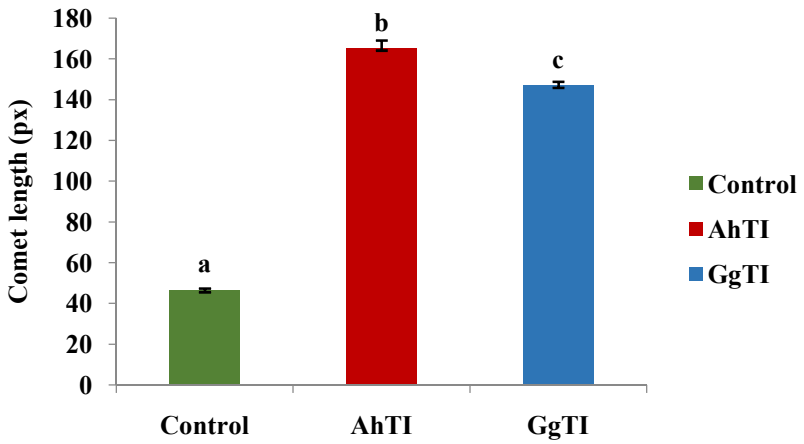
Figure 6.13 Comet assay of skin cancer (A431) cells after treatment with AhTI and GgTI



Evaluation of DNA damage of Skin cancer cells (A431) by comet assay (a) Control, (b) AhTI treated and (c) GgTI treated.

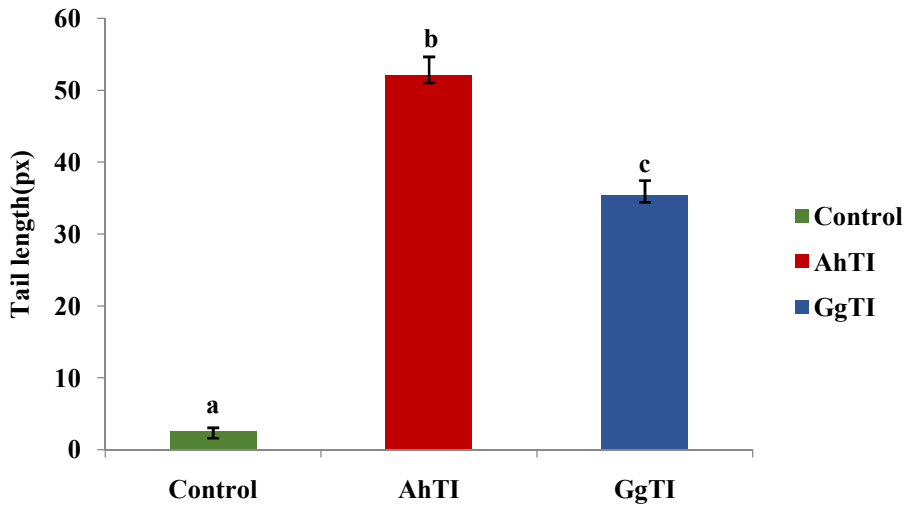
In the Comet assay technique the DNA damage is calculated as the DNA tail length and comet length. The longer the DNA tail length, the more significant the damage. The comet assay (Fig.6.13) revealed that the DNA in the control cells (a) is intact without a DNA tail, whereas the DNA in the AhTI and GgTI-treated cells (b) showed significant damage as the tail lengths show an increase.

Figure 6.14 Comet length of skin cancer cell line (A431) treated with AhTI and GgTI



Graphical representation of Comet length of Skin Cancer Cell line (A431) treated with AhTI and GgTI. (n=3). Error bars indicate Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. Different letters indicate the significant differences between the groups. Fvalue for Control=420; AhTI=834 and GgTI=541.

Figure 6.15 Tail length of skin cancer cell line (A431) treated with AhTI and GgTI



Graphical representation of Tail length of Skin Cancer Cell line (A431) treated with AhTI and GgTI. (n=3). Error bars indicate Standard Deviations, (not visible when smaller than symbol size). $p<0.05$ considered statistically significant. Different letters indicate the significant differences between the groups. Fvalue for Control=247; AhTI=737 and GgTI=523.

In the comet assay technique the DNA damage is calculated as the DNA tail length and comet length. The longer the DNA tail length, the more significant the damage. The comet assay revealed that the DNA in the control cells (a) is intact without a DNA tail, whereas the DNA in the AhTI and GgTI-treated cells (b) showed significant damage as the tail length was increased. The Fig.6.14 shows the Comet length and the Fig.6.15 shows tail length of the AhTI and GgTI treated Skin cancer cell lines. In the control cells the length of the comet is 46.53 ±0.760px and the tail length is 2.6 ±0.45. The AhTI and GgTI treated cells showed a comet length of 165.11±3.90 and 146.75±2.00.

The tail length is 52±2.63 and 35.41±2.01 respectively. The Opitka Pro5 CCD camera and an Olympus CKX41 Inverted Epifluorescent Microscope were used for the imaging of the slides. The length of the comet and tail was calculated by TriTek comet scoring software.

6.4 DISCUSSION

The current chapter describes the anti cancer activity of purified trypsin inhibitor proteins from *A.hirsutus* (AhTI) and *G.gummi-gutta* (GgTI) seeds against skin cancer cell lines. MTT assay, LDH leakage assay and Neutral red uptake assay for the determination of cytotoxic activity of AhTI and GgTI were done. Soybean trypsin inhibitor (STI) protein, a purified trypsin inhibitor protein available commercially was used as a standard to compare the anticancer potential with the AhTI and GgTI. The DNA damage of the cells was detected by comet assay and apoptosis were observed by the method of Annexin V FITC flowcytometry.

PIs have potent activity against several types of cancers (Correa, 1981: Joanitti *et al*., 2010; Magee *et al*., 2012). In certain cell lines, it has been discovered that several plant PIs exhibit tumor-suppressing properties. A trypsin inhibitor protein isolated and purified by affinity chromatography from winged bean were used to human chronic myeloid leukaemia cells (Bhattacharjee *et al*., 2022). According to Fernandes and Banerji (1996), topical application of a *Viciafaba* L. (Field bean) PI can successfully suppress skin carcinogenesis. BWI-1 (buckwheat inhibitor) and BWI-2a, a PI of the potato inhibitor family have been shown to limit the growth of T-acute

lymphoblastic leukaemia (T-ALL) cells such as JURKAT and CCRF-CEM, as well as human normal blood lymphocytes (Park and Obha, 2004).

In this study, direct examination of skin cancer cells using an inverted phase contrast microscope (Olympus CKX41 with an Optika Pro5 CCD camera) was done for the detection of changes in cell morphology (24 h treatment) with various concentrations of AhTI, GgTI and STI. The cells treated with all the TIs showed morphological changes including shrinkage, blebbing and granulation. A well-defined characteristic of apoptotic cell death is plasma membrane blebbing (Sharma *et al*., 2004). The morphological changes associated with necrotic cell death, however, are cell swelling brought on by a loss of plasma membrane integrity (Sharma *et al*., 2006). Both these morphological changes are remarkable in the skin cancer cells treated with AhTI and GgTI, particularly at their higher concentrations.

In the AhTI (Fig.6.1), GgTI (Fig.6.3) and STI (Fig.6.5) treated cells, phase contrast microscopy images of colon cancer cells revealed significant morphological changes including cell shrinkage, blebbing, rounding, granulation and vacuolization in the cytoplasm after different concentrations of the same. Vacuolization often follows cell death. The mechanism and origin of cytoplasmic vacuolization are unknown (Youhua *et al*., 2020). After 24h, all of the treated plates were examined under an inverted phase contrast tissue culture microscope to record the results. The normal cells (L929) underwent the same process as well. However, when normal cells were exposed to

various concentrations of the TIs for 24h, no distinctive changes were found, as demonstrated by the results in Fig.6.2, Fig.6.4 and Fig.6.6.

As cytotoxicity end points, we investigated lysosomal activity (NR uptake), mitochondrial function (MTT reduction) and membrane damage (LDH leakage assay). All the analysis showed concentration dependent significant changes in the skin cancer cells after treatments with AhTI, GgTI and STI. The result of MTT assay showed an increase in cell death in a concentration dependent manner. The basic idea behind the MTT test is that metabolically active cells can convert the water-soluble, yellow tetrazolium salt MTT into non-water-soluble purple formazan crystals. This conversion is carried out by the succinate dehydrogenase system of the active mitochondria (Altman *et al*., 1976). AhTI showed more decrease in cell viability than that of GgTI and STI. The MTT results (Fig.6.7 and Fig.6.8) shows AhTI and GgTI to be powerful enough to kill the skin cancer cells based on the IC_{50} values 10.875µg/mL and 15.625µg/mL respectively. The IC_{50} of STI was calculated as 28.125µg/mL. Hence it is proved that both the TIs are more efficient than Soybean TI against skin cancer progression. Both of the TIs (AhTI and GgTI) have been found to be more efficient than the soybean TI. STI's IC_{50} was determined to be 28.125µg/mL However; it was 10.875g/mL and 15.625µg/mL for AhTI and GgTI, respectively.After a 24h treatment with different concentrations of AhTI, GgTI and STI, the normal cells (L929) showed no detectable cell death. After being exposed to various concentrations (6.25µg/mL 100µg/mL) of AhTI, GgTI and STI, approximately 98% of the cells were still alive. The cell death is around 97-98% after exposure with

highest concentration (100µg/mL), after treatment at same time period (24h). Further research was restricted to cancer cells because the TIs have no impact on the morphology or viability of normal cells.

The LDH enzyme is released significantly more from AhTI-treated cells than from GgTI-treated cells, according to the results of the LDH leakage experiment (Fig.6.9). In comparison to AhTI and GgTI, STI causes less LDH leakage. Higher TI concentrations were shown to cause more leakage in the cells. AhTI, GgTI, and STI were treated at a minimum concentration of 6.25µg/mL and maximum of 100µg/mL.The maximal LDH release measured at the concentration of 100µg/mL for AhTI, GgTI and STI was 0.0963±0.0008U/mL, 0.078±0.0012U/mL and 0.044±0.0005U respectively.As a result of increased glycolysis in cancer cells, mitochondrial LDH may contribute to the acceleration of oxidative phosphorylation (de Bari *et al*., 2018).

The neutral red uptake assay also was conducted to check the cytotoxicity of the AhTI, GgTI and STI. Following a 24h treatment period with AhTI, GgTI and STI, the Neutral Red Uptake Assay results (Fig.6.10) showed that there is a concentration-dependent reduction in the cell viability of the skin cancer cells; AhTI and GgTI were more effective than STI. The result of the neutral red uptake assay with AhTI revealed a cell viability of 23.70% at the highest concentration (100µg/mL), GgTI showed 26.40% and STI 28.76%. All the three TIs are found to have the potential to kill cancer cells.

However, this study establishes that AhTI is more effective than GgTI and STI at killing skin cancer cells. A study by Waheed*et al*., (2013) suggested that the crude extract of *Viburnumfoetens* L. showed growth inhibitory activity against colorectal cancer cells. The study was based on the results of the MTT assay and the neutral red uptake assay.

One of the popular techniques for identifying apoptotic cells is the Annexin V-FITC flowcytometry analysis. In apoptosis, the cell actively takes part in its own mortality owing to the working processes. It exhibits nuclear chromatin condensation, vacuoles in the cytoplasmic membrane, compact cytoplasm, DNA fragmentation and the development of apoptotic bodies as its defining morphological features (Baba *et al*., 2007). The analysis of skin cancer cells after treatment (24h) with AhTI and GgTI were done with IC_{50} concentrations (10.875µg/mL and 15.62µg/mL, respectively). Only 43.58% of the cells in the AhTI-treated skin cancer cells were still alive after 24h compared to 99.40% of the cells in the untreated control.In the AhTI-treated group, it was found that 33.49% of early- and 22.03% of late-apoptotic cells, only 68.02% of the GgTI-treated skin cancer cells were still alive after 24h (Fig.6.11; Table 6.1), in contrast to 99.40% of the cells in the untreated control cells.It was shown that 14.38% of the apoptotic cells were late apoptotic/dead, while 17.02% of them were early apoptotic. A total of 31.40% of the cells had died. When skin cancer cells were exposed to the IC_{50} concentrations of AhTI and GgTI (10.875g/mL and 15.625g/mL,

respectively) for 24h the DNA damage that resulted was examined using the comet test. The slides were imaged using an Olympus CKX41 Inverted Epifluorescent Microscope and an Opitka Pro5 CCD camera. The Tritek comet scoring programme was used to determine the length of the comet and tail. For assessing DNA strand breakage (single or double) in eukaryotic cells, the comet assay, also known as single-cell gel electrophoresis, is a straightforward technique that is regarded as one of the most reliable (Collins A. R, 2014). The DNA damage is estimated using the DNA tail length and comet length in the comet assay technique. The extent of the damage increases with the length of the DNA tail. The DNA in the control cells is intact without a DNA tail, according to the comet assay, whereas the DNA in the AhTI and GgTI-treated cells (Fig.6.13) showed considerable damage as the length of the DNA tail increased. Fig.6.14 displays the comet length and the Fig.6.15 the tail length of skin cancer cell lines treated with AhTI and GgTI. Under usual conditions, the comet assay has a restricted resolution of 10-800 kb (Glei *et al*., 2009).

There has been a constant and severe need for the creation of novel anticancer medications that are intended to kill cancer cells, even if natural products have long been a rich source of cancer therapies. Finding a novel and potent medical therapy that can both prevent and treat cancer is therefore essential (Tickenbrock *et al*., 2006). A wide range of side effects are possible with existing melanoma therapy (Cheng *et al*., 2018). In the future, it will be more beneficial to

concentrate on isolating natural compounds as anticancer agents, which may reduce the risk of such side effects and also be easily available. This chapter communicates the anticancer potential of the isolated TI proteins from *A.hirsutus* and *G.gummi-gutta* against skin cancer (A431) cell lines. These TIs from natural sources poses a significant promise as future anti-cancer medicines.

Chapter 7

Studies on the effect of AhTI and GgTI on colon cancer (HT29) cell line

Colon cancer arises from polyps, which are growths in the inner lining of the colon that can be flat, elevated or on a stalk in the large intestine. Adenocarcinomas that develop from glandular epithelial cells make up the majority of colorectal neoplasms that have been identified. When limited to the bowels; colon cancer is an extremely treatable and frequently curable condition. Treatment for colorectal cancer is based on the size, location, and extent of the cancer's dissemination. A significant issue and a frequent cause of death is recurrence after surgery.

The trypsin inhibitor proteins isolated from *A.hirsutus* and *G.gummi-gutta* was purified by ammonium sulphate fractionation, dialysis, ion exchange chromatography and size exclusion chromatography (Gel filtration chromatography). The TI activity was confirmed by activity staining by reverse zymography. The homogeneity of the TI protein was confirmed by SDS-PAGE and its molecular weight was confirmed by MALDI-TOF/TOF. The stability of the TI protein at different temperatures and pH levels was also determined and presented in earlier chapters. This chapter contains a summary of the effectiveness of isolated, purified TI proteins against colon cancer (HT29) cell lines.

7.1 OBJECTIVES

The main focus of this part of the study is to investigate the anticancer potential of purified trypsin inhibitor proteins against colon cancer (HT29) cell lines. The specific objectives of the study is to check the cytotoxicity of the purified TI proteins (AhTI and GgTI)

against coloncancer cell lines by MTT assay, LDH leakage assay, and neutral red uptake assay. The next aim is to find out the DNA damage of the cells after the treatment with isolated TI proteins and to detect the apoptosis-inducing capacity of TI proteins by flow cytometry analysis. Soybean Trypsin Inhibitor (STI) was used for comparison in this part, since it is the well-studied TI from plant sources.

7.2 MATERIALS AND METHODS

Various cytotoxicity assays were used to identify the colon cancer cells' antiproliferative activity. To assess the cytotoxicity and viability of the cells after treatment with AhTI, GgTI, and STI we performed the MTT assay, LDH leakage assay and Neutral red uptake assay. Colon cancer (HT29) cell line apoptosis was detected by Annexin V FITC flow cytometry analysis following AhTI and GgTI treatments. For the measurement of DNA damage, the comet assay was also carried out.

All the methods mentioned in this chapter for cell maintenance and various assays for assessing cytotoxicity and apoptosis were described in Chapter VI, except that colon cancer cell line (HT-29) is used here, instead of skin cancer cell line (A431).

Cell lines Used

1. Normal (L929): derived from normal subcutaneous areolar adipose tissue of mouse.
2. Colon Cancer (HT29): Human Colorectal adenocarcinoma.

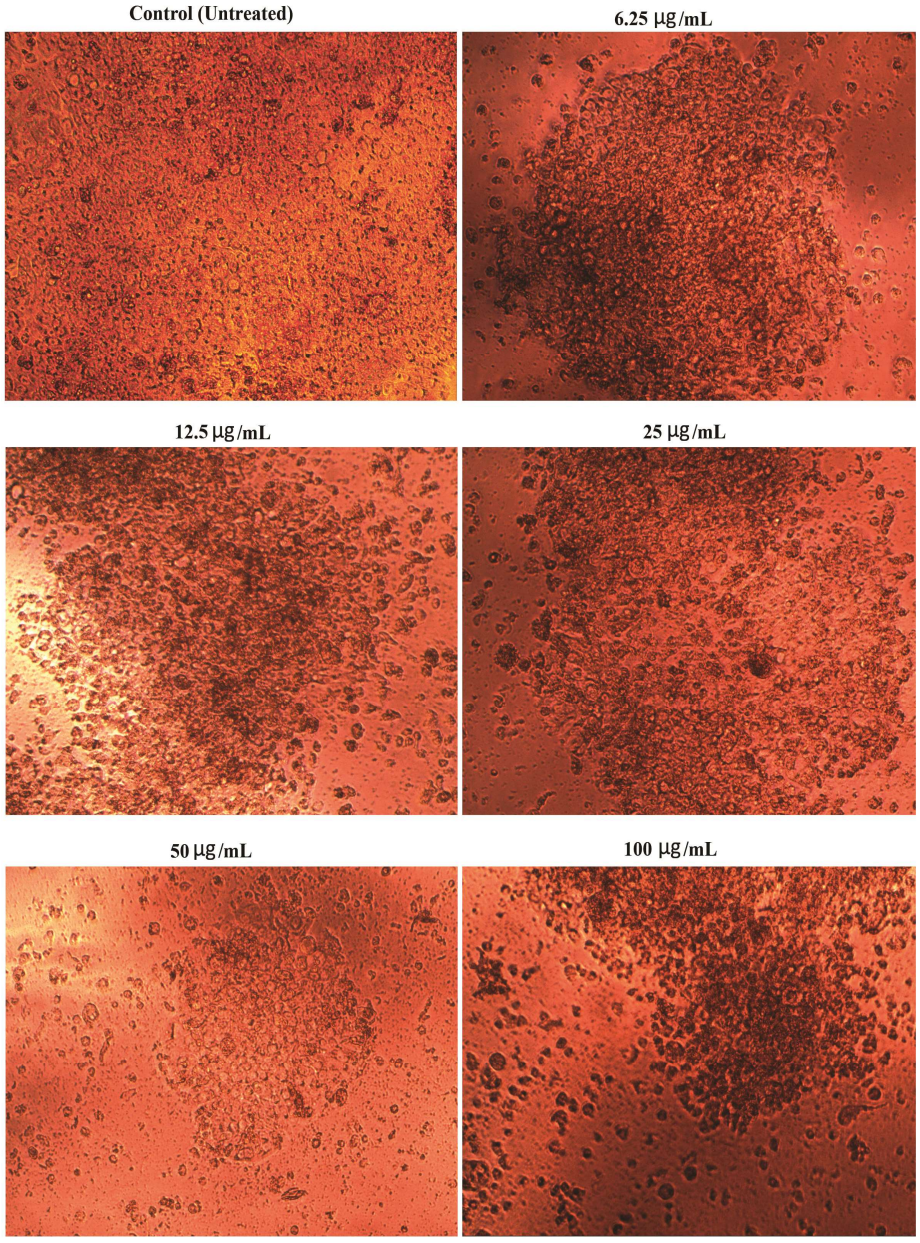
The National Centre for Cell Sciences (NCCS), Pune, India, originally provided the cancer cells (HT-29 and Normal cell (L929) which were cultured in DMEM, or Dulbecco's modified Eagles media (Sigma-Aldrich, USA).

7.3 RESULTS

7.3.1 Effect of AhTI, GgTI and STI on cell morphology

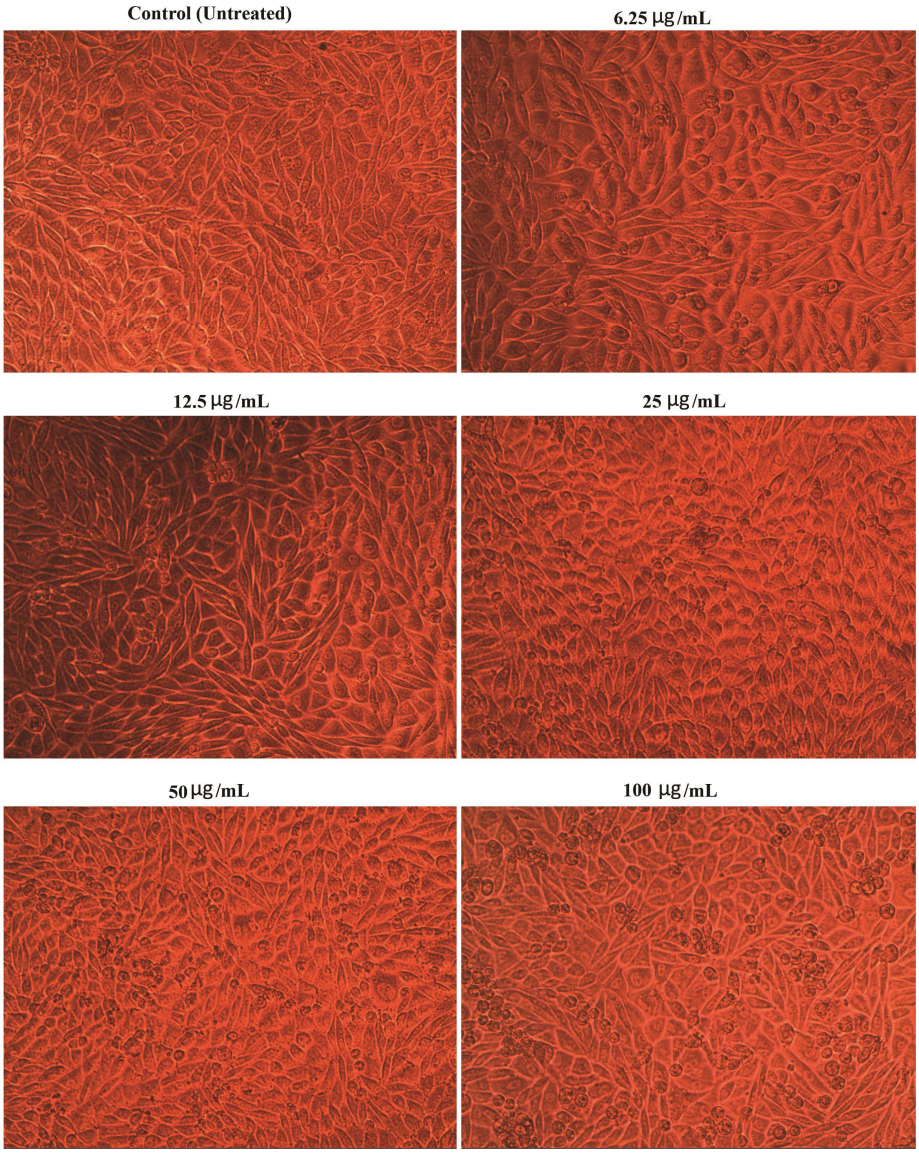
The colon cancer cell line was treated with various concentrations of AhTI (6.25, 12. 5, 25, 50 and 100µg/mL in 100µL of 5% DMEM) to determine the cytotoxic effects. After 24h of incubation, cells were subjected to direct examination of the cells using an inverted phase contrast microscope (Olympus CKX41 with an Optika Pro5 CCD camera). Normal cells (L929) were also used to see if it caused any cytotoxicity to them. Fig.7.1 shows the results.

Figure 7.1 Colon cancer (HT29) cells treated with different concentrations of AhTI



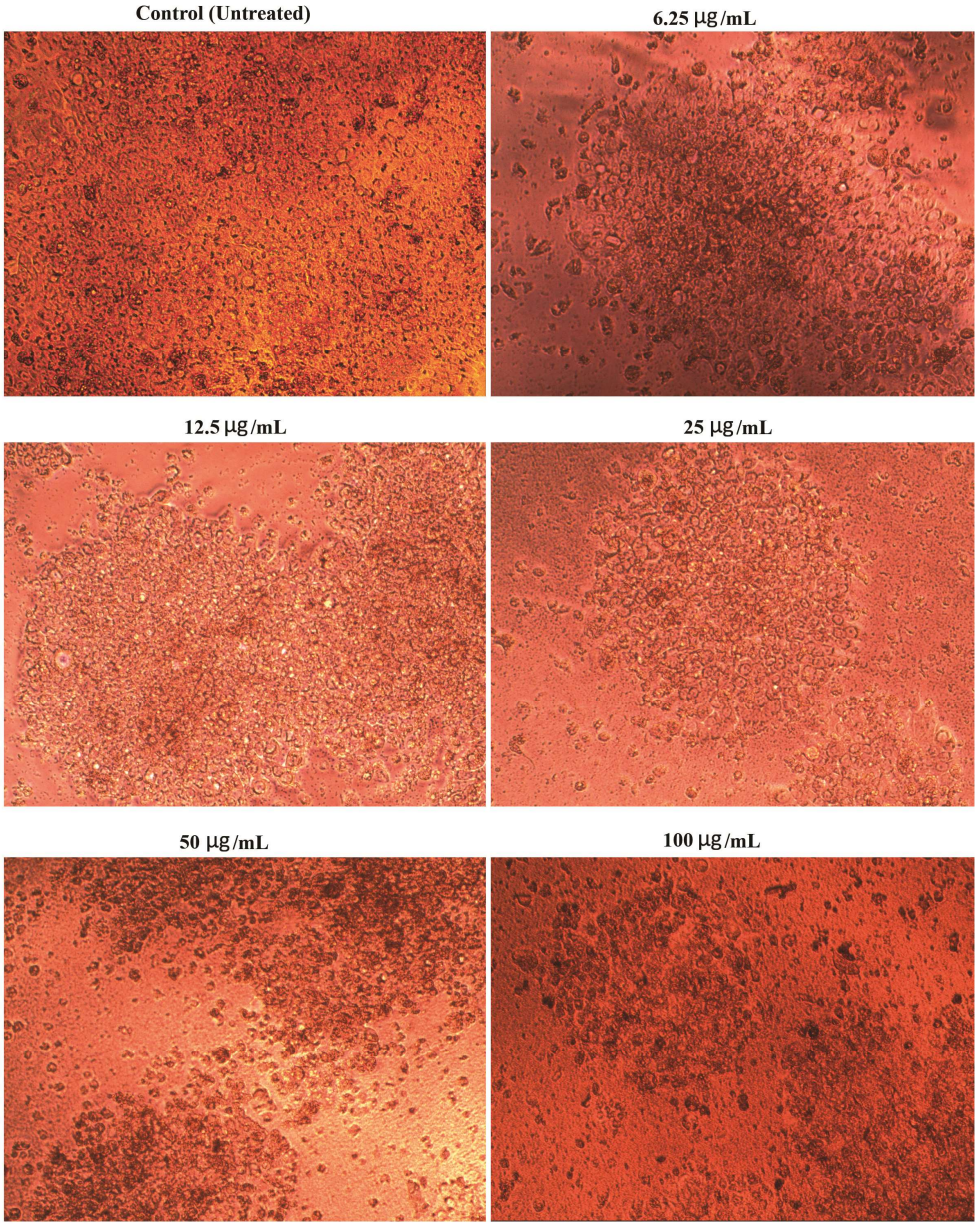
Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 7.2 Normal cell (L929) treated with different concentrations of AhTI



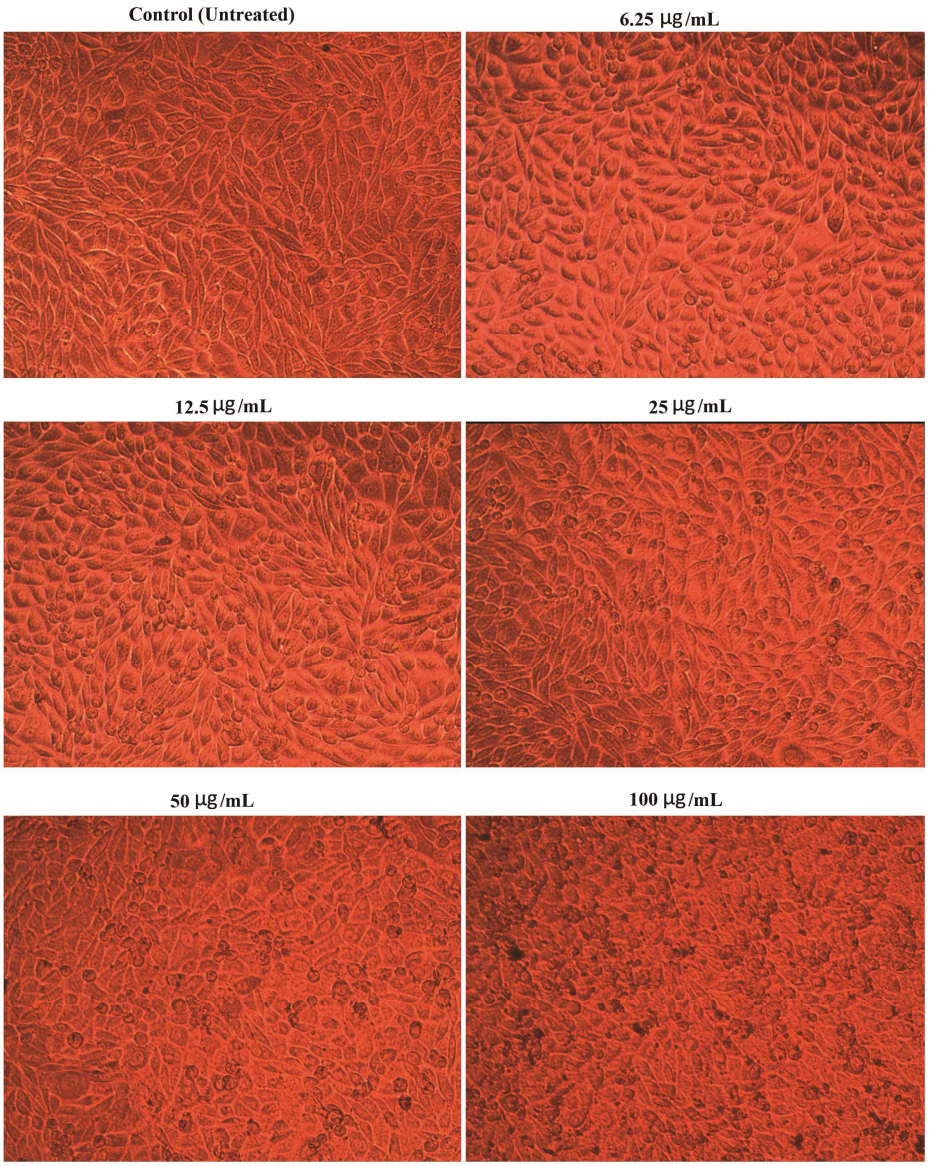
Images are taken using Inverted phase contrast microscope. (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 7.3 Colon cancer cells (HT29) treated with different concentrations of GgTI



Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification).

Figure 7.4 Normal cell (L929) treated with different concentrations of GgTI



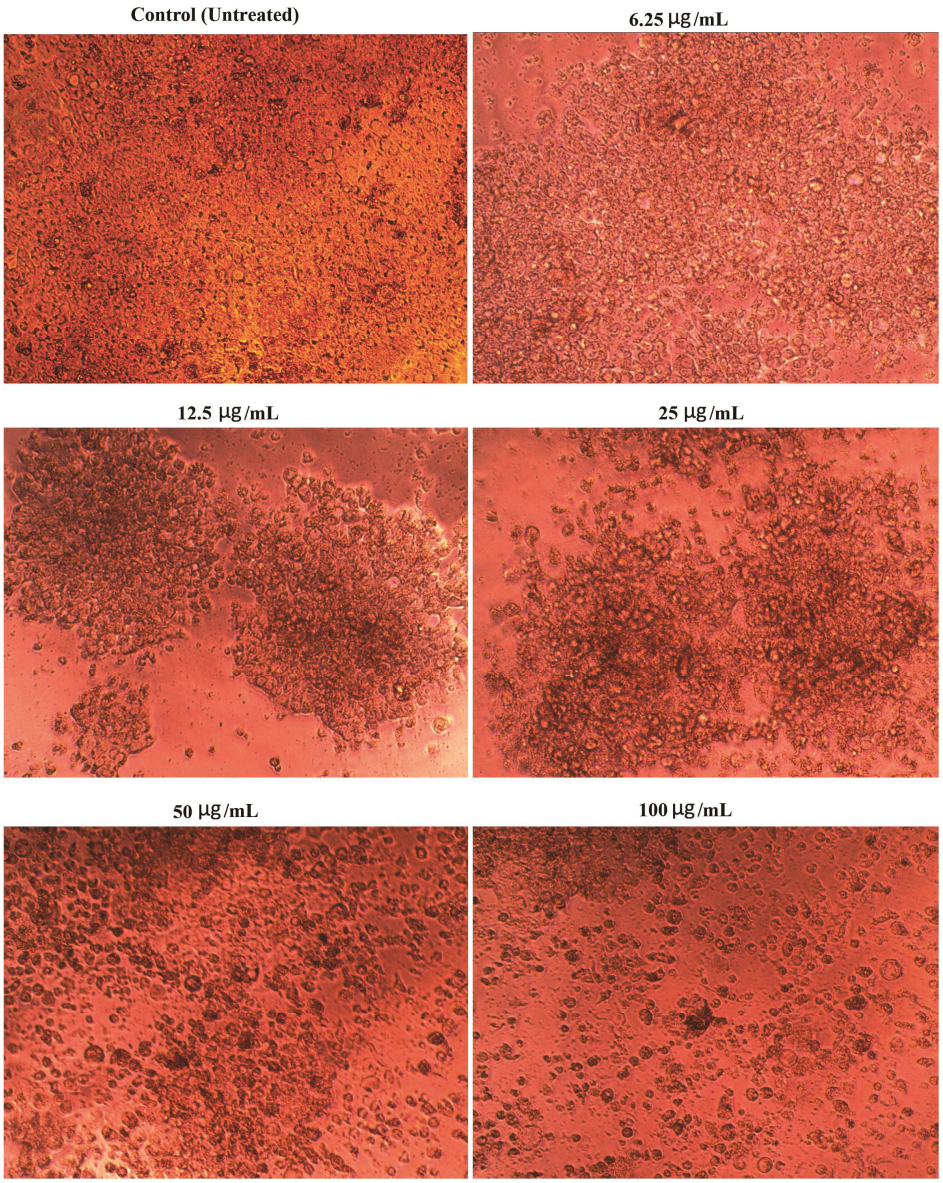
Images using Inverted phase contrast microscopy (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification)

After 24h of treatment with AhTI at various concentrations, phase contrast microscopy images of colon cancer cells indicated (Fig.7.1) considerable morphological changes in the AhTI-treated cells, including cell shrinkage, blebbing, rounding, granulation, and vacuolization in the cytoplasm. After 24h, all of the plates were viewed and recorded under an inverted phase contrast tissue culture microscope. Based on the results (Fig.7.2) of normal cells (L929) treated with AhTI at various concentrations for 24h, no detectable changes were found.

The Colon cancer cell line was treated with various doses concentrations of AhTI (6.25, 12.5, 25, 50 and 100µg/mL in 100µL of 5% DMEM) to determine the morphological changes. After 24h of incubation, cell viability was evaluated by direct examination of the cells using an inverted phase contrast microscope (Olympus CKX41 with an Optika Pro5 CCD camera).

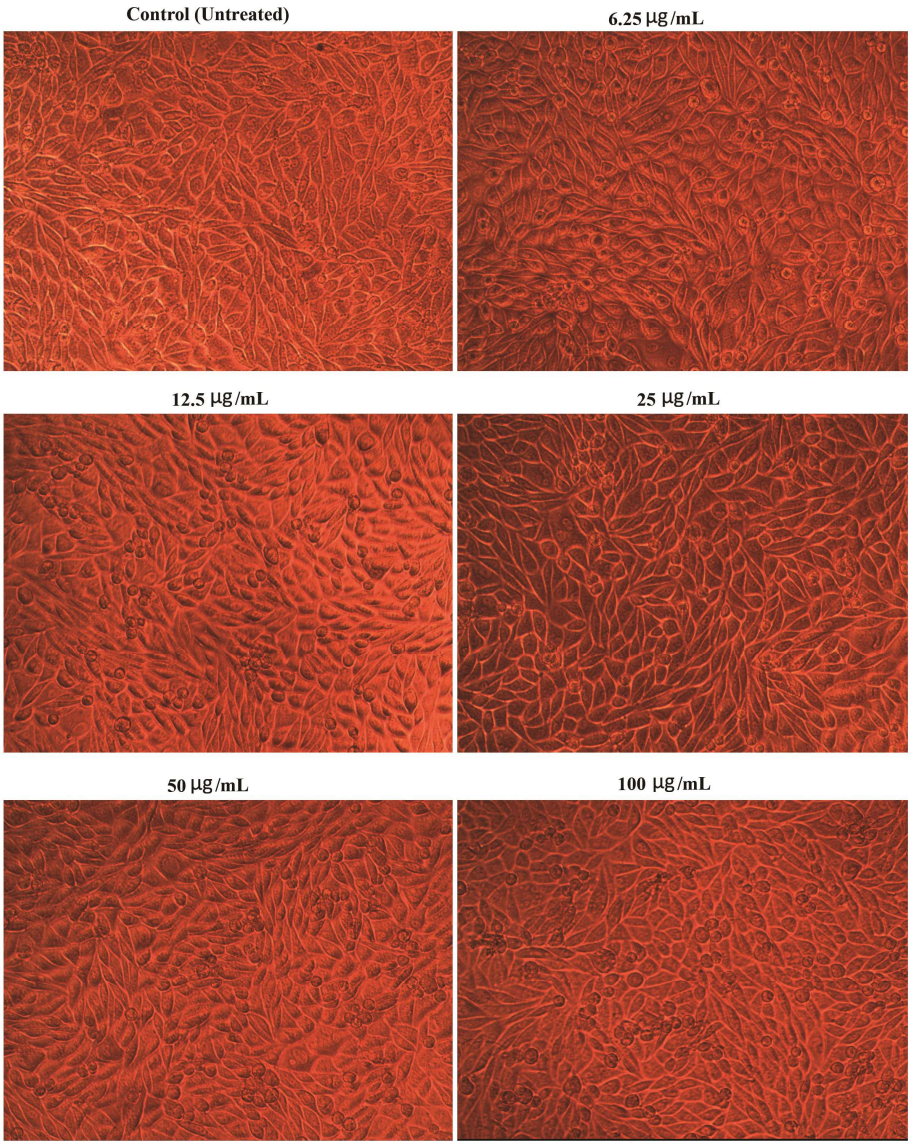
Signs of cytotoxicity were noticed after 24h of treatment with GgTI at various concentrations (6.25-100µg/mL) with colon cancer cells (Fig.7.3). These alterations included cell shrinkage, blebbing, rounding, granulation and vacuolization in the cytoplasm. After 24h, an inverted phase contrast tissue culture microscope was used to observe each plate and images were taken. The effects of normal cells (L929) treated (24h) with GgTI at various concentrations were not significantly affected (Fig.7.4).

Figure 7.5 HT29 cells treated with different concentrations of STI



Images using Inverted phase contrast microscopy (Olympus CKX41 with Optika Pro 5CCD camera). (10x Magnification).

Figure 7.6 L929 cells treated with different concentrations of STI



Images are taken using Inverted phase contrast microscope (Olympus CKX41 with Optika Pro5 CCD camera). (10x Magnification)

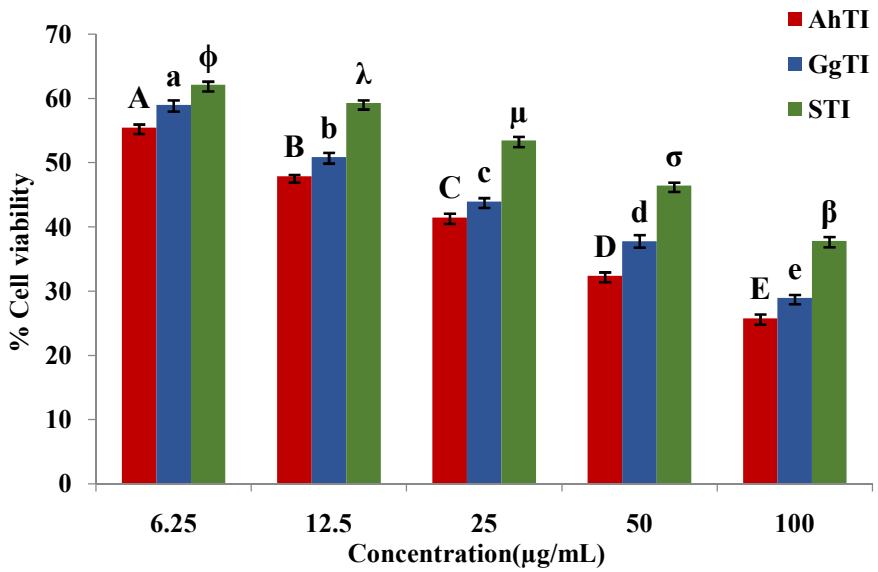
Cytotoxicity is observed in phase contrast microscopic images of colon cancer cells after 24h of treatment with STI at various concentrations (6.25-100µg/mL) as evidenced by significant morphological changes such as cell shrinkage, blebbing, rounding, granulation, and vacuolization in the cytoplasm (Fig.7.5). The results of normal cells (L929) treated with STI for 24h at various concentrations show no noticeable changes (Fig.7.6).

7.3.2 MTT assay

i. Colon cancer (HT29) cell lines

The determination of the antiproliferative effect of colon cancer cells (HT29) by MTT assay was carried out according to the procedure given by Talarico *et al*. (2004). Colon cancer cells were treated with purified AhTI and GgTI for a 24h incubation period. For comparison, the cell lines were treated with pure commercially available soybean trypsin inhibitor (STI).

Figure 7.7 MTT assay of colon cancer cells (HT29) after 24h treatment



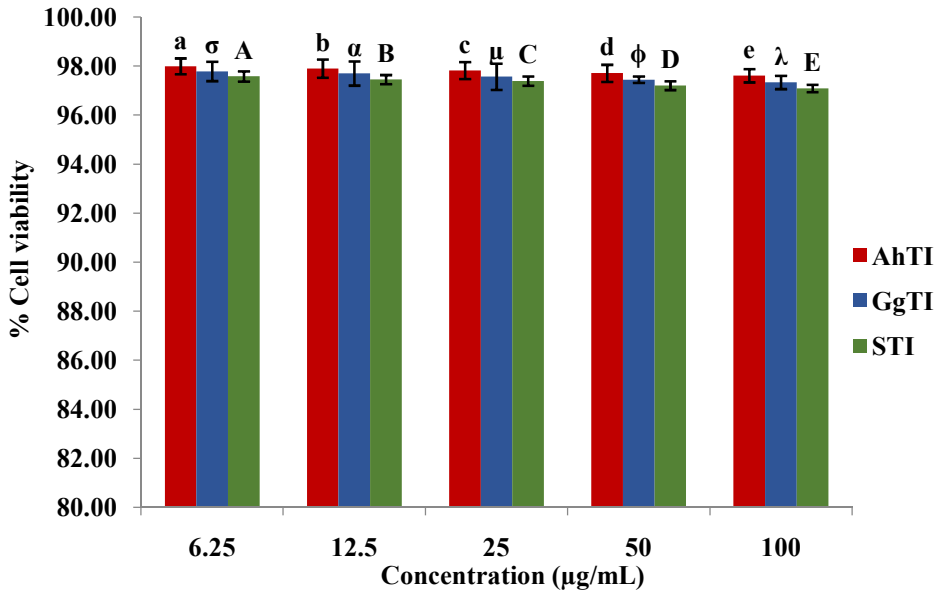
Percentage viability of HT29cells treated with different concentrations of AhTI, GgTI and STI; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. F value for AhTI=$1.7x10^3$; GgTI=868.9 and STI=750.9.

The result (Fig.7.7) indicates that there is a concentration dependent decline in cell viability following treatment with AhTI, GgTI and STI. The samples used had a range of concentrations, from 6.25g/mL at the lowest to 100g/mL at the highest.The cell viability after 24h was correspondingly 55.46±0.472%, 47.89±0.20%, 41.47±0.58%, 32.38±0.57% and 25.77±0.58% when AhTI was treated at concentrations of 6.25, 12.5, 25, 50 and 100µg/mL. The IC_{50} concentrations determined for colon cancer cells were 10.625µg/mL for AhTI, 13.75µg/mL for GgTI and 26.25µg/mL STI.

ii. Normal (L929) cell lines

AhTI, GgTI, and STI were also applied to normal cells obtained from mice subcutaneous areolar adipose tissue in order to study cytotoxicity. After a 24h incubation period, the cells were analysed.

Figure 7.8 MTT assay of normal cells (L929) after 24h treatment



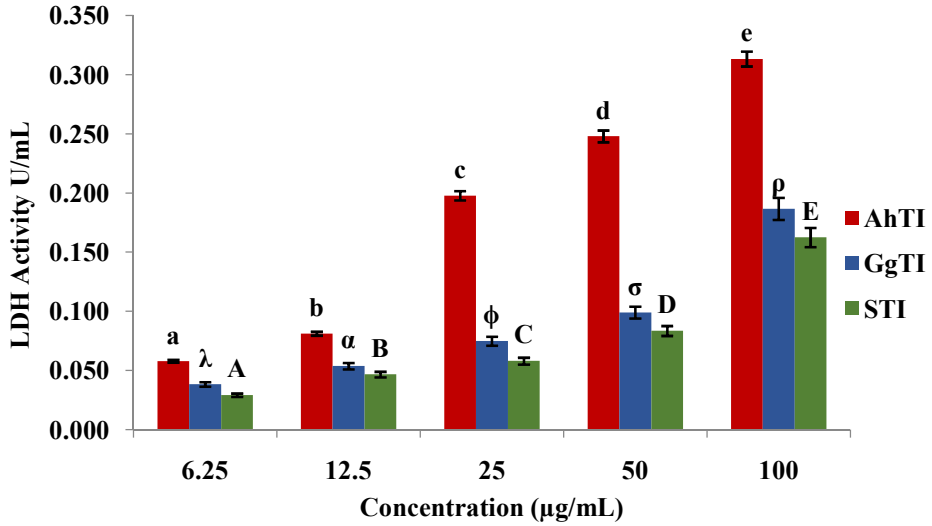
Percentage viability of cells treated with different concentrations of AhTI, GgTI and STI withL929 cells; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. Fvalue for AhTI=0.661; GgTI=0.736 and STI=3.39.

According to the results of the MTT experiment performed on L929 cells (Fig.7.8), following a 24h treatment period with different concentrations of AhTI, GgTI, and STI, there was no considerable cell death in the normal cells (L929). After being exposed to lower to higher (6.25-100µg/mL) doses of AhTI, GgTI, and STI, approximately 97-98% of the treated cells were alive.

7. 3. 3 LDH leakage assay

Following Andry and Erivch's procedure, the LDH leakage assay was conducted (2006). All three samples AhTI, GgTI, and STI were added to colon cancer cells in order to analyse the release of LDH, an enzyme marker for cellular plasma membrane damage. By monitoring the activity of cytoplasmic enzymes produced by injured cells, cytotoxicity is frequently assessed. All cells contain the stable cytoplasmic enzyme lactate dehydrogenase (LDH) (Kumar *et al*., 2018).

Figure 7.9 LDH leakage assay of colon cancer cells (HT29) after 24h treatment



Percentage viability of cells treated with different concentrations of AhTI, GgTI and STI withL929 cells; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. F value for AhTI=$1.4x10^3$; GgTI=$7.7x10^3$ and STI=$2.1x10^3$.

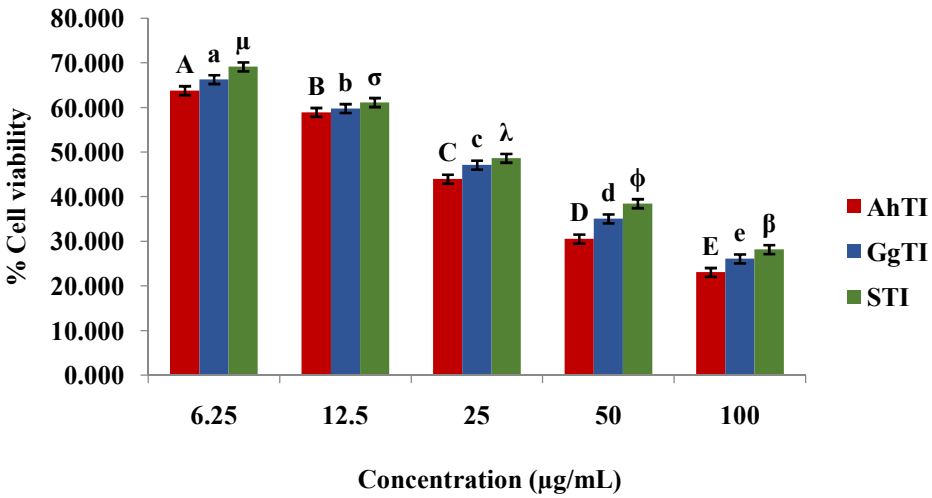
Based on the results of the LDH leakage experiment (Fig.7.9), AhTI treated cells released significantly more LDH enzyme than GgTI

treated cells did. Comparing the STI treated cells to AhTI and GgTI treated cells it can be observered that the LDH leakage shows a lesser value. The cells treated with higher concentrations of AhTI, GgTI and STI exhibited the greatest leakage. The highest LDH activity measured was 0.313±0.003U/mL for AhTI (100µg/mL-Maximum concentration), while it was 0.187±0.002U/mL and 0.163±0003U/mL in cells treated with corresponding concentrations of GgTI and STI respectively.

7.3.4 Neutral red uptake assay

The viability assay known as the neutral red uptake (NRU) assay measures how well living cells can bind and assimilate neutral red (NR) (Borenfreund *et al*., 1984).

Figure 7.10 Neutral red uptake of colon cancer cells (HT29) after 24h treatment



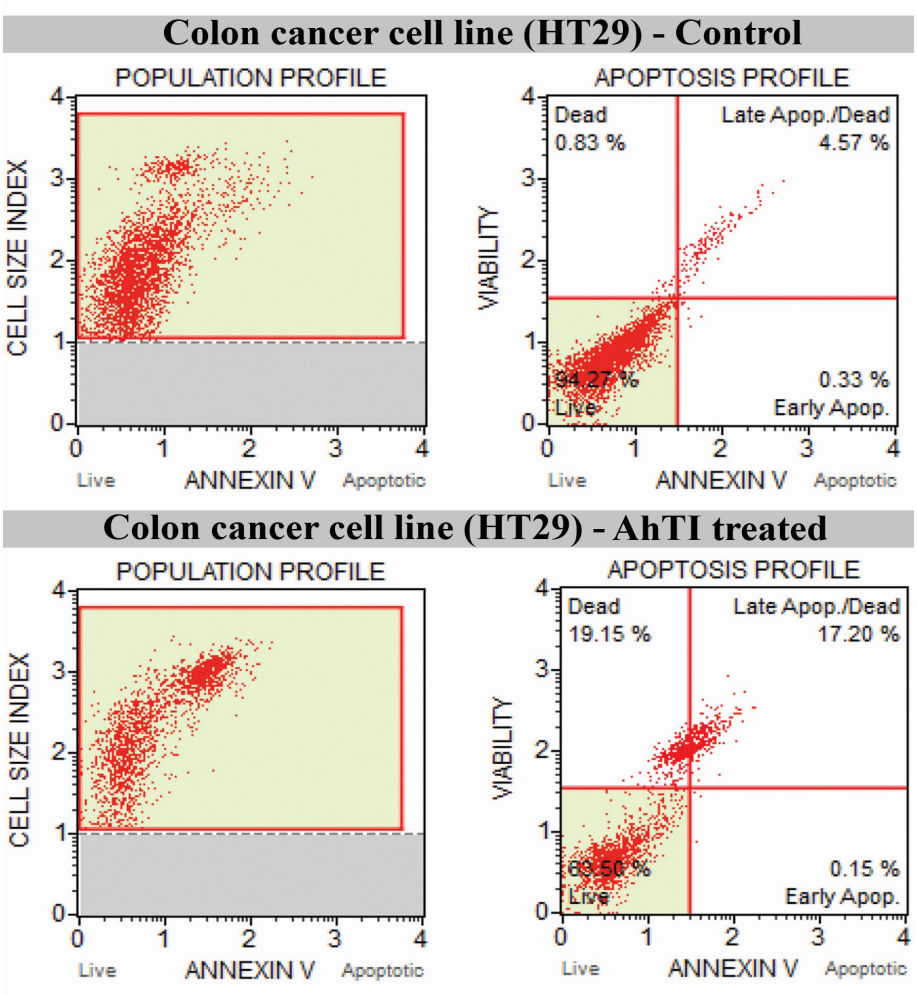
Percentage viability of cells treated with different concentrations of AhTI, GgTI and STI withHT29 cells; Error bars indicate Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. F value for AhTI=2.31x10^3; GgTI=2.29x10^3 and STI=2.04x10^3.

Neutral red uptake assay results (Fig.7.10) showed that treatment with AhTI, GgTI and STI for 24 h decreased the viability of colon cancer cells in a concentration-dependent manner. AhTI and GgTI have shown more efficacy than STI. After a 24h treatment period with AhTI it is observewd that cell viability decreased to 23.074±0.378% at the highest concentration (100µg/mL). It was 63.79±0.741% when treated with the lowest dosage (6.25µg/mL). The cell viability after treatment with GgTI was 66.29±0.752 at lowest concentration (6.25µg/mL) and 26.11±0.200% cell viability was observed after treatment with its highest concentration (100µg/mL). The treatment with STI showed (6.25µg/mL) a cell viability of 69.150±0.927% and it was reduced to 28.19±0.70% in a concentration dependent manner when increased the concentration to 100µg/mL.

7.3.5 Annexin V-FITC apoptosis of colon cancer cells by flow cytometry analysis

The Annexin V-FITC is a common method used for detecting apoptotic cells. The colon cancer cells were treated (24h) with both the samples AhTI and GgTI. The IC_{50} concentration of AhTI (10.625µg/mL) and GgTI (13.75µg/mL) and STI (26.25µg/mL) were used for the same as done in the MTT assay. Fluorochrome-labeled Annexin V is one of the most widely used cytofluorometric stains (Vermes *et al*., 1995).

Figure 7.11 The Annexin V-FITC-Flowcytometry profile of colon cancer cells after AhTI treatment



Lower Left (LL): Live cells, Lower Right (LR): Early Apoptotic, Upper Right (UR): Late apoptotic/Dead, Upper Left (UL): Debris

The colon cancer (HT29) cells treated with AhTI (IC_{50} concentrations) for 24h and stained with Annexin V-FITC and 7-AAD. Population and apoptosis profile of Control and and AhTI treated cells.

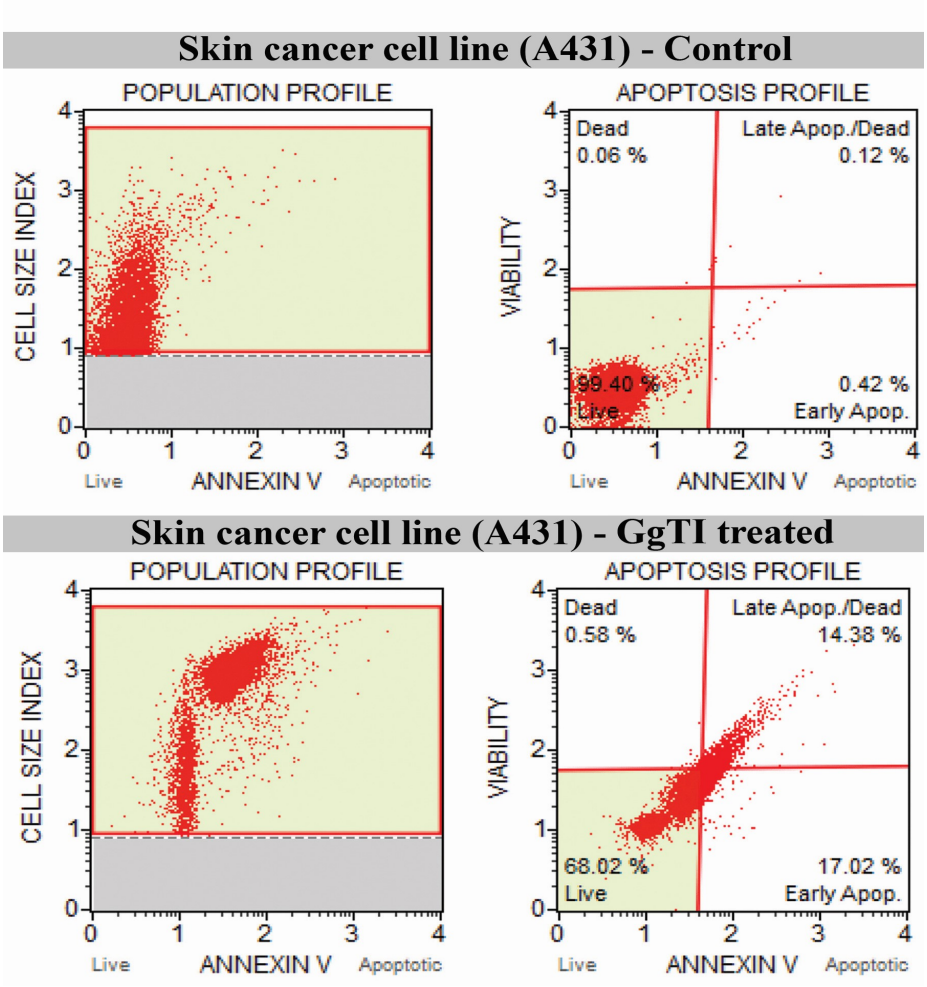
Table 7.1 Cells gated in Flowcytometry profile

Colon Cancer Cells (HT29) - Control			Colon Cancer Cells (HT29) - AhTI Treated		
	Cell Conc. (Cells / mL)	% Gated		Cell Conc. (Cells / mL)	% Gated
Live (LL) :	5.15E+06	94.27 %	Live (LL) :	7.56E+06	63.50 %
Early Apoptotic (LR) :	1.82E+04	0.33 %	Early Apoptotic (LR) :	1.79E+04	0.15 %
Late Apop./ Dead (UR) :	2.49E+05	4.57 %	Late Apop./ Dead (UR) :	2.05E+06	17.20 %
Debris (UL) :	4.55E+04	0.83 %	Debris (UL) :	2.28E+06	19.15 %
Total Apoptotic :	2.67E+05	4.90 %	Total Apoptotic :	2.07E+06	17.35 %

% of Gated cells (Live, Early Apoptotic, Late apoptotic or dead, debris and Total apoptotic cells) of untreated control and AhTI treated colon cancer cells.

In the Annexin V-FITC - flow cytometry analysis (Fig.7.11; Table 7.1), 99.27% of the cells in the non-treated control cells were alive, while only 63.50% of the cells in the AhTI-treated colon cancer cells were alive after 24h. 0.15% early apoptotic, 17.20% late apoptotic/dead cells were detected. The total apoptotic cells detected was 17.35%.

Figure 7.12 The Annexin V-FITC Flowcytometry profile of colon cancer cells after GgTI treatment



Lower Left (LL): Live cells, Lower Right (LR): Early Apoptotic, Upper Right (UR): Late apoptotic/Dead, Upper Left (UL): Debris

The colon cancer (HT29) cells treated with AhTI (IC_{50} concentrations) for 24h and stained with Annexin V-FITC and 7-AAD. Population and apoptosis profile of Control and and AhTI treated cells are presented.

Table 7.2 Cells gated in Flowcytometry profile

Colon Cancer Cells (HT29) - Control			Colon Cancer Cells (HT29) - GgTI Treated		
	Cell Conc. (Cells / mL)	% Gated		Cell Conc. (Cells / mL)	% Gated
Live (LL) :	5.15E+06	94.27 %	Live (LL) :	4.07E+06	75.10 %
Early Apoptotic (LR) :	1.82E+04	0.33 %	Early Apoptotic (LR) :	1.81E+04	0.33 %
Late Apop./ Dead (UR) :	2.49E+05	4.57 %	Late Apop./ Dead (UR) :	6.04E+05	11.13 %
Debris (UL) :	4.55E+04	0.83 %	Debris (UL) :	7.29E+05	13.43 %
Total Apoptotic :	2.67E+05	4.90 %	Total Apoptotic :	6.22E+05	11.47 %

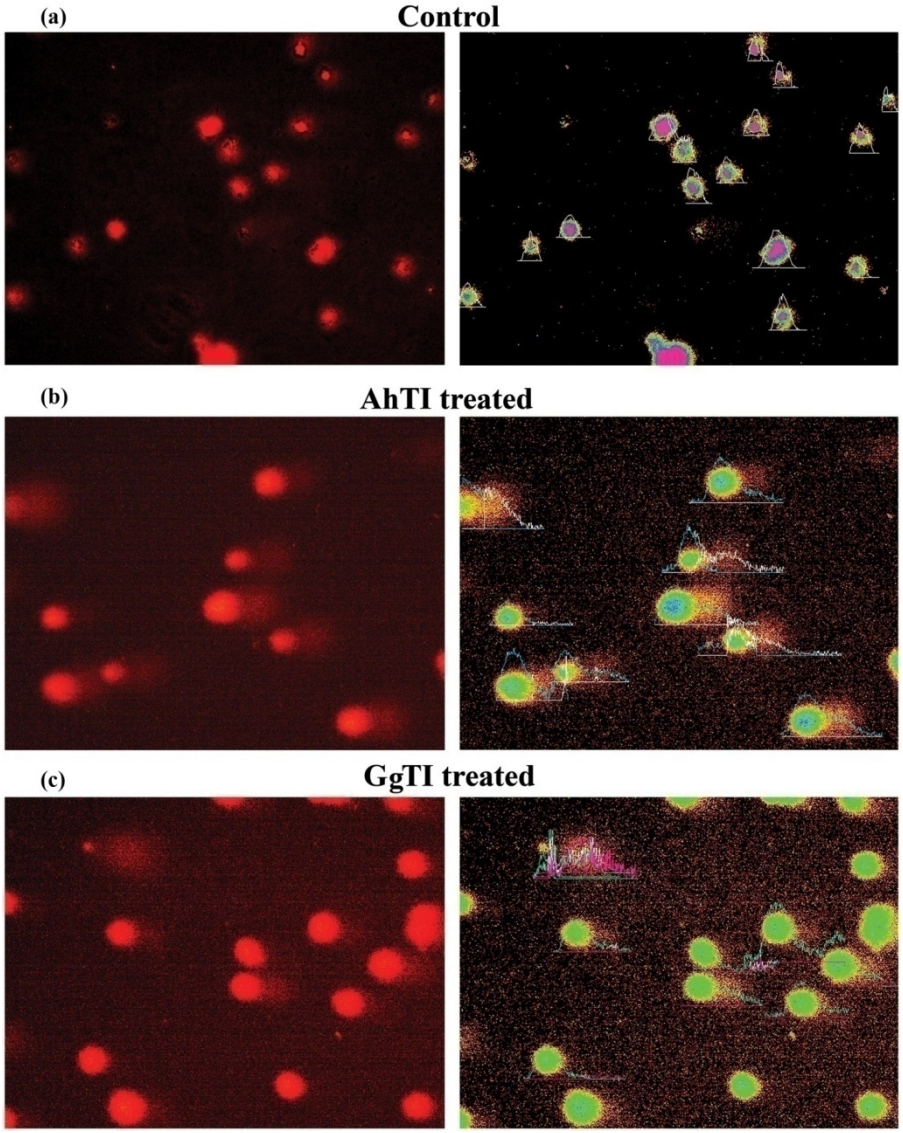
The % of gated Live, Early Apoptotic, Late Apoptotic or Dead, Debris and total apoptotic cells.

The untreated control group has 94.27% cells. The cells after GgTI treatment showed that 75.10% cells were alive, 0.33% cells were of early apoptotic and the total apoptosis was 11.47%.

7.3.6 Comet assay for the evaluation of DNA damage in colon cancer (HT29) cell lines

The comet assay has been shown to be a very useful method for assessing DNA damage in cells in many investigations by researchers. It is now well-established that the comet assay test is an effective method for examining DNA damage and repair (Speit *et al*., 1999). This assay was used to examine the DNA damage that occurred in colon cancer cells that had been exposed to AhTI (IC_{50} concentrations) for 24 h as well as untreated control cells. The TriTek programme was used to measure and calculate the comet and tail lengths.

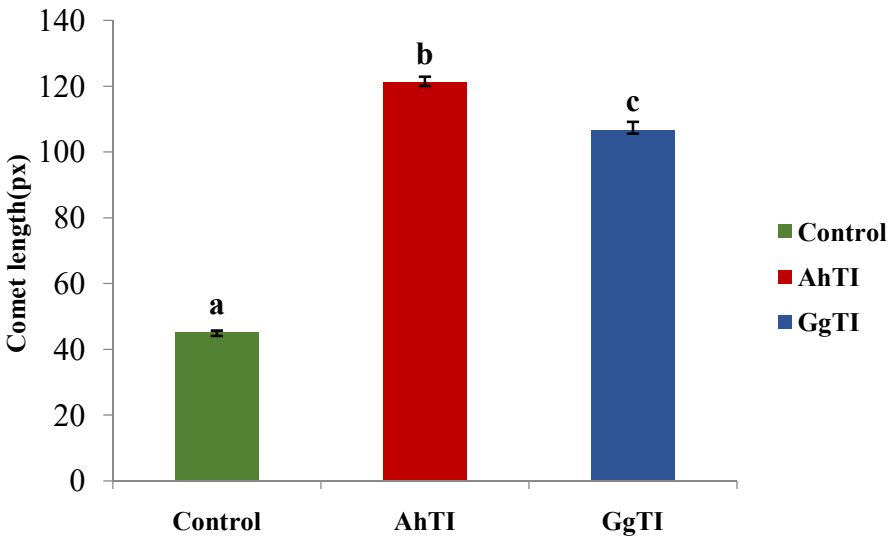
Figure 7.13 Comet assay colon cancer (HT29) cell lines after treatment with AhTI and GgTI



Evaluation of DNA damage of Colon cancer cells (HT29) by comet assay, (a) Control, (b) AhTI and (c) GgTI treated.

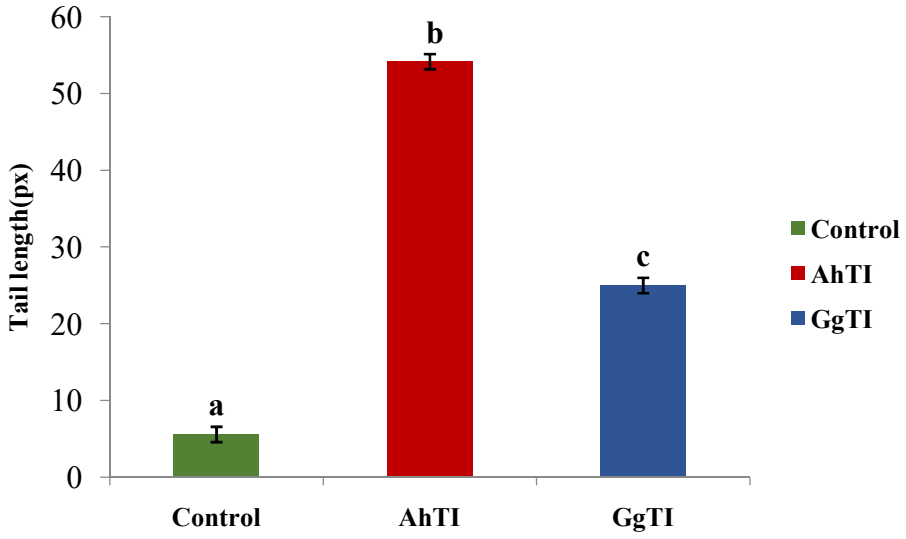
In the Comet assay technique the DNA damage is calculated as the DNA tail length and comet length. The longer the DNA tail length, the more significant the damage. The comet assay (Fig.7.13) revealed that the DNA in the control cells (a) is intact without a DNA tail, whereas the DNA in the AhTI-treated cells (b) showed significant damage as the tail length was increased.

Figure 7.14 Comet length and tail length of colon cancer cell line (HT29) treated with AhTI and GgTI



Determination of comet length of colon cancer cells (HT29) after treatment with IC_{50} concentrations of AhTI and GgTI. Error bars indicates Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. Fvalue for Control=478; AhTI=869 and GgTI=774.3.

Figure 7.15 Tail length of colon cancer Cell line (HT29) treated with AhTI and GgTI



Determination of tail length of colon cancer cells (HT29) after treatment with IC_{50} concentrations of AhTI and GgTI. Error bars indicates Standard Deviations, (not visible when smaller than symbol size). Different alphabets/symbols indicate the significant differences between the groups. Fvalue for Control=172; AhTI=942 and GgTI=568.2.

DNA damage is determined using the DNA tail length and comet length measurement in the comet assay technique. The extent of the increased length of the tail indicates increased DNA damage. The DNA in the control cells (Fig.7.13) is intact without a DNA tail, whereas the DNA in the AhTI and GgTI treated cells (Fig.7.13) showed considerable damage as the length of the DNA tail increased. Fig.7.14 and Fig.7.15 display the comet length and tail length respectively of the TI treated cell lines. In the control cells the length of the comet is 45.13 ± 0.59px and the tail length is 5.6 ± 0.68 px. The AhTI and GgTI treated cells showed a comet length of 121.09±1.82 px

106.64 ± 2.59 px. The tail length is 54.18 ± 2.168 pxand 25 ± 1.910px respectively. The slides were imaged using an Olympus CKX41 Inverted Epifluorescent Microscope and an Opitka Pro5 CCD camera 10x Magnification. The Tritek comet scoring software was used to determine the length of the comet and tail.

7.4 DISCUSSION

Different PIs have anti-proliferative activity against colon Billings *et al*., 1990; Caccialupi *et al*., 2010; Clemente *et al*., 2012). Field bean trypsin inhibitors, for example, are trypsin inhibitors that have the potential to suppress B16-F10 melanoma cell lung metastasis in mice (Banerji *et al*., 1998). It is claimed that the Bowman-Birk family of inhibitors, found in soybeans and other legumes, may be nutritionally significant anti-carcinogens, especially with regard to colon cancer (Yavelow *et al*., 1983). The 14.3kDa *Coccinia grandis* PI (CGPI) protein was found in the plant's leaves. CGPIs inhibited growth in colon cell lines in a dose-dependent manner. In this chapter we focus on the studies of the anticancer potential of the TI proteins isolated from the seeds of *A.hirsutus* (AhTI) and *G.gummi-gutta* (GgTI) against colon cancer (HT29) cell lines.

The result of phase contrast microscopy images of colon cancer cells revealed significant morphological changes in the AhTI (Fig.7.1), GgTI (Fig.7.3) and STI (Fig.7.5) treated cells with different concentrations including cell shrinkage, blebbing, rounding, granulation and vacuolization in the cytoplasm. An inverted phase contrast tissue culture microscope was used to visualize and record all

of the plates after 24h. The same procedure was done with the normal cells (L929) also. But there are no detectable alterations discovered in normal cells when applied different concentrations of AhTI for 24h (Fig.7.2). The normal cells (L929) were also treated with AhTI (Fig.7.2), GgTI (Fig.7.4) and STI (Fig.7.6) which showed no characteristic changes. All of the plates were observed and recorded using an inverted phase contrast tissue culture microscope. In malignanat cells the cytoplasm is scanty and intensely coloured, or, on the other hand, is pale, and the malignant cell has a big nucleus that is uneven in size and shape, prominent nucleoli, and other morphological characteristics may be seen (Baba *et al*., 2007).

Following treatment with AhTI, GgTI and STI, the MTT assay result (Fig.7.7) showed that there is a concentration-dependent decrease in cell viability. From 6.25g/mL at the lowest concentration to 100g/mL at the highest, the samples used had a range of concentrations. After 24h of treatment with AhTI at concentrations of 6.25µg/mL, 12.5µg/mL, 25µg/mL, 50µg/mL, and 100µg/mL, the cell viability was respectively, 55.46±0.472%, 47.89±0.20%, 41.47±0.58%, 32.38±0.57% and 25.77±0.58%. The IC_{50} values for AhTI, GgTI and STI were 10.625µg/mL, 13.75µg/mL and 26.25µg/mL, respectively for colon cancer cells. The MTT experiment was carried out on L929 cells, and the results are shown in Figure 7.8. After a 24h treatment period with different concentrations of AhTI, GgTI and STI, there was no significant cell death in the normal cells (L929). Approximately 97-98% of the treated cells were generally alive after receiving dosages of lower to higher (6.25g/mL-100g/mL)

of AhTI, GgTI and STI. The coenzyme nicotinamide adenine dinucleotide (reduced form) (NADH), which is produced by glycolysis in the cytoplasm and oxidative phosphorylation in the mitochondria, has lately been postulated as the primary reducing agent responsible for MTT reduction (Van Tonder *et al*., 2015). Yaffe *et al*. (2013) also noticed that treatment of HRT-18 colorectal carcinoma cells with various concentrations of piperine significantly inhibited the proliferation of cancer cells in a concentration- and time-dependent manner.

The LDH leakage experiment supports the results of MTT assay. The experiment reveals that AhTI treated cells considerably leaked more LDH enzyme than GgTI-treated cells did (Fig.7.9). The LDH leakage was decreased in the STI treated cells when compared to AhTI and GgTI-treated cells. Reports suggest that the treatment of melanoma cells with Juglone, a chief constituent of walnut led to a considerable concentration dependent rise in LDH levels supporting their MTT assay results (Aithal *et al*., 2009). Results from the neutral red absorption assay (Fig.7.10) demonstrated that colon cancer cells were less viable after being exposed to AhTI, GgTI, and STI for 24h in a concentration-dependent manner. STI has not been as effective as AhTI and GgTI.

After 24h only 63.50% of the cells in the AhTI-treated colon cancer cells were still alive in the aforementioned Annexin V-FITC flow cytometry examination (Fig.7.11; Table 7.1), compared to 99.27% of the cells in the control cells that had not been treated.The

protein Annexin V is used to count or measure apoptotic or dead cells (Huerta *et al*., 2007). Apoptotic cells that were 0.15% early and 17.20% late were found to be dead cells. 17.35% of all cells were discovered to be apoptotic. Apoptosis differs from necrosis in that chromatin does not flocculate, mitochondria are not enlarged, and the cell membrane is impermeable to stains (Baba *et al*., 2007).

The DNA damage increases in significance as DNA tail length increases. The result of comet assay (Fig.7.13), DNA in control cells (a) is undamaged as evidenced by the absence of DNA tail, whereas DNA in AhTI-treated cells (b) demonstrated considerable damage as shown by the increased length of the tail. In the comet assay method, the DNA damage is assessed using the DNA tail length and comet length. According to Kent *et al*., (1995), in this method, the comet's head is a spherical mass of intact DNA, while its tail is made up of damaged DNA (DNA loops around strand breaks). The amount of the tail's lengthening suggests greater DNA damage. According to the comet assay results, the DNA in the control cells (Fig.7.14) is unaffected without a DNA tail, whereas the DNA in the GgTI-treated cells (Fig.7.14) revealed significant damage, since the length of the DNA tail increased. The comet length (Fig.7.15) and tail length (Fig.7.16) of colon cancer cell lines treated with AhTI and GgTI were visualised in an Opitka Pro5 CCD camera with 10x Magnification.

As the second-leading cause of cancer-related deaths globally, colorectal cancer is the primary killer of patients with gastrointestinal cancer and the third-most frequent malignancy overall in both men and

women (Granados *et al*., 2017). A literature search led to the inclusion of 172 experimental investigations and 71 clinical cases involving 190 plants. The findings showed that the best plants for preventing colon cancer include grapes, soybeans, green tea, garlic, olives and pomegranates. Fruits, seeds, leaves and plant roots were used as *in vitro* and *in vivo* models in these investigations (Aiello *et al*., 2019). We found that the trypsin inhibitor proteins isolated from the seeds of *G.gummi-gutta* and *A.hirsutus* were effective in killing skin and colon cancer cells in a concentration dependent manner.

Chapter 8

Summary and Conclusion

Proteases have a crucial role in the development of cancer, making them prospective targets for therapeutic development. One of the main therapeutic approaches for the treatment of cancer involves controlling the activities of proteases using PIs. Serine PIs can control the proteolytic activities of serine proteases at various phases of metastasis.

In the present study we have screened five different plant seeds for the isolation of an effective trypsin inhibitor. The plants selected for the studies are *Syzygium cumini*, *Bauhinia acuminata*, *Cascabela thevetia*, G*arcinia gummi-gutta* and *Artocarpus hirsutus.* Different extraction media were tried to select the ideal one. Among the different extractants used, 50mM Sodium phosphate buffer with pH 7.6 was found to be the most suitable one for extraction. Among the different plant seeds *Bauhinia acumianata* (707.59±26.33TIU), *A.hirsutus* and *G.gummi-gutta* showed TI activity. The seed extracts that showed higher TI activity- *A.hirsutus* (4748.70±61.99TIU) and *G.gummi-gutta* (5142.59±67.36TIU) were used for further purification and studies.

Germinated seeds of both *A.hirsutus* (after 30 days of germination) and *G.gummi-gutta* (after 3 months of germination) were also checked for TI activity. The germinated seeds of both the AhTI and GgTI showed lesser inhibitory activity than the corresponding non-germinated ones. The TI activity of germinated seeds and non-germinated seeds of *A.hirsutus* were 12.97±0.47TIU and 73.67±0.55TIU respectively. In the case of *G.gummi-gutta* the TI activities were 85.28±0.63TIU in the dry seeds and 21.01±0.65TIU in the germinated seeds. Both the plant seeds showed a reduced TI

activity after seed germination. Hence for further studies, only the non-germinated (dry) seeds were used, in both the cases.

The TI proteins after extraction with 50mMSodium phosphate buffer (pH-7.6) were purified by Ammonium sulphate precipitation and dialysis, Ion exchange chromatography and size exclusion chromatography. The ammonium sulphate precipitation was carried out as 0-30%, 30-60%, 60-90% fractions. The 30-60% fraction showed highest activity for both AhTI (439.69±5.11TIU/mg protein) and GgTI (96.1±4.88TIU/mg protein). The highest activity fraction was then subjected to Ion exchange chromatography using DEAE cellulose as the resin. The specific inhibitory activity of the pooled fraction collected during ion exchange chromatography was 458.67±5.049TIU/mg protein (AhTI) and 259.26±2.66TIU/mg protein (GgTI). After Ion exchange chromatography the pooled fraction was further purified by size exclusion chromatography. The Specific inhibitory activity of AhTI and GgTI fraction collected during Size exclusion chromatography was 2048±27.3TIU/mg protein and 281.74±0.131TIU respectively.

The inhibitory activities of both the AhTI and GgTI proteins were confirmed by activity staining by reverse zymography. The molecular weight was determined by SDS PAGE which was further confirmed by MALDI-TOF/TOF analysis. The molecular weight of the AhTI is 14.28kDa and that of GgTI is 7.85kDa.

The thermal stability and pH stability of both the TIs were determined by incubating the purified fractions after gel filtration

chromatography at different temperatures and at different pH conditions. Both the AhTI and GgTI retained the TI activity up to 100°C and highest activity is observed at 37°C. Both AhTI and GgTI showed highest activity at a pH of 7.6. At this optimal pH, AhTI showed 2043.39±17.61TIU and that of GgTI was 278.65±6TIU.

The inhibitor constant (K_I) of the purified TI proteins was determined at two different substrate concentrations from Dixon plot. The nature of inhibition of AhTI and GgTI was found to be of competetive type. The K_I value of AhTI was 0.82µg/mL and GgTI was 0.95µg/mL. Dixon plot was plotted using the software Origin Pro8.

The isolated TI protein's efficacy against skin (A431) and colon cancer (HT29) cell lines was determined. AhTI and GgTI treated (24h) cell lines in a concentration dependent manner were analysed under a phase contrast microscope to detect the morphological changes occurred. The Soyabean trypsin inhibitor (STI) was used as standard. Treatment with various concentrations of AhTI, GgTI and STI showed changes including shrinkage, blebbing and granulation. Any of the three TI proteins showed significant cytotoxic effects to the normal cell lines. But they induced detectable changes in the growth of skin and colon cancer cells. The cytotoxicity exerted by AhTI, GgTI and STI was analysed by MTT assay, LDH leakage assay and Neutral red uptake assay. The MTT assay result of AhTI treated skin cancer cell lines showed more decrease in cell viability than that of GgTI and STI. The IC_{50} values of AhTI and GgTI was 10.875µg/mL and 15.625µg/mL respectively, while the IC_{50} of STI was 28.125µg/mL.

The IC_{50}values for colon cancer cell line was 10.625µg/mL (AhTI) 13.75µg/mL (GgTI) and STI it was 26.25µg/mL.

The results of LDH leakage assay revealed that the AhTI treated skin cancer and colon cancer cells indicated a significant release of LDH enzyme than that of GgTI treated cells. STI leads to lesser LDH leakage when compared to AhTI and GgTI. Increased leakage was found in the cells treated with higher concentrations of TIs.

The results of neutral red uptake assay of the skin cancer and colon cancer cells revealed that there is a decrease in cell viability of the skin cancer cells after treatment (24h) with AhTI, GgTI and STI in a concentration dependent manner. AhTI and GgTI are found to be more effective than STI.

Annexin V FITC was used to determine the apoptotic activity of the skin and colon cancer cells after treatment with IC_{50} concentrations of AhTI and GgTI. Annexin V-FITC - flow cytometry analysis of skin cancer cell lines reveals 99.40% of cells in the non-treated control and only 43.58% of cells in the AhTI-treated group were alive after treatment for 24 h. In that case 33.49% were early apoptotic and 22.03% were late apoptotic/dead cells. The total apoptotic cells detected was 55.52%. In the case of GgTI treated cells, only 68.02% of the cells in the skin cancer cells were alive after 24h, in contrast to 99.40% of the cells in the untreated control cells. 17.02% of the apoptotic cells were early and 14.38% were late or dead. 31.40% of cells had undergone cell death.

The flow cytometry analysis of colon cancer cell lines showed that 99.27% of the cells in the non-treated control cells were alive, while only 63.50% of the cells in the AhTI-treated colon cancer cells were alive after 24h of treatment. In this case 0.15% early apoptotic, 17.20% late apoptotic/dead cells were detected. The total apoptotic cells detected was 17.35%. After GgTI treatment 75.10% cells were alive and 0.33% cells were of early apoptotic and the total apoptosis was 11.47%.

Comet assay was performed to determine the DNA damage of the skin and colon cancer cells after treatment (24h) with IC_{50} concentrations of AhTI and GgTI. The increased length of tail is an indication of DNA damage. The Opitka Pro5 CCD camera and an Olympus CKX41 Inverted Epifluorescent Microscope were used for the imaging of the slides to detect the length of the comet and tail which was calculated by TriTek comet scoring software. The more the length the more is the DNA damage occurred. AhTI treated skin cancer cells showed more tail length than that of GgTI treated cells. The tail length of AhTI treated skin cancer cells was 52±2.63px and that of GgTI treated was 35.41±2.01px.

In the instance of colon cancer cell lines also the AhTI treatment displayed more DNA damage as the tail length was augmented in size than that of GgTI treated cells. The tail length of AhTI treated colon cancer cells was 54.18±2.168px while that of GgTI treated cells was 25±1.910px.

In the current study we found that the seeds of both *A.hirsutus* and *G.gummi-gutta* contain an effective trypsin inhibitor each with molecular weights of 14.28KDa and 7.85kDa respectively. Both the trypsin inhibitor proteins (AhTI and GgTI) own the nature of competitive type of inhibition and they exhibited potent activity against skin cancer (A431) and colon cancer (HT29) cell lines.

We can hence conclude that AhTI and GgTI are promising candidates for the development of active drugs for human colon and skin malignancies. We believe that this study will be helpful to individuals involved in cancer research and that it will promote the expansion of ethnomedicine; because it has revealed a potential plant source of a medicinally valuable trypsin inhibitor that is cost-effective as well.

Future Prospects

Two dominant trypsin inhibitors were isolated from the seeds of *Artocarpus hirsutus* Lam. (AhTI) and *Garcinia gummi-gutta* (L.) Roxb.(GgTI) and they were successfully purified and characterised. Their anticancer activity against skin cancer (A431) and colon cancer cell lines was also determined. Both of the TIs showed significant cancer killing activity in both the cell lines. The study can be extended in the future to explore the amino acid sequence of both the TI proteins by MALDI TOF/TOF-MS analysis. This will be helpful for the subsequent computational docking studies to detect the affinity of the trypsin inhibitors to human trypsin enzyme. Cell cycle analysis of the TI treated cancer cells can be performed to observe DNA damage and apoptosis that interrupt cell cycle progression and the phase at which the arrest of cell growth occurs. These *in silico* and molecular level studies may be very useful in the field of pharmacological research for developing suitable and cost effective drug against these types of cancers or trypsin associated diseases and for possible collaboration with pharmaceutical companies. Most of the trypsin inhibitors of plant origin flopped in early clinical trial phases because of their nonspecific toxicity and lack of pharmacological efficacy, even if the side effects of the initial forms of these inhibitors vanished with the emergence of future drugs. New applications that selectively modulate proteases and support their planned mode of delivery are therefore highly regarded.

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