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INTRODUCTION

Cancers are caused by uncontrolled cell development that is the second biggest cause of death in the world, after cardiovascular disease. On a global scale, cancer is the superior origin of mortality, and it is completely culpable for preventing humans from living longer lives. Chemotherapy, along with surgery and radiation therapy, have been used to treat cancer, although side effects such as nephrotoxicity and neurotoxicity have been shown to have a negative impact on patient survival. As a result, another type of medicine with fewer or no hazardous adverse effects is required. Plant-based natural ingredients with medicinal value have sparked interest in regulating cancer progression and have the potential to lead to the development of new cancer-prevention medications (Rajendran *et al*., 2021).

For the discovery of novel effective medications, chemotypes, and pharmacophores, nature serves as an endless and inexhaustible resource. Medicinal herbs are widely used around the world and have become increasingly important in the treatment of ailments in recent years. Bioactive chemicals, which are components of the plant's chemical core, play a therapeutic role. Natural substances have played an essential role in the treatment and prevention of many illnesses since ancient times, providing the backbone of the traditional therapeutic system. Furthermore, terrestrial plants continue to dominate modern therapeutic techniques, as plant-derived substances make up a major portion of today's pharmacological drugs, particularly in the fields of antibiotherapy and chemotherapy. In the realm of oncology, the use of herbal medications has been increasingly embraced as a supplemental or alternative option. As a result, several novel cytotoxic chemicals are identified from plants every year, presenting new cancer-fighting opportunities. Many scientists are focusing their efforts on studying naturally occurring molecular entities that could be valuable to the pharmaceutical sector (Dehelean *et al*., 2021).

The present study aims to isolate and identify the flavonoid and acetogenin rich fractions from *Annona muricata* leaves and to investigate the antioxidant activity with the focus on the anticancer action of the *Annona muricata* ethanolic leaf extract and its chromatographically separated fractions. A brief introduction about the research layout of the current study is discussed in this chapter.

In the human body, free radicals are formed during metabolic reactions in living cells. Environmental contaminants, oxidative stress, synthetic pesticides, harmful radiation, water contaminated with pollutants, and different medicinal drugs and procedures are all known to enhance the generation of free radicals. Due to their rapid reactivity nature, free radicals can undergo sequential events including free radical chain reactions with biomolecules namely proteins, triglycerides and fats, and nucleotides, causing damage to the entire organisms as these biomolecules are building blocks and, as a result, to body tissues. Furthermore, free radicals have been linked to ageing and several diseases and disorders of chronic nature caused due to the presence of excess oxidants (Zheng *et al*., 2022).

ROS stands for reactive oxygen species, which are unstable, reactive, partially reduced oxygen compounds generated because of the metabolic activities. ROS production and its increased level causes damage in tissues and cells, and this imbalance state of oxidants is referred as oxidative stress (Peng *et al*., 2022). In biological systems, oxidative stress is a condition where an imbalance exist between the body's ability to detoxify the detrimental impact of oxidants by effective neutralisation by the antioxidant molecules and the generation of free radicals; oxidant induced stress shows a significant function in biological processes. Hence, the impact of oxidative stress on cancer incidence, progression, and treatment is determined by the amount and types of radicals produced and are divided into two categories: reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Oubaidy *et al*., 2021).

ROS including superoxide anion, singlet oxygen, hydroxyl radical, hydrogen peroxide and hypochlorous acid are small intracellular molecules in cell signalling which is required for a variety of activities in both normal and malignant cells. ROS is required for a variety of cellular functions in normal somatic cells, including immunological defence mechanisms and obligatory secondary signalling. ROS levels in cancer cells are elevated as a result of both external and internal pathways (Yang *et al*., 2018).

Reactive nitrogen species are peroxynitrite and NO_2 intermediates. High, extended levels of NO, superoxide, and precursors of peroxynitrite have been associated to cancer, inflammatory illnesses and tissue damage (Ronzio, 2020).

Although aerobic creatures must thrive, oxygen can be a potentially hazardous molecule. When oxygen is lowered during biochemical reactions important to living organisms, intermediate metabolic products known as ROS are produced, which cause oxidative damage to numerous tissues. Because they cause oxidative death in all living

creatures that metabolise molecular oxygen, ROS is called "oxidant" or "free radical." Free radicals are so harmful to the body and are extremely short-lived molecules that disturb the structure of high-energy electrons by removing other electrons from their orbits (Kukurt *et al*., 2021).

Increased mitochondrial respiration causes oxidant induced stress, which is authenticated as a disproportion between harmful ROS formation and the cell's potential to neutralise such radical precursors. Electron leakage from complex I and III of the electron transport chain causes oxygen decline and the consequent production of ROS. Peroxisomes (fatty acid oxidation) and the endoplasmic reticulum (protein oxidation) are other subcellular sites where ROS are produced as derivatives of enzymatic processes by xanthine oxidases, NADPH oxidases, cyclooxygenases and lipoxygenases (Greenwood and Witney, 2021).

ROS are created as a consequence of oxidative metabolism, primarily mitochondrial respiration, as well as in response to xenobiotics or cytokines. The bulk of ROS in the cell is produced by the mitochondrial electron transport chain, which generates energy. The oxidation of NADH and $FADH_2$ at the electron transport chain's complexes I (NADH dehydrogenase) and III (coenzyme Q and cytochrome C oxidoreductase) produces superoxide ($O_2^{\bullet-}$). The enzyme complex NADPH oxidase is the second major generator of ROS (Tauffenberger and Magistretti, 2021).

Antioxidants are chemicals that help to prevent oxidation. Thiols and ascorbic acid are antioxidants that prevent these sequence reactions. To maintain a balanced oxidation condition, plants and animals possess complex systems of overlapping antioxidants (Salehi *et al*., 2018).

Endogenous antioxidants, including glutathione (GSH), glutathione peroxidase (GPx), CAT, and SOD, are found in our bodies, while exogenous antioxidants, such as phenolics, carotenoids, vitamins (E and C), and minerals, can be obtained from food as a primary source or as a supplement in other circumstances. To maintain cellular redox equilibrium, both endogenous and exogenous antioxidant systems function together (Bekhet and Eid, 2021).

The socioeconomic standards, and mortality rate of the population are all affected by health issues. Cancer is a significant contribution to these global challenges, with epidemiological research indicating that over 14 million new cases are diagnosed each year, with an annual fatality rate of roughly 8 million. When cells develop resistant to chemo or

radiotherapy, the increase in mortality risk is connected to a surge in tumour recurrence (Sung *et al*., 2021).

Cancer is a disease typified by aberrant cell growth and the potential to invade and metastasize. Changes in the genes that regulate normal body processes are one of the components implicated in cancer initiation. Given the steady rise in cancer incidence worldwide, as well as the growing problem of drug resistance, there is a growing interest in various cancer prevention strategies (Ranjan *et al*., 2019).

ALL is an abnormal lymphoid cell proliferation which can infect the bone tissue, bloodstream, and extranodal sites. A series of complex process leads to T-cell acute lymphoblastic leukemia, in which genetic defects accumulate and disturb the normal control of thymopoiesis cell formation, development, multiplication, and longevity. This condition's genetics are highly variable, with anomalies in the chromosomes discovered in nearly every patient (Malard and Mohty, 2020).

T-cell acute lymphoblastic leukemia (T-ALL) is a rare and aggressive form of acute lymphoblastic leukaemia (ALL), accounting for about 20% of adult and 10-15% of paediatric ALL cases. T-cell malignancy can strike at any stage of their maturation in the thymus, resulting in a high degree of disease heterogeneity (Drobna *et al*., 2018).

Persistent illness, bone aching, swollen lymph glands, petechia, and dyspnea because of mediastinum enlargement are all indications of ALL. Acute lymphoblastic leukaemia is diagnosed using WHO categorization recommendations from 2016, which include morphology of cell, immunophenotyping, heredity, and analysing chromosome structures. Immunohistochemistry seems to be the standard method for progenitor assessment, categorization, and identification of features essential to the evaluation of residual disease, whereas microscopy-based morphological identification of lymphoblasts can evaluate peripheral blood and bone marrow involvement.

Traditional cytogenetics should be performed on every patient, and this should be supplemented with molecular cytogenetic technique or RT-PCR for the discovery of certain genomic anomalies, such as cryptic translocations that are not detectable by traditional cytogenetic techniques. Flow cytometer can also be used to detect aneuploidy. Recent developments in next-generation genomics have made whole genome sequencing conceivable, and diagnostic procedures may be obsolete once this methodology becomes widely available and affordable (Malard and Mohty, 2020).

Complementary and alternative medicine (CAM) refers to treatments that are outside of the scope of conventional medicine. These treatments have the potential to be employed as therapeutic and preventative agents in the treatment of physical and mental diseases. Prior to the emergence of modern medicine, they were commonly utilised to treat ailments for generations. In some countries, CAM has been utilised in conjunction with existing therapeutic approaches to support and assist patient recovery since the dawn of modern medicine. Natural supplements, physical exercises, and mental relaxation techniques are examples of these types of treatments (Yajid *et al*., 2018).

Natural products are becoming more popular in cancer treatment because they are thought to be more biological friendly, which means they are more co-evolved with their target locations and less hazardous to normal cells (Seca and Pinto, 2018). Phenolic components, organosulphur compounds, polyphenolic compounds, glycosides, and vitamins are examples of phytochemical metabolites. Despite significant advances, some tumours still have a bad prognosis, and research is being focused on using non-toxic dosages of plant-extracted chemicals. The discovery and use of natural chemotherapeutic agents paved the way for a new therapeutic approach that relied on natural molecules and drugs (Forni *et al*., 2019).

Flavonoids are a type of polyphenolic chemical that can be found naturally in plant-based diets. In plants, around 6000 flavonoids have been identified. They have a phenylbenzopyrone ring and are divided into subgroups based on their chemical structures, such as isoflavones, flavones, anthocyanins, flavonols, flavanonals, and chalcones. These chemicals are helpful to humans because they have the ability to interact with a variety of biological targets, including antioxidant, anti-inflammatory, antiviral, and anticancer effects (Rajendran *et al*., 2021).

Surgery, radiation, and chemotherapy are the three basic aspects of cancer medical treatment. This sort of treatment is frequently associated with a slew of negative side effects and health risks, including nausea, loss of appetite and weight, anaemia, spinal cord injury, renal damage, mucositis. These adverse effects have a major negative impact on one's quality of life of those receiving these medicines. It's also crucial to remember that only about a quarter of treatments result in a complete response, thus multiple cycles of these therapies are frequently required. As a result, modern medicine increases cancer patients' life expectancy at the expense of their quality of life; as a result, a great number of people with this illness turn

to medicinal plants to treat the cancer and/or lessen the side effects of current medical treatment (Ortega and Campos, 2019).

Human epidemiological data demonstrated a stronger association between a plant-based diet and a reduced risk of heart disease and cancer. Polyphenols are powerful anticancer drugs that work through a variety of mechanisms, including inhibiting one or more biochemical pathways and biomolecules that aid in the treatment of cancer cells. Targeting the NF-κB signalling pathway using natural techniques that affect the expression of thousands of genes is one of the viable strategies for treating lymphomagenesis. To treat cancer cells, natural chemicals promote apoptosis, angiogenesis, metastasis, autophagy, cell proliferation, and cell cycle arrest (Batool *et al*., 2021).

Annona muricata is a small perennial tree in the family of Annonaceae, that is widely grown and distributed throughout the tropical and subtropical regions. *Annona muricata*, also known as soursop, graviola, guanabana, or Brazilian paw-paw, is a large, glossy dark green plant with delicious green heart-shaped fruits. Soft curved spines wrap the fruit's surface, and black seeds are distributed in a creamy surface with a distinct aroma and flavour. *Annona muricata* fruits have already been extensively used as confectionary products, and a variety of preparations, especially decoctions of the plant parts have already been broadly used in herbal medicine by local people in tropical Africa and South America to treat a variety of conditions, including leukemia. *Annona muricata* therapeutic properties against various human malignancies and microbial pathogens are frequently investigated in *in vitro* culture and preclinical animal model systems for their ability to target specific disease, while exhibiting almost no effect on normal cell viability. Over 212 phytochemical components, various classes of acetogenins, isoquinoline alkaloids, phenolics, lipids, and various active metabolites have been found in *Annona muricata* extracts found in various plant sections, are responsible for antioxidant, anti-inflammatory, anticancer and antimicrobial effects. (Rady *et al*., 2018).

Phytochemical research on various portions of *Annona muricata* have yielded 212 secondary metabolites, including annonaceous acetogenins, isoquinoline alkaloids, flavanoids, and megastigmanes, which have been isolated and characterised to date. Acetogenins, a distinctive set of derivatives of lipids generated from the polyketide pathway that belong to the Annonaceae family, have been found in abundance in *Annona muricata* leaves and they are the most often utilised parts for a range of ethnomedicinal purposes. The most common bioactive molecules in the Annonaceae family, as well as *Annona muricata*,

are acetogenins. About 120 acetogenins have been recorded from the parts of *Annona muricata* in prior phytochemical studies, with roughly 46 acetogenins found in the leaves. Acetogenin is a long aliphatic chain interlinked to a γ-lactone α ring and terminally substituted by β-unsaturated methyl; in other situations, it is a ketolactone, with tetrahydrofuran positioned along with the hydrocarbons chain and a specific number of oxygen groups. Despite the fact that acetogenins with two adjacent or nonadjacent THF rings have been described, the majority of acetogenins previously identified in *Annona muricata* are mono-THF rings. Acetogenins are also linear and can include one or two epoxy groups. Phenolic compounds are the most important phytochemicals since the majority of them are water soluble, and aqueous infusion is the most widely utilised extract in traditional medicine. The leaves of *Annona muricata* were used to isolate 34 phenolic chemicals. In addition to the phytochemicals mentioned, antioxidant vitamins, organic amides, and aromatic oils have been identified in *Annona muricata* leaves. (Wahab *et al*., 2018).

Flavonoids are secondary plant metabolites with antibacterial, antiviral, antioxidant, anti-allergic, and anti-inflammatory properties. They are responsible for the colour and perfume of flowers. Polyphenolic compounds interact with several pathways during carcinogenesis such as initiation, progression and dissemination of cancer (Abotaleb *et al*., 2019).

Flavonoids are potent antioxidants that protect plants from the effects of the environment. As a result, epidemiological and experimental study to determine if they can help with a variety of acute and chronic human ailments has received a lot of interest. Flavonoids have been proven to have anti-inflammatory, immunomodulatory, and significant anticancer properties according to the *in vitro* and *in vivo* investigations (Kopustinskiene *et al*., 2020).

The study of phytochemical synergism improves the efficacy of anticancer drugs. Rather than a single biological reaction, the potential of chemo preventive phytochemicals to prevent tumour growth is the consequence of a mixture of several diverse intracellular effects. Combination therapy may also produce therapeutic synergy between separate drugs at the appropriate dose, lowering their individual toxicity and concentration. Individual anti-carcinogen therapeutic doses may be naturally incorporated from plant sources (Gupta, 2021).

Polyphenol-rich extracts and phenolic compounds have a higher likelihood of acting as therapeutic agents that are also chemo preventive. In the case of extracts, they have demonstrated prospective results from diverse phenolic and natural proportions of synergistic interactions among them, in addition to their complementary biological activities. According to published data, anticancer polyphenols' activity is dependent on a number of mechanisms, including inhibition of signal transduction pathways, regulation of tumour suppressor genes, cell cycle arrest, downregulation of oncogenes, impairment of angiogenesis, differentiation induction, and apoptosis induction. With these many modes of action performed by phenolic compounds, it is possible to choose the best anticancer agent (Gupta, 2021).

One technique for treating cancer is to gain control over or maybe stopping the uncontrolled multiplication of cancer cells. It's a really effective approach to employ the cell's natural dying mechanism. The most effective non-surgical treatment is apoptosis-targeting. Evasion of apoptosis is a signature of cancer and is independent of the genesis or type of cancer, making it a suitable target for a variety of cancers (Pfeffer and Singh.2018).

Patients are suffering from significant side effects from current apoptosis-inducing chemotherapies. As a result, finding less hazardous medications is a top priority, and natural products are likely to help in the creation of apoptosis-modulating drugs (Herrera *et al*., 2019).

Apoptosis is a type of cell death that differs from necroptosis, autophagy, ferroptosis, pyroptosis, netosis, and necrosis. Apoptosis causes morphological changes in cells, such as nuclei and mitochondria shrinking, membrane blebbing, or the tying off of cell content by sections of the plasma membrane, lowers cell size and functionality over time (Valentin *et al*., 2018).

BCL-2 dependency has been found in both cell lines and patient samples of Acute Lymphoblastic Leukemia, and high BCL-2 expression has been linked to a sluggish response to therapy. BCL-2 and BCL-xL are expressed at higher levels in Acute Lymphoblastic Leukemia cells than in normal B cells, hence inhibition of BCL-xL and BCL-2 may be advantageous (Valentin *et al*., 2018).

Acetogenins' ability to inhibit NADH oxidase has also been proven to be significant for their anti-tumour activity. Acetogenins have also been demonstrated to inhibit ATP generation in mitochondria. This type of activity has been demonstrated to be active in contrast to malignant cells that produce more ATP than normal cells, therefore restricting

cancer cells' ability to grow. Surprisingly, acetogenin toxicity was only seen in cancer cells, with only minor effects on normal cells (Yajid *et al*., 2018).

In view of this, the current study was designed to determine the synergistic effect and apoptosis induction of flavonoid and acetogenin enriched fractions of *Annona muricata* leaves in Molt-3 cells and Peripheral Blood Lymphocytes under *in vitro* conditions.

Hypothesis of the study

Present study was designed to test the following hypotheses:

Null hypothesis (H_0): Flavonoid and acetogenin enriched fractions of *Annona muricata* leaves does not possess synergistic interaction and apoptosis induction in Molt-3 cells and Peripheral Blood Lymphocytes.

Alternative hypothesis (H_A): Flavonoid and acetogenin enriched fractions of *Annona muricata* leaves possess synergistic interaction and apoptosis induction in Molt-3 cells and Peripheral Blood Lymphocytes.

Hence, the present study was formulated with the following objectives to test the above mentioned null and alternate hypotheses

- To perform bioassay guided fractionation, Isolation and characterization of Flavonoid and Acetogenin enriched fractions from *Annona muricata* leaves
- To evaluate and compare the antioxidant potential of Ethanolic crude extract (ECE), Flavonoid enriched fraction (FEF), Acetogenin enriched fraction (AEF) and combination of FEF and AEF of *Annona muricata* leaves against a team of radicals
- To evaluate the synergistic effect and anticancer activity of ECE, FEF, AEF and combination of FEF and AEF of *Annona muricata* leaves using Molt-3 and its normal counterpart Peripheral Blood Lymphocytes (PBL)
- To analyse ADME properties and molecular docking of compounds present in FEF and AEF with genes involved in apoptotic induction and targets from leukemia pathway

A brief review relevant to the present study is presented in the next chapter.