A study on phytochemical analysis and cytotoxic activity of ethanolic extract of *Annona muricata*

M.Kavitha

(15PBCOO5)

A Thesis submitted to

Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore – 641043

In Partial Fulfillment of the Requirements for the

Degree of Master of Science in Biochemistry

April, 2017

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10/04/2017
Signature of the Head of the Department

Signature of the Supervisor

ACKNOWLEDGEMENT

Every work on its backdrop has the blessing of ***GOD ALMIGHTY****. Therefore I submit my reverential gratitude at the feet of lord Almighty.*

I am grateful to ***Dr. (Thiru) P.R. Krishna Kumar,*** *Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for providing all facilities necessary for the study.*

My sincere debt to ***Dr. (Tmt) Premavathi Vijayan,*** *Vice Chancellor, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for providing the opportunity and exposure to the world of knowledge.*

My sincere thanks to ***Dr. (Tmt) S.Kowsalya,*** *Registrar, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for administrative support and for providing adequate help required to carry out the work.*

My sincere thanks to ***Dr. (Tmt) A. Parvathi****, Dean, Faculty of Science, Avinashilingam Institute for Home Scienece and Higher Education For Women, Coimbatore for her guidance and affectionate support, expert suggestions throughout the project.*

My sincere thanks to ***Dr. (Mrs.) S. Annapoorani,*** *Professor and Head, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore for, her guidance and moral support, encouragement given throughout the study period.*

Any attempt at any level cannot be satisfactorily completed without the support and guidance of learned people, I would like to express my immense gratitude to my guide ***Dr.R.Nirmaladevi****, Assistant Professor, Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for the opulent guidance rendered at every stage of the dissertation. She showed me different ways to approach a research problem and the need to be persistent to accomplish any goal. Without her dynamic guidance, valuable suggestions, untiring help, meticulous efforts and enduring support, this study would never have seen the light of the day.*

*I heartfully thanks to **Ms.G.T.Iswariya Research Scholar** Department of Biochemistry, Biotechnology and Bioinformatics for her constant support and tremendous care rended for carrying out of my thesis successfully.*

*I express my sincere thanks to all the **Staff members** of the Department of Biochemistry, Biotechnology and Bioinformatics, Avinashilingam Institute for Home Science and Higher Education for Women, Coimbatore, for their timely help and valuable suggestions rendered during the period of the study.*

*I whole heartedly thanks to my father **Mr.T.K.Manoharan, Mrs.M.Sumithra** and my sisters, **Ms.M.Gayathri** and **Ms.M.Sivaranjani** and my brother **Mr.M.Aravindhan** for their emotional support to complete the research work successfully.*

*I am indebted to all **my friends, research scholars** and **lab assistants** for their entiring help and support.*

*Passionally, I would like to my special thanks to all **my seniors** and **juniors** for their encouragement and making this study a smooth sail.*

Lastly, I would like to express my special thanks to all hands during the course of the study for the help rendered in the successful completion of the study.

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1.0 INTRODUCTION

Oxygen is most important for all living cells. It can induce several illnesses in human such as cardiovascular diseases, neurodegenerative disorders, Alzheimer‟s, Parkinson‟s, diabetes, cancer, liver cirrhosis, obesity and metabolic syndrome. During mitochondrial respiration, electrons are given in steps to oxygen, process that leads to the formation of ROS and secondary to RNS. Oxygen is the ultimate electron acceptor in the electron flow system that yield energy. Problems can occur when the electron flow becomes uncoupled, generating free radicals (Erzsebet *et al*., 2016).

Recently there is an increasing awareness among people about the role of free radicals in health and disease. Free radicals are continuously produced by the normal metabolism in the body. Free radicals are generated in the mitochondria during the energy generation process in the presence of oxygen. Oxygen (O_2) is an essential element for cellular function and life. It plays an important role in a series of biochemical reaction occurring in the respiratory chain, which is responsible for most of the production of adenosine triphosphate (ATP), which provides the energy required for a multitude of cellular reaction and function (Bhattacharya, 2015). Free radicals of most concern in biological system are derived from oxygen. Oxygen is an element in-dispensable for aerobic life. Cells need oxygen to generate energy in the mitochondrial electron transport chain. The oxygen is a toxic gas that produces oxygen radicals during the ATP synthesis in the mitochondria. About 90% of oxygen taken up in the lungs is utilized by the mitochondria to produce ATP. The reminder (~ 10%) of oxygen is used in metabolism by various oxidizing enzyme, which catalyze oxidation of diverse chemical compounds (Santo *et al*., 2014).

Free radicals are chemical species containing one or more unpaired electron in its balance shell and therefore it is unstable and unpairing of molecular oxygen leads to the formation of unstable free radical. The radicals are highly reactive and leads to the formation reactive oxygen species. Most of the biological molecules are non-radicals. When a free radical react with a non-radical, a new free radical is generated. Most common free radicals are O^{2-}, H_2O_2, $^{\cdot}OH$, ROOH, $RO^{\cdot}$, ROO^{-}, HOCl are called oxidants. Reactive oxygen species is a collective term used for a group of oxidants which are either free radicals or molecular species capable of generating free radicals.

Free radicals are responsible for tissue damage. An imbalance between the production of reactive oxygen species and the biological systems ability to detoxify the reactive intermediate or easily repair the resulting is called oxidative stress (Kayal *et al*., 2015).

Free radicals have several roles which can be enumerated as:

1. Generation of ATP (universal energy currency) from ADP in the mitochondria: oxidative phosphorylation
2. Detoxification of xenobiotics by Cytochrome P450 (oxidizing enzymes)
3. Apoptosis of affected or defective cells
4. Killing of micro-organisms and cancer cells by macrophages and cytotoxic lymphocytes
5. Oxygenases (eg. COX: cyclo-oxygenases, LOX: lipoxygenase) for the generation of prostaglandins and leukotrienes, which have many regulatory functions.

Reactive oxygen species are formed in minute quantities during physiological cell metabolism. However, at high concentration, excessive free radical production or decreased capacity of endogenous antioxidant leads to the formation of oxidative stress. Oxidative stress, defined as “an imbalance between oxidants and antioxidants in favour of the oxidants, which is potentially cause damage” is associated with higher risk of many disease including diabetes mellitus, hypertension, obesity and inflammation (Nourien *et al*., 2016). Oxidative stressed condition have the potential of causing damage to cellular macromolecules including carbohydrates, proteins, lipids and nucleic acids there by disturbing the functional and structural integrity of biological cells. Oxidative stress plays important roles in the pathogenesis of diseases such as cancer, neurological disorders, atherosclerosis, hypertension, ischemic disease, diabetes, acute respiratory syndrome, fibrosis, pulmonary disease and asthma (Ojezele *et al*., 2016).

Leukemia is a progressive and malignant disease of the blood forming organs, marked by distorted proliferation and development of abnormal white blood cells and

their precursors in the blood and bone marrow. Leukemia is a "neoplasmic proliferation of one particular cell type" such as granulocytes, monocytes, lymphocytes, or infrequently red blood cells. The countries with highest incidence of leukemia cancer includes Denmark, Luxembourg, Italy, Belgium, France, Latvia, Germany, New Zealand and Australia. Although the cause of leukemia is unknown in most of the patients, several factors that are associated with increased risk of developing the disease that includes age, prior chemotherapy, gender, inherited syndrome, ionizing radiations, infection by certain viruses and smoking (Pokharel, 2012).

Leukemias are broadly classified into four main categories based on type of white blood cell and characterization of the disease. Based on type of blood cells affected, it is classified as myelogenous leukemia and lymphocytic leukemia, Based on characterisation of the disease includes acute leukemia and chronic leukemia (Saultz and Garzon, 2016).

Myelogenous leukemia develops from myeloid cells. The disease can either be acute or chronic. Lymphocytic leukemia may either be acute or chronic that can be arise from lymphocytes or lymphoblasts in bone marrow.

Acute myeloid leukemia (AML) is a cancer of the blood and bone marrow. It usually progresses quickly if it is not treated. The disease accounts for about 10,600 new cases of leukemia each year, and it occurs in both adults and children.

Other names for AML include:

- Acute myelogenous leukemia
- Acute myeloblastic leukemia
- Acute granulocytic leukemia
- Acute non-lymphocytic leukemia.

Acute lymphocytic leukemia (ALL) is a type of cancer in which the bone marrow makes too many lymphocytes, which is a type of white blood cell. Acute lymphocytic leukemia, which is also called acute lymphoblastic leukemia, accounts

for about 3,800 new cases of leukemia each year. Although acute lymphocytic leukemia is the most common type of leukemia in young children, it can also affect adults (Pokharel, 2012).

Oxidative stress caused by reactive oxygen species is associated with the pathogenesis of a numerous diseases such as diabetes, cancer, atherosclerosis, coronary artery diseases and other degenerative diseases. ROS causes tissue damage including intracellular protein, lipids, DNA and oxidation of membrane bound and mitochondrial enzymes. The use of antioxidants derived from plants such as flavonoids and polyphenols has been most valuable in combating diseases. Antioxidants are the chemical substances which prevent the free radicals damage in the body. Numerous research are going globally focused on investigating natural antioxidants of plants origin (Nair *et al*., 2016).

India has a long history and strong base for Ayurveda, which is the traditional herbal medical system. Herbal plants play an important role in the prevention and treatment for human diseases. People have been using plants as a traditional medicine for thousand years ago. Plants which possess these medicinal properties is also known as medicinal plants (Samrot *et al*., 2016). Plants have been associated with the development of human civilization around the whole world. However, plants are considered as rich sources of phytochemical ingredients which enable them to have medicinal value. Medicinal plants are a potential source for the development of new herbal drugs. Plants produce wide variety of phytochemical compounds which have been known to perform most biological activities such as antioxidant, anticancer and antimicrobial activities (Shakya, 2016).

Recently lots of attention has been devoted to natural oxidants and their health benefits. Plants produce various antioxidant compounds as protection against reactive oxygen species and free radicals. Reactive oxygen species are various species of activated oxygen leading to oxidative damage to tissue. Free radicals in the cell may occur due to various external factors such as ultraviolet radiation, chemical reactions and some metabolic processes. Accumulations of these radicals cause considerable diseases, such as cardiovascular diseases, cancer, aging, inflammatory diseases. Different parts of plant (root, leaf, flower, fruit, stems, bark) have been used

successfully to treat many diseases. Their antioxidant and antimicrobial activity effect many physiological processes in the body, thus protecting against free radicals and undesirable microorganisms, phenols are commonly found in plants and have been reported to have antioxidant activity (Kazazic *et al*., 2016).

Free radicals attack the antioxidants producing other specific free radicals with low reactivity, which participate less in the reactions of initiation of peroxidation. Antioxidant compounds are classified after their type of action into *scavengers* and *"preventive" antioxidants*:

− Scavengers are compounds that prevent oxidative stress by capturing free radicals

− "Preventive" antioxidants are compounds that act by sequestrating the ions of transition metals, preventing the Fenton reaction (Butnariu, 2012).

Enzymic antioxidants play an important role in cellular defense against reactive oxygen species and Non-enzymic antioxidants also play an important role in second line defense mechanism against damage induced by oxidative stress (Muthu, 2015).

Many plants that are used as natural remedies possess antioxidant properties. These plants contain phytochemicals, which are non-nutritive plant chemicals that have protective or disease preventive properties. Most phytochemicals have antioxidant activity and are suspected to reduce the risk of developing certain types of cancer and other diseases related to reactive oxygen species. The use of antioxidants for the prevention of damage caused by reactive oxygen species there by assumes great importance for health and traditional medicine (Curiac et al., 2016).

A plant cell produces two types of metabolites: primary metabolites involved directly in growth and metabolism (carbohydrates, lipids and proteins), and secondary metabolites considered as end products of primary metabolism and are not involved in metabolic activity (alkaloids, phenolics, sterols, steroids, essential oils, lignins and tannins). Secondary metabolites are substances which are produced by plants as defense chemicals (Irchhaiya *et al.,* 2014).

Annona muricata belongs to family *Annonaceae* and is a fruit tree with a long history of traditional use. It is also known as Soursop, Graviola and Guanabana. It is an evergreen plant that is mostly distributed in tropical and subtropical regions of the world. All portions of the *A. muricata* tree are extensively used as traditional medicines against an array of human ailments and diseases, especially cancer and parasitic infections. The leaves are employed to treat cystitis, diabetes, headaches, insomnia and it is deployed as an ethnomedicine against tumors and cancer (Moghadamtousi *et al*., 2015).

Annona muricata contains phytochemicals that are responsible for their healing properties. The phytochemical screening of this plant revealed the presence of flavonoids (group of polyphenolic antioxidants), saponins, tannins, glycoside and alkaloids. Flavonoids are a large group of plant polyphenol secondary metabolites and can be found widely in the leaves. It is already well recognized that flavonoids possess anti-tumoral, anti-ischemic, anti-allergic, anti-inflammatory and anti-bacterial activities. Moreover, flavonoids show strong antioxidant capacities through scavenging oxygen free radicals promote anti-oxidase or inhibit oxidative enzymes (Nourian *et al*., 2016).

The objectives of the present study are as follows

- To screen the phytochemical constituents present in the ethanol extract of leaves of *Annona muricata*.
- To assess the cytotoxic activity of the ethanol extract of leaves of *Annona muricata*.

REVIEW OF LITERATURE

Medicinal plants

Reactive Oxygen Species

Free radicals

Oxidative stress

Diseases caused by free radicals

Aging

Atherosclerosis

Neurodegenerative Diseases

Ischemia- reperfusion

Rheumatoid Arthritis

Cancer

Leukemia

Antioxidants

Types of antioxidants

Secondary metabolites

Annona muricata

Medicinal plants

Plants and plant products are part of the vegetarian diet and a number of them exhibit medicinal properties. Medicinal plants have been used for many years traditionally. Plants have attained status of natural source of new potent antimicrobial agents. Medicinal plants are used as ethnomedicine in different countries around the world and are the sources of Natural products providing unlimited opportunity for new drugs because of readily available medicinal diversity (Mohammed *et al*., 2014).

Plants, which have one or more of its parts having substances that can be used for treatment of diseases, are called medicinal plants. Medicines derived from plants are widely popular due to their safety, easy availability and low cost. Herbal medicines may include whole plant or parts of plant mostly prepared from leaves, roots, bark, seed and flowers of plants. They are administered orally, inhaled or directly applied onto the skin. Medicinal herbs are more important to the health of an individual and community. The medicinal value of these plants lies in bioactive phytochemical constituents that produce definite physiological action on the human body. Some of the most important bioactive phytochemical constituents are alkaloids, essential oils, flavonoids, tannins, terpenoids, saponins and phenolic compounds (Ullah *et al.*, 2014).

Plant alone act as a major role of phytochemical compounds which have been known to perform most biological activities such as antioxidant, antimicrobial and anticancer activities. India is endowed with a rich wealth of medicinal plants. From earliest times, mankind has used hundreds of medicinal plants in an attempt to cure diseases and relieve physical suffering. Medicinal properties of plants have been mentioned even in the oldest “Rig Veda” which is becoming popular throughout the developed world, as people want to treat illness. Medicinal plants are the “back bone” of traditional medicine. Medicinal plants have curative properties due to presence of various complex chemical substances in different composition like alkaloids, glycosides, corticosteroids and essential oils. These medicinally valuable compounds obtained from the medicinal plants are called as biomedicines (Singh, 2015).

Medicinal plants besides therapeutic agents are also a big source of information for a wide variety of chemical constituents which could be developed as drugs with precise selectivity. These are the reservoirs of potentially useful chemical compounds which could serve as newer leads and clues for modern drug design. Thus these medicinal plants could potentially serve as a remedy for several diseases that are associated with ROS (Vijyalakshmi and Ravindra, 2012).

Reactive oxygen species

Oxygen is an element indispensable for life. When cells use oxygen to generate energy, free radicals are created as a consequence of ATP production by the

mitochondria. These products are called reactive oxygen species that result from the cellular redox process and play a dual role as both toxic and beneficial compounds. At low or moderate levels, ROS exert beneficial effects on cellular responses and immune function. At high concentrations, they generate oxidative stress, a deleterious process that can damage all cellular structures. ROS induced oxidative stress is associated with the chronic diseases such as cancer, coronary heart disease and osteoporosis. Free radicals attack all major classes of biomolecules, mainly the polyunsaturated fatty acids of cell membranes. The oxidative damage of PUFA, known as lipid peroxidation particularly destructive, because it produced as a self-perpetuating chain reaction (Kabel, 2014).

Molecular oxygen is relatively harmless and it is essential for living cells in order to obtain energy. However, some reactive species are derived from oxygen during aerobic metabolism (as a byproduct of mitochondrial respiration), or from environmental conditions (pollution, smoke, radiation and drugs). ROS exhibit two unpaired electrons in different orbitals at their highest energy level, which makes them susceptible to the formation of radicals (Kaludercic *et al*., 2016).

Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. All are capable of reacting with membrane lipids, nucleic acids, proteins and enzymes, and other small molecules, resulting in cellular damage (Mohammed *et al*., 2015).

Free radicals

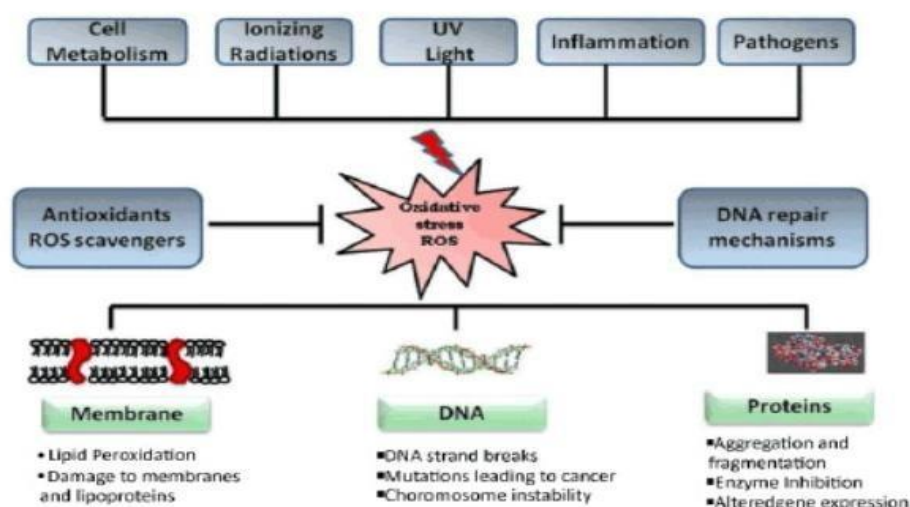
A free radical is any chemical species that contains a single (unpaired) valence electron in the outermost electron orbital. In human body, several metabolic reactions, inflammatory process can occur during which, free radicals react with cellular compounds and can cause damage to the proteins, lipids, carbohydrates and nucleotides in the tissue thus leading to the cell damage and destruction. Release of free radical poses the major endogenous damage in the biological system. It is often associated with various degenerative diseases and disorder such as cancer, cardiovascular disease, decline in immunofunctioning and aging (Sinha *et al*., 2015).

Free radicals are fundamental to any biochemical process and represent an essential part to aerobic life and metabolism. Reactive Oxygen Species and Reactive Nitrogen Species are products of normal cellular metabolism. The most common includes superoxide anion, hydrogen peroxide, peroxyl radicals and reactive hydroxyl radicals and the nitrogen derived free radicals are nitricoxide and peroxynitrite anion. These reactive species play an important role in pathogenesis of several oxidative stress related diseases like cancer, cardiovascular diseases, rheumatoid arthritis, ulcerative colitis and neurological degenerative diseases. Excessive production of free radicals causes direct damage to biological molecules such as DNA, protein, lipids and carbohydrates leading to tumor development and progression (Pavithra, 2015).

Oxidative stress

Oxidative stress is defined as a natural physiological process in the biological systems where the presence of free oxygen radicals overpowers the radical scavenging mechanisms, thus creating an imbalance between the oxidants and the antioxidants (Dhawan, 2014). Normal condition is indicated by the balance between oxidant production and antioxidant defense system. Oxidative stress demonstrates the imbalance between generation and clearance of oxidant. The reported chemical evidence suggests that dietary antioxidants help in diseases prevention (Palipoch and Koomhin, 2015).

Figure 1: Oxidative stress (Sharma, 2014)



Diseases caused by free radicals

Diseases caused by free radicals may be due to conditions like „mitochondrial oxidative stress" or „inflammatory oxidative stress". In addition, xanthine oxidase-induced formation of reactive oxygen species has been associated in ischemia-reperfusion injury. Oxidative stress has been implicated in the pathology of chronic inflammatory diseases such as rheumatoid arthritis as well as other diseases like diabetes, cancer, cardiovascular diseases, ischemia-reperfusion injury, neurodegenerative diseases, liver disorders and aging (Umamaheswari *et al*., 2015).

The recent growth in the knowledge of free radical and ROS in biology is producing a medical revolution that promises a new age of health. Reactive oxygen species have been implicated in the etiology of a host of a degenerative disease including cardiovascular disease, diabetes, cancer, Alzheimer disease and other neurodegenerative disorders, and aging (Sihombing *et al.,* 2015).

Aging

Free radicals which are produced endogenously either due to the body"s metabolic processes or due to environmental contaminants including tobacco smoke leads to the oxidation in living cells when these are accumulated in our body. Thus, the accumulated free radicals cause oxidative stress and the damage to the cellular structure that leads to premature aging and age-related diseases (Bolarin *et al*., 2016)**.** This is a complex process and is defined as the gradual biological impairment of normal function. It involves a series of morphological and functional changes taking place over time. There is a gradual decline in living organisms with accumulation of cellular and molecular damages of tissues and organs leading to mortality and morbidity. This may be due to changes made to cells (dividing cells such as fibroblasts and differentiated cells such as neurons). These changes affect functional ability of organs (such as heart, kidney and lungs), biological systems (such as the reproductive, digestive and nervous system) and ultimately the organism as a whole (Jyoti *et al*., 2016).

Atherosclerosis

Atherosclerosis is a complex process involving the deposition of plasma lipoproteins and the proliferation of cellular elements in the artery wall. This chronic condition advances through a series of stages leading to the atherosclerotic plaques formation that provide a barrier to arterial blood flow and may contribute to clinical events. Considerable evidence has been gathered in support of the hypothesis that free-radical-mediated oxidative processes and its specific products play a key role in atherogenesis. At the centre of this hypothesis are low-density lipoproteins (LDL), which as part of their normal circulation, occasionally leaves the antioxidant- replete plasma, entering the sub-endothelial space of arteries, where LDL lipids are oxidized. The oxidized form of LDL (oxLDL) is capable of initiating processes that contribute to the formation of atherosclerotic lesions. LDL is taken up by macrophages and induces the release of factors that recruit other cells and stimulate smooth muscle cell proliferation. LDL may also upregulate expression of cellular adhesion molecules that facilitate leukocyte binding. All of these events speed up the formation of plaque, which may results heart attack and stroke in many patients. The oxidation theory is supported by the presence of LDL within atherosclerotic lesions and the correlation between the sensitivity of LDL to oxidation and risk of CVD. Moreover, LDL oxidization can be inhibited by nutritional antioxidants. Several epidemiological evidences and interventional studies correlate higher level of antioxidant-rich food uptake with lower incidence of Coronary Heart Disease (Rahman *et al*., 2012).

Neurodegenerative Diseases

A growing body of evidence indicates that free radicals are involved in the initiation of cellular injury observed in neurodegenerative diseases which are characterized by loss of specific neuronal populations. This is often accompanied by intraneuronal damage, as well as extracellular accumulation of fibrillary materials (Dalaen, 2014).

Oxidative stress has been investigated in neurological diseases including Alzheimer"s disease, Parkinson"s disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), memory loss and depression. In a disease such as Alzheimer"s, numerous experimental and clinical studies have demonstrated that oxidative damage

plays a key role in the loss of neurons and the progression to dementia. The production of ß-amyloid, a toxic peptide often found present in Alzheimer‟s patients‟ brain, is due to oxidative stress and plays an important role in the neurodegenerative processes (Durak, 2014).

Ischemia- reperfusion

Reactive oxygen-derived radicals and metabolites are known to play important roles in the pathogenesis of ischemia/reperfusion and anoxia/reoxygenation injury. Free radicals are induced by the reperfusion blood flow in addition the lack of oxygen (O_2) supply to the ischemic cell (Babu *et al.,* 2016).

Rheumatoid Arthritis

Rheumatoid arthritis is an autoimmune disease characterized by chronic inflammation of the joints and tissue around the joints with infiltration of macro phages and activated T cells. The pathogenesis of this disease isdue to the generation of Reactive Oxygen Species and Reactive Nitrogen Species at the site of inflammation. Oxidative damage and inflammation in various rheumatic diseases were proved by increased levels of isoprostanes and prostaglandins in serum and synovial fluid compared to controls. Role of oxidative stress in RA patients is confirmed and indicates that antioxidant supplementation play an important role in controlling oxidative stress and decreasing disease activity in these patients. There is a necessity for therapeutic co-administration of antioxidants along with conventional drugs to such patients (Sultan, 2014).

Cancer

Cancer is a multistage process defined by at least three stages: initiation, promotion, and progression. Oxidative stress interacts with all three stages of these processes. During the initiation stage, ROS may produce DNA damage by introducing gene mutations and structural alterations of the DNA. In the promotion stage, ROS can contribute to abnormal gene expression, blockage of cell to cell communication, and modification of second messenger systems, thus resulting in an increase of cell proliferation or a decrease in apoptosis of the initiated cell population. Finally, oxidative stress may also participate in the progression stage of the cancer process by

adding further DNA alterations, lipid peroxidation and protein oxidation to the initiated cell population (Khanna *et al*., 2014).

Cancer is a proliferation of cell which grows in an uncontrolled manner invading local tissues and spreading widely through the blood. A tumour was called a cancer because of swollen veins around area resembling a crab‟s limbs. Cancer is a major health burden in both developed and developing countries. Anticancer activity effect of the natural and synthetic or biological and chemical agents to reverse, suppress or prevent carcinogenic progression (Eggadi *et al*., 2014).

Cancer is the leading cause of mortality in developed countries and the second most important cause of death in developing countries. Now it is a common disease, and more than one in three people will develop some form of cancer in their lifetime. Tobacco use is one of the common causes of cancer associated with lung, head and neck, gastric, pancreas, kidney and bladder cancer (Arunraj *et al.,* 2014).

Leukemia

In a person with leukemia, the bone marrow produces abnormal white blood cells that are called leukemia cells and leukemic blast cells. The abnormal cells can‟t produce normal white blood cells. Leukemia cells divide to produce copies of themselves. The copies divide again and again, producing more and more leukemia cells. Unlike normal blood cells, leukemia cells do not die when they become old or damaged. Because leukemia cells can build up and crowd out normal blood cells. The low level of normal blood cells can make it harder for the body to get oxygen to the tissues, control bleeding, or fight infections. Leukemia cells can also spread to other organs, such as the lymph nodes, spleen, and brain (https://www.cancer.gov/publications/patient-education/leukemia.pdf)

Types of leukemia

Leukemia is classified based on clinical behavior (acute or chronic) and the primary hematopoietic cell line affected (myeloid or lymphoid). The four principal diagnostic categories are the following (Deliverska and Krasteva, 2013).

- Acute Lymphoblastic Leukemia (ALL)
- Acute Myeloid Leukemia (AML)
- Chronic Lymphocytic Leukemia (CLL)
- Chronic Myeloid Leukemia (CML)

Acute Lymphoblastic Leukemia (ALL)

Acute lymphoblastic leukaemia (ALL) is a malignant neoplasm of the lymphocyte precursor cells. ALL is characterised by aberrations in proliferation and differentiation of lymphoblasts, leading to failure of normal immune response and decreased haematopoiesis. It represents a heterogeneous group with distinct morphology, cytogenetics, and molecular groupings. It is a clonal disease that can be separated by immunophenotyping into a B lineage ALL group (about 75%) and a T cell lineage ALL group (about 25%) and their subtypes according to the stage of maturation/differentiation. Standard cytogenetics, fluorescence *in situ* hybridisation, and reverse transcriptase polymerase chain reaction (RT-PCR) allow the detection of chromosomal translocations and the corresponding gene rearrangement. The identification of these rearrangements has provided critical insights into leukaemogenesis and is currently central to risk stratification. Gene expression profiling, novel molecular techniques, and next-generation sequencing can also recognise newly defined ALL entities (Thomas and Jeune, 2016).

Acute Myeloid Leukemia

AML is a group of infrequent neoplasms responsible for a significant number of cancer related deaths. Its incidence has been stable over the last years at about 3.7 per lakh persons per year in the western world. It is primarily a disease of later adulthood with an increasing incidence with age. The median age at diagnosis is 65 years with a slight male preponderance. Outcome varies greatly according to age at diagnosis due to disease and patient features. Untreated AML is a uniformly fatal disease with a medium survival of 11-20 weeks. The etiology of AML in most cases is unclear. Associated known risk factors include exposure to chemotherapeutic agents particularly alkylating agents, topoisomerase-II inhibitors and taxanes as well

as both therapeutic and non-therapeutic radiations. A higher than average incidence is seen in individuals with Down syndrome, Klinfelter‟s syndrome, Ataxia telangectasia, Kostman syndrome, neurofibromatosis or Fanconi anemia. Exposure to benzenes, pesticides, herbicides and smoking may also plays a role in its development. There is also a greater incidence of AML in individuals with pre-existing hematologic disorders such as the myelodysplastic syndromes or myeloproliferative disorders (www.albertahealthservices.ca).

Chronic Lymphocytic Leukemia

CLL is the most common type of leukemia in western countries. More than 15,000 newly diagnosed cases and ~4,500 deaths are currently estimated. The median age at diagnosis lies between 67 and 72 years. Males are prone to this disease compared to females. As the incidence rate rises with age, the prevalence and mortality of CLL are likely to increase further due to the demographic changes in society in the forthcoming decades. Moreover, the proportion of younger patients with early stage CLL and minimal symptoms seems to increase due to more frequent blood testing. CLL is characterized by the clonal proliferation and accumulation of mature, typically CD5-positive B-cells within the blood, bone marrow, lymph nodes, and spleen. Very recently, it has been reported that in CLL, the capacity to generate clonal B cells might be acquired at the hematopoietic stem cell (HSC) stage. Suggesting that the primary leukemogenic event in CLL might involve multipotent, self-renewing HSCs. The leukemic transformation is initiated by specific genomic alterations causing the deletion of specific micro-RNA genes and increasing the resistance of B cells toward apoptosis (Hallek, 2015).

Chronic myeloid leukemia

It affects myeloid cells and it grows slowly in the cells. Blood tests show an increase in the number of white blood cells. There may be a small number of leukemic blast cells in the bone marrow. The leukemia cell that starts this disease makes blood cells (red cells, white cells and platelets) that function almost like normal cells. The number of red cells is usually less than normal, resulting in anemia. But many white cells and sometimes many platelets are still made. Even though the white cells are nearly normal, their counts are high and continue to rise. This can cause

serious problems, if the patient does not get treatment. If untreated, the white cell count can rise so high and the blood flow slows down and anemia becomes severe (http://www.cancer.org/acs/groups/cid/documents/webcontent/003110-pdf.pdf).

2.6 Antioxidants

Antioxidant is defined as the agent that neutralizes the effect produced by free radical. It is classified into multiple ways by their activity; they can be categorized as enzymatic and non-enzymatic antioxidants. Antioxidants exist in both enzymatic and non-enzymatic forms in the intracellular and extracellular environments. Cellular constituents of the body matter are altered during oxidative stress can be effectively neutralized by enhancing cellular defense in the form of antioxidants. Certain compounds act as *in vivo* antioxidants by raising the levels of endogenous antioxidant defenses. Expression of genes encoding the enzymes such as superoxide dismutase, catalase and glutathione peroxidase increase the level of endogenous antioxidants. The enzymatic antioxidants are produced in our body, whereas most of non-enzymatic antioxidants are obtained from either natural plants or synthetics which are used for the treatment for various diseases (Elumalai, 2016).

Antioxidants may be defined as radical scavengers which protect the human body against free radicals that may cause anemia, asthma and arthritis. Antioxidants inhibit the oxidation of other molecules. Oxidation is a chemical reaction that transfers electron or hydrogen from a substance to an oxidizing agent. Oxidation reaction produces free radicals, in turn these radical can start chain reaction. Antioxidant terminates the chain reaction by removing free radicals intermediates and inhibits other oxidation reaction (Nagananda *et al*., 2013).

.2.7 Types of antioxidants

Antioxidants are classified into two types. They are enzymatic and non-enzymatic antioxidants. Enzymatic antioxidants are superoxide dismutase, catalase, peroxidase, glutathione reductase and polyphenol oxidase. Non-enzymatic antioxidants work by interrupting free radical chain reactions. Few examples of the non-enzymatic antioxidant are vitamin C, vitamin E, plant polyphenol, carotenoids and glutathione (Kumar, 2014).

Enzymatic antioxidants

2.7.1 Catalase

Catalase are highly expressed enzymes, particularly in certain plant cell types, and they are thus an integral part of the plant antioxidative system (Hamid *et al.,* 2012). Peroxisome of aerobic cells contain the enzyme catalase and it is efficient for promoting the conversion of hydrogen peroxide to water. Catalase enzyme has higher turnover rates apart from all other enzymes. Each molecule of catalase get converted into molecules of hydrogen peroxide to water and oxygen for each minute. Catalase is present in all prokaryotes and eukaryotes. With the exception of erythrocytes, it is predominantly located in peroxisomes of all types of mammalian cells where H_2O_2 is generated by various oxidases. Since H_2O_2 serves as a substrate for certain reaction that generate the highly reactive hydroxyl radical, catalase is believed to play a role in cellular antioxidant defense mechanisms by limiting the accumulation of H_2O_2 (Sisien , 2014).

2.5.2. Superoxide dismutase (SOD)

SOD catalysis are assisted by copper, zinc, magnesium, iron and SOD breakdown to form superoxide into oxygen and hydrogen peroxide. They are found mainly in lipid peroxidation (Gupta, 2016).

The balance between SODs and the different H_2O_2 scavenging enzyme activities in cells is considered to be crucial in determining the steady-state level of O_2 and H_2O_2. This balance along with the sequestering of metal ions by ferritin and other metal-binding proteins, prevents the formation of SOD the highly toxic hydroxyl radical via the metal- dependent Haber-Weiss reaction or the Fenton reaction (Chorianopoulou *et al*., 2012).

2.7.3. Polyphenol oxidase

Polyphenol oxidase activity is mainly due to their redox properties. They play an important role in absorbing and neutralizing free radicals, singlet and triplet oxygen, or decomposing peroxides (Prakash and Kumar, 2010). Phenolic antioxidant is divided into five types based on the plant namely phenolic acids, flavonoids,

lignans, stilbenes and tannins which are constituted with one or more aromatic rings and more hydroxyl groups. Polyphenols has potential effect which is linked to the electron donation, metal ion chelating ability and reducing power on the phenolic antioxidants (Bae *et al.,* 2015).

Non-enzymatic antioxidants

Non-enzymatic antioxidants include low-molecular-weight compounds, such as glutathione, vitamins C and E. Which play key roles in the body defense mechanisms. In general, water-soluble antioxidants (e.g., ascorbic acid) react with oxidants in the cell cytoplasm and the blood plasma, while lipid-soluble antioxidants (e.g., α -tocopherol) protect cell membranes from lipid peroxidation (Bind *et al.,* 2015).

Glutathione S-transferase

Gluthathione S-transferase is an enzymatic antioxidant which catalyze the reaction of compounds of -SH group of gluthathione, neutralizing the electrophilic sites and products contain more water-soluble (Rajat and Panchali, 2014).

Glutathione S-transferase is a detoxification enzyme that catalyses the conjugation of glutathione to a wide variety of endogenous and exogenous electrical compounds like chemotherapeutic agents. GSTs are present in human tissues and they have subdivided into atleast eight gene-independent classes. They are Alpha, Pi, Mu, Theta, Zeta, Omega, Sigma and Kappa. Resistant cells have increased detoxification of compounds mediated by high levels of GSH and GST. Evidence suggests that the GST isoenzyme may have additional functions beyond their catalytic role (Henderson *et al*., 2011).

Vitamin C

Vitamin C is the rich source in green vegetables and citrus fruits. Ascorbic acid is a water soluble vitamin, easily oxidized, especially in aqueous solution. It plays an important role in the oxidative stress reaction and in the maintenance of normal physiological functions of the body. It is also involved in wound healing,

tyrosine metabolism, carbohydrate metabolism, iron metabolism, resistance to infections and cellular respiration (Hu *et al.,* 2012).

It has been found in biological fluids which indicate the presence of normal metabolic functions in the body. Ascorbic acid is also called as vitamin C that interact with radicals such as O_2- and HO- in the plasma (Rajat and Panchali, 2014).

2.7.7 Vitamin E

Vitamin perform its functions as antioxidant in the glutathione peroxidase pathway, and it protects cell membranes from oxidation by reacting with lipid radicals produced in the lipid peroxidation chain reaction. This would remove the free radical intermediates and prevent the oxidation reaction. The oxidized α-tocopheroxyl radicals produced in this process may be recycled back to the active reduced form through reduction by other antioxidants, such as ascorbate, retinol or ubiquinol. However, the importance of the antioxidant properties of this molecule at the concentrations present in the body are not clear and the reason vitamin E is required in the diet is possibly unrelated to its ability to act as an antioxidant (Nirmala *et al.*, 2015)

On the basis of their size, it again classified into two types. They are small-molecule antioxidant and large-molecule antioxidant. The small molecule antioxidant neutralize the ROS in a process called radical scavenging and carry them away. The large molecule antioxidants are enzymes (SOD, CAT and GSHPx) and sacrifcial protein (albumin) that absorb ROS and prevent them from attacking other essential protein (Nimse and Pal, 2015).

Recently more attention has been given to natural oxidants and their health benefits. Plants produce various antioxidant compounds as protection to reactive oxygen species and free radicals. ROS are various species of activated oxygen leading to oxidative damage to tissue. Free radicals in the cell may occur due to various external factors such as UV radiation, chemical reaction and some metabolic processes. Its accumulation cause various diseases like cardiovascular diseases, aging, cancer and inflammatory diseases. Different parts of plants (root, stem, leaf, flower, fruit and park) have been used successfully to treat many diseases. Their antioxidant

and antimicrobial activity affect many physiological processes in the body, thus protecting against free radicals and undesirable microorganisms (Kazazic *et al*., 2016).

Secondary metabolites

Medicinal plants produce a vast array of secondary metabolites and such metabolites have been found to have a broad range of therapeutic properties, including antioxidant activities. Through oxidation reactions, living cell generates a number of Reactive Oxygen Species like superoxide, hydroxyl, peroxyl, alkoxy and nitric oxide, which induce oxidative stress and initiate chain reactions leading to cell damage and various diseases while antioxidants prevent the oxidation of other molecules, cancel out the cell-damaging effects of free radicals and lower the risk of different diseases. Several enzymes as well as non-enzymatic secondary metabolic compounds of plant origin are able to scavenge ROS and thus can protect the organism from oxidative damage (Alamgir *et al*., 2014).

Metabolites are the intermediates and products of metabolism. The term metabolites mean small molecules. It involve functions including fuel source, structure signaling, stimulatory and inhibitory effects on enzymes, catalytic activity of their own defense and interaction with other organisms. Plant produce numerous organic compounds, the great majority of which do not appear to participate directly in growth and development. These substances traditionally referred to as secondary metabolites (Tiwari, 2015).

Secondary metabolites includes three main groups: Terpenes (such as plant volatiles, cardiac glycosides, carotenoids and sterols) phenolic (such as phenolic acids, coumarins, lignans, stilbenes, flavonoids, tannins and lignins) and nitrogen containing compounds such as alkaloids and glucosinolates. Traditional separation techniques, various solvent system and spray reagents have been described as having the ability to separate and identify secondary metabolites. Secondary metabolites are compounds bio synthetically derived from primary metabolites. In plant kingdom, they are limited to occurrence and may be restricted to a particular systematic position of genus, species or family. Secondary metabolites are accumulated by plant cell in smaller qualities than primary metabolites. It is also synthesized in specialized cells at

particular developmental stages making extraction and purification difficult (Costa, 2017).

There is an increasing demand for the herbal drug treatment of various ailments and many plant drugs from Ayurvedic system are being explored globally. The biological activities from various clinical and preclinical studies have been included along with some patents arising from these plants (Megraj, 2011).

The exogenous antioxidants are mainly derived from medicinal plants, such as fruits, vegetables, seeds, bark, leaves, flowers and traditional medicinal herbs. Besides, the industries processing agricultural by-products are also potentially important sources of natural antioxidants. These natural antioxidants from plant materials are mainly polyphenols (phenolic acids, flavonoids, anthocyanins, lignans and stilbenes), carotenoids (xanthophylls and carotenes) and vitamins (vitamin E and C). Generally, these natural antioxidants, especially polyphenols and carotenoids, exhibit a wide range of biological effects, such as anti-inflammatory, antibacterial, antiviral, anti-aging, and anticancer (Xu *et al*., 2017).

In recent years, there has been a gradual revival of interest in the use of medicinal plants in developing countries because herbal medicines have been reported safe and without any adverse side effects especially when compared with synthetic drugs. Thus, the search for new drugs with better and cheaper substitutes from plant origin is a natural choice. The medicinal value of these plants lies in some chemical substances that produce a definite physiological action on the human body (Usunobun, 2015).

Annona muricata

Annona muricata commonly known as Graviola or Soursop, belongs to the family of annonaceae. The leaves are lanceolate with glossy and dark green in color had been traditionally used to treat headaches, hypertension, cough, asthma, sedative and used as antispasmodic. Previous reports over the years demonstrated that leaves, bark, root, stem, and fruit seed extracts of *Annona muricata* are anti-bacterial, antifungal, and anti-malarial. This leaf extract was also found to possess antioxidant properties. Among the chemical constituents found in the leaf of *Annona muricata* are

alkaloids, oils and acetogenins. Annonaceous acetogenins, from *Annona muricata* was found to be a promising new anti-tumor and anticancer agent in numerous *in vitro* studies. These acetogenins demonstrated to be selectively toxic against various types of the cells without harming healthy cells (Mohammed, 2016).

Annona muricata is not only a delicious and healthy source but also used in medicinal preparation to treat several diseases. Some literature report says that *Annona muricata* using as anticancer agent. The crushed fresh leaves can be applied on skin eruptions to promote healing. A decoction of the young shoots or leaves is regarded as a remedy for gallbladder trouble, coughs, cataract, diarrhea, dysentery, fever and indigestion. This can be used to cure diabetes by regulating blood sugar, which shows, high effectiveness in endocrine commitments like, kidney, thyroid, pancreas, ovary, prostate, intestines, muscle or breast cancer (Elavarasan *et al*., 2014).

Anti-cancer therapies should involve the determination of novel drug targets that must be highly effective and specific against cancer development and growth. Biological activities of *Annona muricata* includes antifungal, anti-bacterial, anti-malaria and antioxidant activities. It has been showed to have anti-cancer properties on multi drug resistant cancer cell lines. The ability of *Annona muricata* to have selective growth inhibition against a variety of cancer cells including lung carcinoma cell lines, breast solid tumor lines, prostate adenocarcinoma, pancreatic carcinoma cell lines, colon adenocarcinoma cell lines, liver cell lines and human lymphoma cell lines. *Annona muricata* also express analgestic and anti-inflammatory effects, promotes apoptosis (PCD also called programmed cell death) and cytotoxicity on cancer cell that may result from the presence of alkaloids and acetogenin. These acetogenins demonstrate to be selective and toxic against various types of cancer cells without harming normal and healthy host cells (Ioannis *et al*., 2015).

The experimental procedure adopted for the present study is presented in the next chapter.

METHODOLOGY

Medicinal plants contain several different pharmacological active compounds that may act individually, additively or in synergy to improve health. Recently there has been an increase of interest in the potential of plants as the source of antioxidants in reducing free radical induced tissue damage. Unstable reactive oxygen species react rapidly and could destruct the biomolecules such as protein, lipid, DNA and RNA that leads to lipid peroxidation and results in a cell structural damage, tissue injury or gene mutation. Free radicals may be related to various illnesses such as arteriosclerosis, rheumatic arthritis, aging, circulatory disease and neurodegenerative diseases. Alteration of DNA could cause different type of cancer and hundreds of other pathologies.Antioxidants are compounds that inhibit the oxidation processes and protect cells from the harmful effects of freeradicals. The damage generally reduced by endogenous antioxidants. There are two types of antioxidants which are natural antioxidant and synthetic antioxidant. Synthetic antioxidant is man-made antioxidant through chemical process while natural antioxidant is produced by human body or plants (Sihombing *et al*., 2015)

The methodology adopted in the present study is described as follows.

Sample collection and preparation

Preliminary screening for phytochemical constituents

Test for alkaloids

Test for flavonoids

Test for phenolics

Test for saponins

Test for tannins

Test for steroids

Test for terpenoids

Quantitative determination of phytochemical constituents

Spectroscopic analysis

HPLC analysis

Free radical scavenging assays

DPPH radical scavenging assay

Hydrogen peroxide scavenging activity

Cytotoxicity assays

Culturing of Molt-3 cell lines

MTT

SRB

3.1 COLLECTION OF SAMPLE:

The fresh leaves of *Annona muricata* were collected from the region of Coimbatore, Tamilnadu, India.

PREPARATION OF SAMPLE:

Fresh leaves were washed in running tap water till the dirt was removed. About 10g of leaf samples were weighed and homogenized with 50 or 100ml of solvent (ethanol). The supernatant was taken in a pre-weighed beaker and concentrated by incubating at 60˚C in a water bath and the residue was weighed and dissolved dimethylsulphoxide at a concentration of 5mg/ml and stored at 4˚C. Then the extract was filtered and kept for evaporation and it was used for further analysis of the study.

PRELIMINARY PHYTOCHEMICAL SCREENING

The ethanolic extract of *Annona muricata* leaf was prepared and tested for the presence of alkaloids, flavonoids, phenolics, saponins, tannins, glycosides, steroids and terpenoids as per (Khandelwal, 2002).

3.2.1Test for alkaloids

a)Mayer's test

A fraction of the extract was treated with Mayer"s reagent (1.36g of mercuric chlorate and 5g of potassium iodide in 10ml of distilled water) and observed for a cream coloured precipitate.

b) Dragendroff's test

A fraction of extract was treated with Dragendroff"s reagent and observed for the formation of reddish orange precipitate.

c) Wagner's test

A fraction of the extract was treated with Wagner"s reagent (1.27g of iodine and 2g of potassium iodide in 100ml of distilled water) and observed for the formation of reddish brown precipitate.

Test for flavonoids

a) Aqueous NaOH test

To a fraction of the extract 1N aqueous NaOH was added and observed for the formation of yellow- orange colour.

b) Concentrated H_2SO_4 test

To a small fraction of the extract, concentrated H_2SO_4 was added and observed for the formation of orange colour.

c) Schinido's test

To a small fraction of the extract, a piece of magnesium turning was added, followed by concentrated H_2SO_4 and then heated slightly for the formation of dark pink colour which was recorded.

Test for phenolics

a) Ferric chloride test

A fraction of the extract was treated with 5% $FeCl_3$ solution and observed for the formation of deep blue colour.

b) Lead acetate test

A fraction of the extract was treated with 10% lead acetate solution and observed for the formation of white precipitate.

Test forsaponins

a) Foam test

A fraction of the extract was vigorously shaken with water and observed for persistent foam formation.

b) Haemolytic test

A fraction of the extract was added with a drop of blood placed in a glass slide and observed for the haemolytic zone.

Test for steroids

a) Libermann- Buchard test

To a fraction of extract, 2ml of chloroform, followed by 10 drops of acetic anhydride and 2 drops of concentrated H_2SO_4 was added. The appearance of rose red colour which quickly changes from blue to green indicated the presence of steroids.

Test for tannins

a) Braemer's test

To a fraction of extract, a few drops of 10% ferric chloride was added. A dark green blue or brown colour was observed, indicating the presence of tannins.

Test for terpenoids

a) Salkowski test

A fraction of extract was dissolved in chloroform and shaken well with an equal volume of concentrated H_2SO_4. The appearance of red color, in the chloroform layer and green fluorescence in the acid layer indicated the presence of steroid.

QUANTITATIVE DETERMINATION OF PHYTOCHEMICAL CONSTITUENTS

Determination of total alkaloids

Fresh leaves (1g) were extracted with 20ml of 95% ethanol: 28% NH_4OH (95:5) at room temperature overnight. The extract was filtered and concentrated under reduced pressure to a fuzzy residue, which was extracted twice with 1N HCL (10ml each) and then filtered. Alkaloids were liberated at pH 9.8 by the addition of 0.7M Na_2CO_3. The organic extract was dried over anhydrous sodium sulphate to yield the total alkaloids fraction.

Determination of total phenolics

Leaves (1g) were crushed using a mortar and pestle and extracted with 20ml of 80% ethanol at 80˚C for 15minutes. The leaves were extracted respectively till a clarified fraction was obtained.

Determination of total flavonoids

The phenolic extract was further extracted with petroleum ether, when the flavonoids were present in the aqueous fraction.

Determination of total saponins

Fresh leaves (1g) were crushed, transferred to a conical flask and 200ml of aqueous ethanol was added. The mixture was filtered and re-extracted with another 200ml of 20% ethanol. The combined extract were reduced to 40ml over a water bath at about 90˚c. The concentrate was transferred into a 250ml separating funnel, 20ml

of diethyl ether was added and shaken vigorously. The aqueous layer was recovered and the ether layer was discarded. The extraction was repeated twice with the addition of n-butanol. The combined n-butanol extract was washed with 10ml of 5% NaCl. The remaining solution was heated in water bath, evaporated and dried in an oven.

Determination of total steroids

Fresh leaves of about 2g was weighed and added to 10ml of methanol. It was kept in a water bath for 15 min. The mixture was filtered, condensed and used.

Determination of total tannins

The plant material was suspended in methanol and allowed to stand overnight. It was refluxed for 4 hours, then filtered and the residue was washed with methanol. The filtrate was allowed to cool down, observed for any modification and an aliquot of this was used to assay tannins.

Determination of terpenoids

The plant material was suspended in petroleum ether and filtered. The filtrate was condensed and an aliquot of the filtrate was used for the assay.

SPECTROSCOPIC ANALYSIS

3.4.1 HPLC analysis

The ethanol extract of *Annona muricata* leaf was dissolved in an appropriate volume of HPLC grade methanol and 20µl of the sample was injected using Hamilton Syringe into the reverse phase C18 column of the HPLC system (Sigma-Aldrich equipped with PDA detector). The sample analysis was performed at room temperature in the wavelength ranging between 220-800nm at 1000psi and the mobile phase used was 100% HPLC grade methanol 60 minutes at a flow rate of 0.5ml/minute.

FREE RADICAL SCAVENGING ASSAYS DPPH

radical scavenging assay (Mensor *et al.*, 2001)

Principle:

DPPH (1,1diphenyl – 2 – picrylhydrazyl) is a stable free radical containing a single electron in its structure. Antioxidants react with DPPH and convert it to diphenylpicryl hydrazine. The degree of discoloration from deep violet to yellow was measured at 515nm.

Reagents:

1. 1.0 mM DPPH in methanol.
2. Methanol.

Procedure:

3 ml of 1.0mM DPPH in methanol solution was added to the plant extract. DPPH solution with methanol was used as positive control and methanol alone acted as blank. After 30 minutes, the discoloration from deep violet to yellow color was measured at 515nm in a spectrophometer. The % inhibition was calculated by the following formula

$$\text{Scavenging activity (\% inhibition)} = \frac{(A_c - A_e) \times 100}{A_c}$$

Where,

A_c= Absorbance of control

A_e = Absorbance in the presence of plant extract

Hydrogen peroxide scavenging activity (Ruch *et al.*, 1989)

Principle:

Hydrogen peroxide is a weak oxidizing agent and can inactivate a few enzymes by oxidizing its thiol groups. Antioxidants to scavenge the oxidant H_2O_2 was read at 230nm in spectrophometer.

Reagents:

1. Phosphate buffer (0.1 M) (pH – 7.4)
2. 40mM hydrogen peroxide in phosphate buffer.

Procedure:

A solution of H_2O_2 (40mM) was prepared in phosphate buffer. Plant extract at the concentration of 10mg /10µl was added to 0.6ml H_2O_2 solution. The total volume was made upto 3ml. The absorbance of the reaction mixture was recorded at 230nm. The solution containing phosphate buffer with H_2O_2 acts as blank. The percentage of H_2O_2 scavenged by the plant extract was calculated using the formula

$$\text{Scavenging activity (\% inhibition)} = \frac{(A_c - A_e)}{A_c} \times 100$$

Where,

A_c= Absorbance of control

A_e = Absorbance in the presence of plant extract

3.5 CYTOTOXICITY ASSAYS

The extent of cell death in Acute Lymphoblastic Leukemia cells in comparison with imapinib which acts via apoptosis were determined by 3-(4, 5 dimethylthiazole-2) (MTT) and Sulphorhodamine (SRB) assays. Apoptosis is generally characterized by its distinct morphological characteristics such as shrinkage

of cells, segmentation of nucleus condensation and cleavage of DNA into domain sized fragment in most cells followed by nucleolus degradation.

Culturing of Molt-3 cell lines

Molt-3 T-cell Acute Lymphoblastic Leukemic cell line, was purchased from NCCS, Pune, India. It is originally derived from 19 year old male with acute lymphoblastic leukemia (www.atcc.org/products/all/CRL-1552.aspx).

It was cultured using RPMI1640 medium supplemented with 10% FBS and 1mM sodium pyruvate and incubated at 37˚C. The cell count and viability was tested with trypan blue using haemocytometer and 1×10^{6} cells were seeded onto 96 and 6 well plates for cell viability and staining assays respectively, after allowing them to grow for several hours.

Treatment groups

1. Cells alone
2. Cells + imapinib
3. Cells + *Annona muricata* leaf extract

Cytotoxicity test (MTT dye reduction assay)

(Igarashi and Miyazawa, 2001)

Cell viability was evaluated by the reduction of 3-(4,5- dimethylthiozol-2-yl)-2,5-diphenyltetrazolium bromide. MTT is a water-soluble tetrazolium salt that is reduced by metabolically viable cells to a colored water insoluble formazan salt. Live cells convert MTT into its formazan derivative, the number of surviving cells can be determined by the amount of MTT formazan produced, which is measured in a microtiter plate reader at 650nm.

Reagent

- RPMI 1640 medium
- Yeast Peptone Dextrose medium

- Phosphate Buffer Saline (PBS) – pH-7.4
- MTT-3mg/ml in PBS
- Isopropanol in 0.04N HCl (acid-propanol)

Procedure

The treated Molt-3 cells and *Saccharomyces cerevisiae* cells were incubated with 50µl of MTT at 37ºC for 3 hours after centrifugation. After incubation, 200µl of PBS was added to all samples. The liquid was then carefully aspirated. Then 200µl of acid propanol was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Anthos 2020, Australia). The optical density of the control cells were fixed to be 100% viabile and the percent viablity of the cells in the treatment groups were then calculated.

Cytotoxicity assay (Sulphorodamine assay)

(Skehan *et al.*, 1990)

SulphoRodamine B (SRB) is a bright pink amino xanthene dye with two sulphonic acid groups. Under mild acidic conditions, SRB binds to protein basic amino acids in TCA fixed cells to provide a sensitive index of cellular protein content, which is directly proportional to cell viability. The SRB assay provides a sensitive measure of drug-induced cytotoxicity and is useful in quantitating clonogenecity and is well suited to high volume, automated drug screening.

Reagents

- RPMI 1640 medium
- Yeast Peptone Dextrose medium
- 40% TCA
- Sulphorodamine B (SRB) - 0.4% in 1% TCA
- 1% acetic acid
- 10mM Tris (pH 10.5)

Procedure

The treated Molt-3 cells and *Saccharomyces cerevisiae* cells were collected by centrifugation and washed with PBS. An aliquot of 350µl of ice-cold 40% TCA was layered on the top of the treated cells where and incubated at 4ºC for one hour after which they were washed 5 times with 200µl of ice cold PBS. The PBS was removed and SRB (350µl) was added to each tube and left in contact with the cells for 30 minutes at room temperature. After which they were washed 4 times with 1ml portion of 1 % acetic acid to remove the unbound dye, then 350µl of 10mM Tris (pH 10.5) was added to each tube to stabilize the protein bound dye. The pellet was shaken gently for 20 minutes on a gyratory shaker. The debris was spun down and the absorbance of the tris layer in each group was transferred to a 96-well plate and read in a microtiter plate reader at 490nm. The cell survival was measured as the percentage absorbance compared to the control (untreated cells).

Results will be presented in the next chapter.

RESULTS AND DISCUSSION

The results of the present study entitled **"A study on phytochemical analysis and cytotoxic activity of ethanolic extract of *Annona muricata*"** are discussed as follows.

Plants have been the subject of human curiosity and use for thousands of years. Traditional people relied on medicinal plants to combat various ailments. Bioactive natural products from plant sources have enormous economic importance as they can be used as drugs, lead compounds, biological or pharmaceutical tools, feed stock products, excipients and nutraceuticals (Ladan *et al*., 2014). Plants and herbal extracts have formed important position in modern medicine, due to their chemical and medicinal contents. Their secondary metabolites represent a large reservoir of structural moieties which work together exhibiting a wide range of biological activities (Devanaboyina *et al*., 2013).

In recent times, focus on plant research has increased all over the world and a large body of evidence and knowledge has accumulated in the literature to show immense potential of medicinal plants used in various medical, pharmaceutical, cosmetic and agrochemical applications. An advantage of natural bioactive molecule is that they have a milder side effects on the body in comparison to chemically synthesized drugs. With the increasing acceptance of herbal medicines as alternative form of health care delivery, the screening of medicinal plants for bioactive compound is imperative (Ladan *et al*., 2014).

Annona species commonly known as „Custard-Apple‟ belongs to the family Annonaceae and was cultivated in many tropical countries all over the world, for its edible fruits. Among these, *Annona muricata* L. (Soursop or Graviola) is a naturally occurring plant seen in Central America and in Southern part of India, traditionally used to treat various ailments. Fruits and fruit juice of *A. muricata* were taken internally to treat worms and parasites, fever, to increase mother‟s milk after child birth and as an astringent for diarrhea and dysentery. The leaves of the plant are found to be anti-spasmodoic (George *et al*., 2015). With this background the leaf extract of *Annona muricata* was subjected to the phytochemical screening and its anticancer effect was also studied using Molt-3 Tcells of Acute Lymphoblastic Leukemic origin.

Phytochemical analysis of the ethanol extract of *Annona muricata* leaves

Preliminary phytochemical studies are helpful in finding out chemical constituents in the plant material that may well lead to their quantitative estimation. Thus, in the present study phytochemical screening for the candidate plant was carried out and the results of the preliminary phytochemical analysis of the ethanol extract of *Annona muricata* is tabulated in Table 1. It showed the presence of all the secondary metabolites including alkaloids, flavonoids, phenols, terpenoids, tannins, saponins and steroids thus showing that the leaf extract is rich in phytochemical constituents.

Quantitative phytochemical analysis was performed in order to determine the major phytoconstituents present in the ethanol extract of *Annona muricata* leaves. The concentration of the secondary metabolites present in the leaf extract was analyzed by calculating the dry weight of the alkaloids, flavonoids, phenols, terpenoids, tannins, saponins and steroids and it is found to be 0.65, 0.01, 0.15, 0.03, 0.39, 0.62 and 0.45g respectively. Among all the phytochemicals tested, alkaloids represent the predominant phytoconstituent of *Annona muricata* leaf as shown in Table 1.

Table 1: Phytochemical analysis of the ethanol extract of *Annona muricata* leaves

S.No.	Phytochemical constituents	*Annona muricata* leaf extract	Yield (g)/g leaf tissue
1.	Alkaloid	+	0.65
2.	Phenolics	+	0.15
3.	Flavonoids	+	0.01
4.	Saponins	+	0.62
5.	Steroids	+	0.45
6.	Terpenoids	+	0.03
7.	Tannins	+	0.39

(+) - Presence of phytoconstituent

The phytochemical screening of the ethanol extract of *Tithonia diversifolia* dry flowers revealed the presence of tannins, flavonoids, phenols while alkaloids and saponins were absent (Gama *et al*., 2014). The ethanol

extracts from leaves, stem bark and flower of *Moringa pterygosperma* contained a number of phytochemicals such as alkaloids, flavonoids, glycosides, phenols, saponins, steroids and tannins when compared to aqueous extract (Bargah, 2015). Petroleum ether, chloroform and water extracts of *Mimusops elengi* were tested for its phytochemical constituents. The results showed the presence of alkaloids in both petroleum ether and chloroform extracts while flavonoids, terpenoids, saponins and phenols were present only in chloroform extract. Steroid was present in both petroleum ether and aqueous extracts and tannin was present only in petroleum ether extract while carbohydrates, protein and amino acids were present in all the extracts (Kalaiselvi *et al*., 2016). The preliminary phytochemical analysis of an ethanol extract of the whole plant of *Tridax procumbens* indicated the presence of alkaloids, tannins, flavonoids, saponins, and phenolic compounds (Parvin *et al*., 2015). In accordance with the above results reported the ethanol extract of *Annona muricata* leaves possessed considerable amount of phytonutrients which might be responsible for their medicinal value and therefore used as traditional medicine. Further quantitative analysis was also performed to identify the predominant metabolite present in the leaf extract.

Madhu *et al*. (2016) reported that among the 10 different medicinal plants tested the petroleum ether extract of *S. saponaria* plant fruit pericarpic extract contained high concentrations of steroids. *Bauhinia tomentosa L.* flower extract possessed high flavonoid content than alkaloids and saponins (Sathya *et al*., 2013). The results of the quantitative analysis of the aqueous and methanol extract of root, stem and flower of *Taraxacum officinale* showed saponins, flavonoids, alkaloids, phenols were highly concentrated in the stem, root and flower, with the higher concentration of flavonoids in the flower extracts (Mir *et al.,* 2013).

Phenolic compounds are a category of phytonutrients with strong antioxidant properties and the relationships between phenolic content and antioxidant activity have been reported in many studies. The antioxidant activity of phenolic compounds is due to their ability to scavenge free radicals donate hydrogen atoms or electrons or chelate metal cations (Saravanan and

Parimelazhagan, 2014). Thus the results of phytochemical analysis confirmed the presence of major secondary metabolites in the ethanolic extract of *Annona muricata*. Presence of these phytoconstituents might be responsible for their therapeutic potential.

HPLC analysis of the ethanol extract of *Annona muricata* leaves

The HPLC analysis of the ethanol extract of *Annona muricata* leaves includes recording of the chromatograms, retention time of individual peaks and the absorption spectra (recorded with a photodiode array detector) using C18 RP column (Schimazdu). The leaf extract showed the presence of one major peak and one minor peak with retention time of 2.80 and 3.12 as shown in Figure 2. Figure 3 shows the HPLC chromatogram of the standard quercetin with retention time of 2.88 and 7.38. This indicates the presence of compounds similar to quercetin or the derivatives of quercetin in the ethanol extract of *Annona muricata* leaves. More spectral and chromatographic analysis need to be carried out to determine the nature of phytoconstituents present in the leaves of *Annona muricata.*

Figure 2: HPLC profile of the ethanol extract of *Annona muricata* leaves

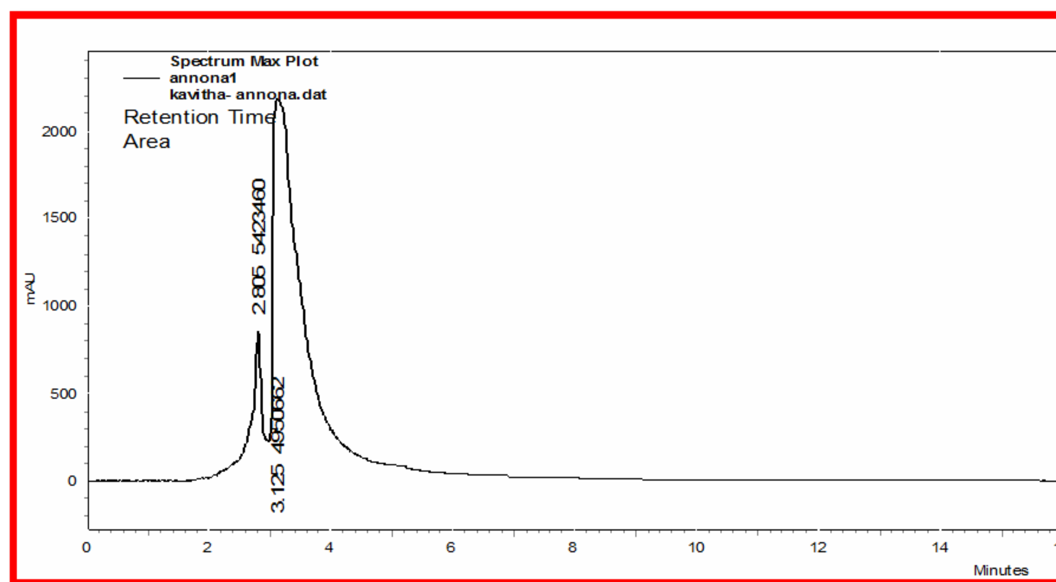
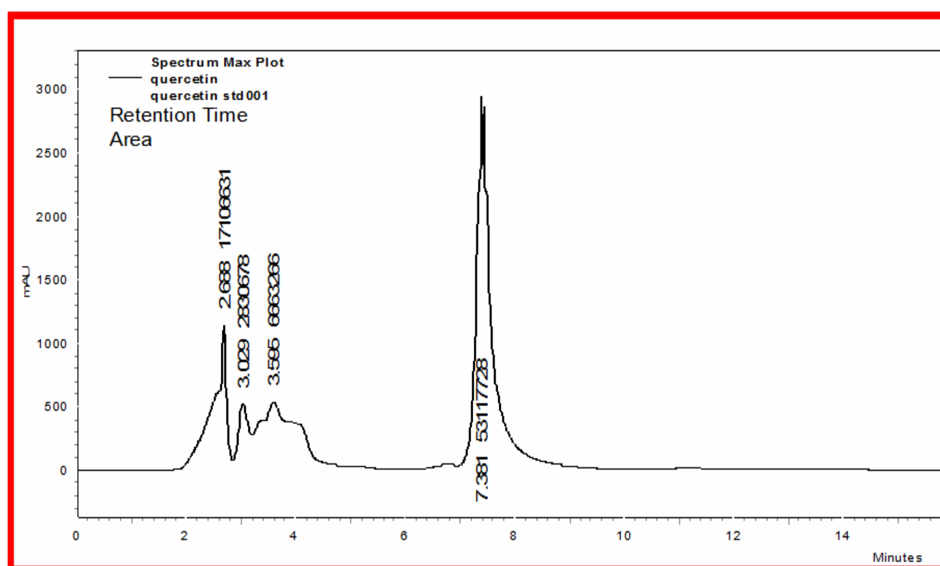


Figure 3: HPLC profile of the standard quercetin



In order to determine phenolic composition in berry leaves extracts, the HPLC assay was performed by Radovanovic *et al*. (2015). The results showed the presence of phenolic acids, flavonols and flavan-3-ols such as hydroxybenzoic, hydroxycinnamoyl, gallic, caffeic, ellagic and chlorogenic, epicatechin, catechin, epicatechin gallate and procyanidin B2, quercetin-3-glucoside, rutin, kaempherol-3-glucoside, quercetin, luteolin-3-glucoside and myricetin were present in the berry leaves extract (Radovanovic *et al*., 2015). Chung *et al.* (2016) found that there are 23 phenolic compounds present in fruit, leaves and root of *Panax ginseng* Meyer. The HPLC analysis of *Gardenia jasminoides* extracts confirmed the presence of gallic acid, catechin, rutin hydrate and quercetin. Out of the four phenolic compounds catechin was found in the highest concentration in the *Gardenia jasminoides* extracts (Uddin *et al*., 2014). In agreement with these studies, the HPLC analysis of *Annona muricata* leaves showed the presence of standard quercetin.

From the results of the phytochemical analysis, it is evident that the leaves of *A. muricata* are found to be a potent source of secondary metabolites. Further, to determine the pharmacological effect of these

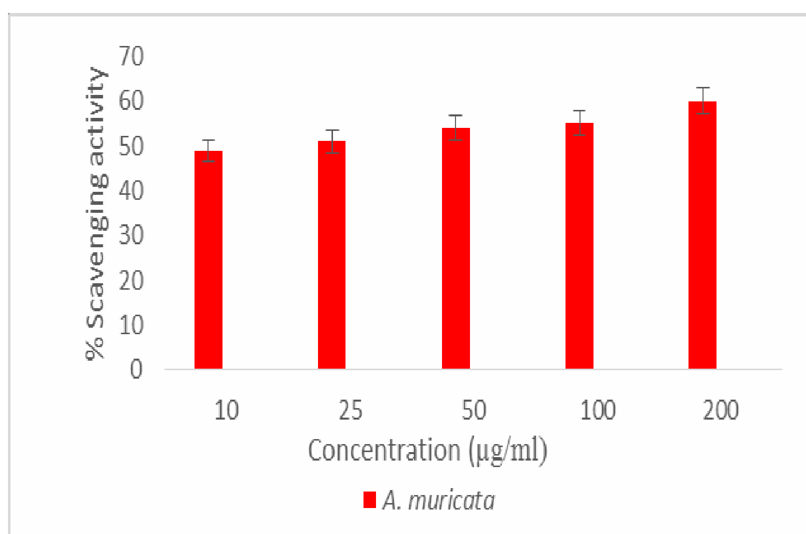
phytoconstituents present in the leaf extract of *A. muricata*, cytotoxicity assays (MTT and SRB) were performed using Molt-3 cell line.

DPPH radical scavenging activity of the methanol extract of *A. muricata* leaves

Because of their high reactivity, most free radicals react rapidly with oxidizable substrates. Methods used for evaluation of radical trapping properties often utilize stable free radicals as indicators for radical scavenging abilities, among which 1,1 diphenyl- 2-picrilhydrazyl radical (DPPH), have gained the highest popularity. From the methodological point of view the DPPH method is recommended as easy and accurate with regard to measuring the antioxidant activity of extracts (Neelambika and Leelavathi, 2015).

The results revealed that the ethanol extract of *A. muricata* leaves showed significant scavenging activity. The maximum scavenging activity is seen at the concentration of 200µg/ml which is presented in the Figure 4. The result demonstrates that leaf extract possess good antioxidant potential in a concentration dependent manner.

Figure 4: DPPH radical scavenging activity of the methanol extract of *A. muricata* leaves



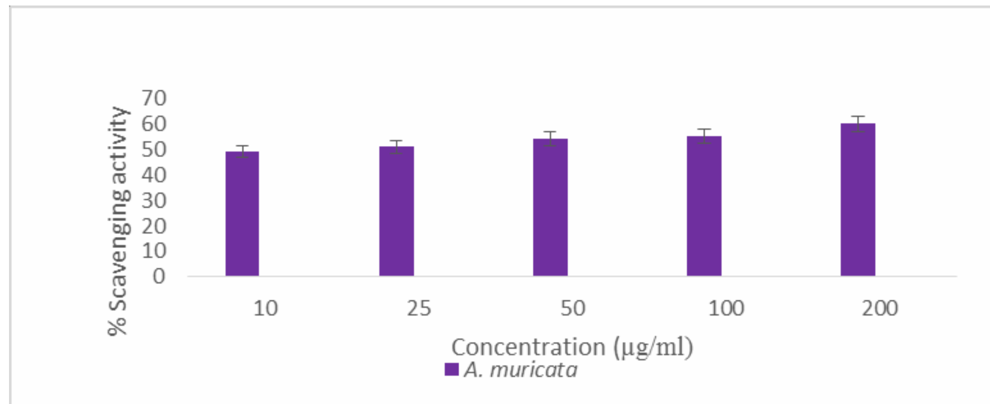
The methanol extract of *B.sensitivum* L. is tested for DPPH scavenging activity and found to possess strong antioxidant activity when compared with quercetin (Pallab, *et al*. 2013). Adeosun *et al.* (2016) showed that *Phoenix dactilyfera L.* exhibited stronger scavenging activity against DPPH radical. Among the two plants tested for the antioxidant activity *Blepharis maderaspatensis* ethyl acetate extract shows highest antioxidant capacity followed by *Blepharis molluginifolia* alcoholic extract and also reported that these plants are rich in phenols, flavonoids, glycosides, alkaloids, saponins, diterpenes, triterpenes and phytosterols. The activity of these plants is because of their biologically active secondary metabolites (Neelambika and Leelavathi, 2015). Thus the antioxidant activity of the extracts may be due to the presence of polyphenolic compounds such as flavonoids and tannins and this, in turn, may be attributable to the hydrogen or electron donating ability of the group present in the structure.

Hydrogen peroxide scavenging activity of the methanol extract of *A. muricata* leaves

Hydrogen peroxide is a biologically relevant, non-radical reactive oxygen species and is inevitably generated as a by-product of normal aerobic metabolism. However, when concentration increases under stress conditions, H_2O_2 could be detrimental for cells and, furthermore, could be converted into other ROS such as hydroxyl radicals. Thus, elimination or neutralization of H_2O_2 is crucial for the cells.

The ethanol extract of *A. muricata* leaf was subjected to oxidative stress by adding H_2O_2. The leaf extract was able to scavenge H_2O_2 to considerable extent. At lower concentration of 10µg, the leaf extract was able to scavenge H_2O_2 moderately. When the concentration is increased, the per cent scavenging is also increased in a concentration dependent manner. The result is depicted in Figure 5.

Figure 5: Hydrogen peroxide scavenging activity of the methanol extract of *A. muricata* leaves



The aqueous extract of *Azhadirachta indica* leaf shows higher antioxidant activity found by hydrogen peroxide assay. Scavenging activity of H_2O_2 by *Morinda citirfolia* root and butylated hydroxyl toluene (BHT) as reference was not remarkably different and was shown to be 82% and 92% repectively (Pal *et al*., 2012). Among the extracts, petroleum ether, chloroform and ethanol extract of X. strumarium L. showed potential antioxidant activity (Kosanic *et a*l., 2011). Numerous reports have already proven that nutritive phenols play a significant role in protecting mammalian and bacterial cells from cytotoxicity induced by H_2O_2, indicating that the observed activity of plants extracts could be due to the presence of phenols (Gul *et al.,* 2013). Thus indicating that the phytoconstituents present in the *A. muricata* leaves might be responsible for H_2O_2 scavenging activity.

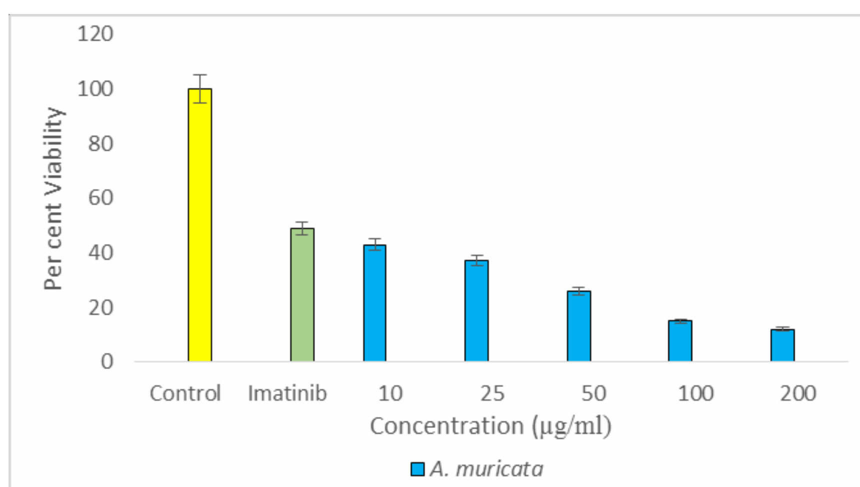
Effect of ethanol extract of *Annona muricata* on Molt-3 cell line as determined by MTT assay

Cell survival was usually determined by tetrazolium 3-(4,5-dimethylthiozoly-2)-2,5-diphenyltetrazolium bromide (MTT) assay. The assay is based on the capacity of mitochondrial dehydrogenase enzymes in living cells to convert the yellow water-soluble substrate 3-(4,5- dimethylthiazol-2-y1)-2,5-diphenyl tetrazolium bromide (MTT) into a dark blue formazan product that is insoluble in water. Viable cells are able to reduce the yellow MTT under tetrazolium ring cleavage to a water-insoluble purple-blue formation which precipitates in the cellular cytosol and can be dissolved

after cell lysis, whereas cells being dead following a toxic damage, cannot transform MTT. This formazan production is proportionate to the viable cell number and is inversely proportional to the degree of cytotoxicity. The reaction is mediated by dehydrogenases enzymes associated with the endoplasmic reticulum and the mitochondria (Senthilraja and Kathiresan, 2015).

In order to determine the cytotoxic effect of the leaf extract of *Annona muricata*, MTT assay was performed in leukemic cells (Molt-3 cell line) in comparison with the standard chemotherapeutic drug Imatinib at a concentration of 50µg/ml. The experiment was carried out using varying concentrations of leaf extracts ranging from 10, 25, 50, 100 and 200µg. The viability of the control cells were fixed as 100%. The results revealed that ethanol extract of *Annona muricata* leaves are able to induce cell death even at lower concentration of 10µg/ml. When Molt-3 cells are treated with increasing concentration of extract, the viability was found to be decreased in a dose dependent manner. The results showed that the leaf extract exhibited good cytotoxic effect towards the leukemic cell line. The data also revealed that 50% viability was observed at lower concentration (10µg/ml) while the viability was decreased to a greater extent of about 12% at the higher concentration of 200 µg/ml as shown in the Figure 6.

Figure 6: Effect of ethanol extract of *Annona muricata* on Molt-3 cell line by MTT assay



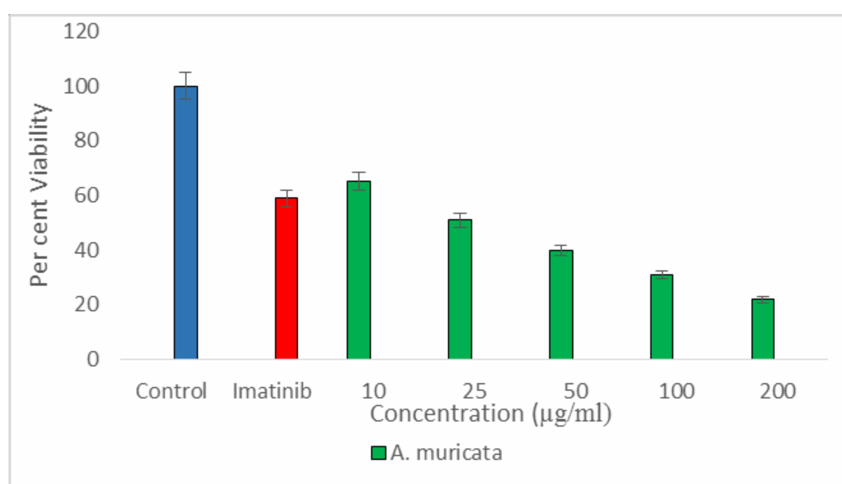
Semsri et al. (2015) demonstrated that the *c*rude ethanol extract of *Moringa oleifera* Lam. leaves inhibited cell proliferation in a dose- and time-dependent manner thereby exhibiting distinct cytotoxic effect on K562 (leukemic) cells. Garbi *et al.* (2015) investigated the cytotoxic activities of *Bauhinia rufescens* extracts against MCF-7 cell line. The data revealed that the treatment with petroleum ether and methanol of *Bauhinia rufescens* leaf extract induced cell death in MCF-7 cells. Findings by Ali *et al*. (2014) suggest that among the plants screened, the potential cytotoxic activity was found to be exhibited by the ethanol extract of *Lavandula dentata* (Lamiaceae). Similar results were observed in the present study. The results showed that *Annona muricata* leaf extract exhibited good cytotoxicity towards Molt-3 cell line thereby proving its apoptosis inducing property.

Effect of ethanol extract of *Annona muricata* on Molt-3 cell line as determined by SRB assay

Sulphorodamine B is a bright pink aminoxanthine dye with two sulfonic groups. Under mild acidic conditions, SRB binds dye to basic amino acid residues in TCA (Trichloro acetic acid) fixed cells to provide a sensitive index of cellular protein content that is linear over a cell density range of visible at least two order of magnitude (Patel *et al*., 2009).

The anti-proliferative SRB assay was performed to assess growth inhibition by the *Annona muricata* leaf extract. The results demonstrated that the ethanol extract of *Annona muricata* leaves showed significant cytotoxic effect against Molt-3 cell line. When the cells were treated with different concentration of the leaf extract, cell death was observed in a dose dependent manner. It is evident that the leaf extract was able to induce cell death to a considerable extent. Upon treatment with leaf extract, the cytotoxic effect was observed even at lower concentration of 10µg/ml and was increased gradually at the higher concentration of 200 µg/ml which was comparable with the standard drug imatinib as shown in Figure 7.

Figure 7: Effect of ethanol extract of *Annona muricata* on Molt-3 cell line by SRB assay



In vitro cytotoxic potential of *Holarrhena antidysenterica* extracts (ethanol and hot water extracts) was evaluated against 14 human cancer cell lines. The results showed that ethanol extract displayed maximum anti-proliferative effect in 8 different cancer cell lines while the hot water extract did not show any activity (Sharma *et al*., 2014). *In vitro* antiproliferative activity by Dzoyem *et al.* (2013) revealed that, extract from the bark of *F. heitzii* and extract from *H. lyrata* roots had significant cytotoxic activity on THP-1 (leukemic) and PC-3 (prostrate) cell line respectively indicating its anticancer activity. Doshi and Une (2015) screened the crude petroleum ether extracts (*Benincasa hispida* and *Carissa congesta*) and ethanolic extract of (*Polyalthia longifolia*) for its *in vitro* cytotoxicity activity using different cell lines (human colon cancer HCT15, human breast cancer MCF7 and human leukemia Molt-4). Similarly, the results of the present study revealed that *Annona muricata* leaf extract exhibited cytotoxicity against Molt-3 cell line in a concentration dependent manner.

The results of both MTT and SRB assays revealed the cytotoxic effect of the candidate plant extract in leukemic cell line. Administration of imatinib caused a steep decline in the cell survival. It is clearly evident that the increase in the concentration of the extract could cause Molt-3 cells to die at a greater extent. However, the results are comparable with the results obtained from the standard drug imatinib. Thus, it becomes imperative to assess whether the leaf extract is causing

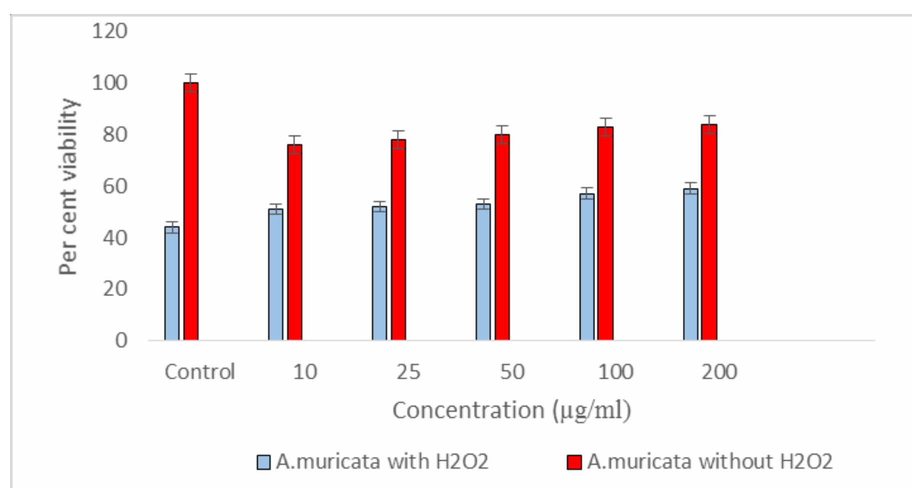
damage to normal cells also. For this, *Saccharomyces cerevisiae* cells were chosen and tested under *in vitro* conditions.

Effect of ethanol extract of *Annona muricata* on *S. cerevisiae* as determined by MTT assay

Saccharomyces cerevisiae is a primitive eukaryotic, non-pathogenic fungi whose complete genome has been sequenced. Yeast cells have remarkable similarities to mammalian cells at the molecular and organelle level, and several yeast proteins are functionally interchangeable with highly homologous human proteins. Thus, it is not surprising that using yeast cells as a model system provides relevant contribution to understand the molecular mechanisms underlying oxidative stress and apoptosis (Kiruthika and Padma, 2013).

In the present study, the normal/ untransformed cells are treated with varying concentrations of the extract and the results showed that the cell viability was found to increase with the increasing concentration. Further, treatment with H_2O_2 caused a steep decrease in the cell survival rate and upon administration of the ethanol extract of *A. muricata* leaves there is an improvement in the cell viability indicating its protective property in normal/ untransformed cells. As the concentration increases, the cell viability also increased in a dose dependent fashion as given in Figure 8.

Figure 8: Effect of ethanol extract of *Annona muricata* on *S. cerevisiae* by MTT assay

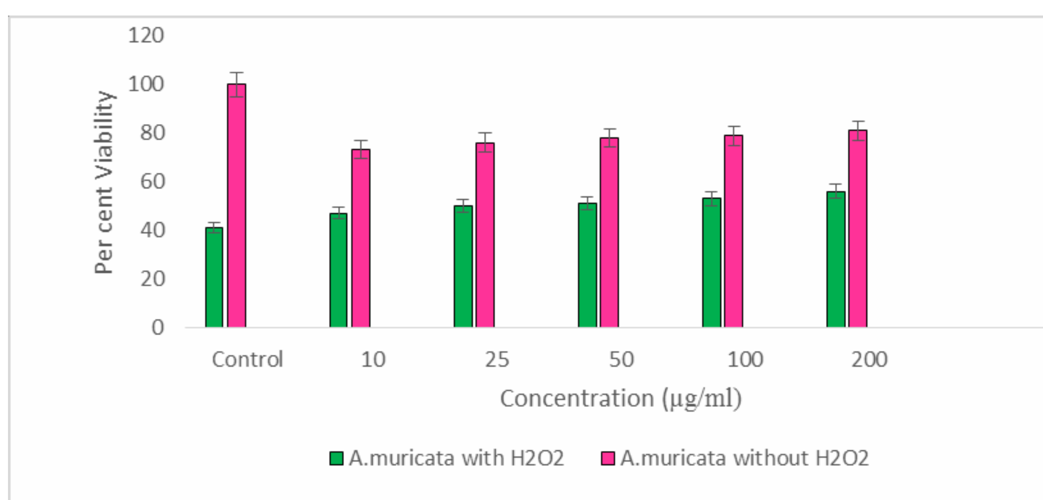


Kiruthika and Padma, (2013) reported that the methanol extract of *Zea mays* was able to protect the yeast cells from oxidative damage followed by aqueous and chloroform extract. Venkateswarlu *et al.* (2015) compared the anticancer effect of petroleum ether extract of *Cynodon dactylon* with normal, Vero cell line using MTT assay and found that the petroleum ether extract was non-toxic to Vero cells but toxic to HEP-2, HELA and MCF-7 cell line. The results indicated that methanol extract of leaves and rhizomes of *Curcuma amada* efficiently reduced the oxidative stress caused by hydrogen peroxide treatment in yeast cells (Skehan*et al.,* 2013). The results of the present study is found to be in agreement with these findings.

Effect of ethanol extract of *Annona muricata* on *S. cerevisiae* as determined by SRB assay

In the SRB assay, the normal/ untransformed yeast cells were subjected to oxidative stress by H_2O_2. Upon addition of the leaf extract, the oxidative damage induced by the H_2O_2 in the normal/ untransformed yeast cells was greatly reduced. The viability of the normal/ untransformed yeast cells is increased with the increasing concentration of the leaf extract, as shown in Fighure 9.

Figure 9: Effect of ethanol extract of *Annona muricata* on *S. cerevisiae* by SRB assay



Dwivedi *et al*. (2013) evaluated that the ethanol and chloroform extracts of *Cissus quadranqularis* exhibited more cytotoxicity towards HeLa cell line and less

toxicity towards normal monkey kidney cell line VERO as estimated by MTT assay. The results showed that all the three flower (*C. pulcherrima)* extracts increased the cell viability of untransformed cells subjected to oxidative stress and also influenced the process of apoptosis induced *in vitro* (Yamuna and Padma, 2016). The cytotoxic effect of the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp was assessed in normal/untransformed cells (*Saccharomyces cerevisiae*). The result revealed that the methanol extract of *Citrus limetta* and *Citrus sinensis* pulp exhibited significant anticancer activity towards Molt-3 cell line while rendering protection to untransformed *S. cerevisiae* cells from oxidative damage (Iswariya *et al*., 2016).

MTT and SRB assays were carried out using yeast cells. Treatment with the standard oxidant H_2O_2 reduces the cell survival rate whereas administration of *A. muricata* plant leaf extracts could reverse the cytotoxic effect exerted by H_2O_2. No significant change observed in the plant extract alone treated group indicates that the leaf extract by itself is not toxic to the normal/untransformed yeast cells. However,these extracts were able to cause significant cell death when tested in Molt-3 cell lines. Thus, the results evidenced a differential response (ie) the leaf extract is decreasing the rate of cell survival when administered in Molt-3 leukemic cells along with imatinib. However, rendering protection to yeast cells. This differential response evoked by the leaf extract of *A. muricata* may be due to the presence of phytoconstituents such as alkaloids, flavonoids, phenols, tannins and saponins. Llagas *et al*. (2014) reported that the ethylacetate, chloroform, and crude ethanol extracts of *Ficus pseudopalma* (FP) leaf exhibited cytotoxicity against human prostate cancer PRST2 cell line. They also have stated that the anticancer activity of *Ficus* species have been attributed to its naturally occurring compounds such as terpenoids and flavonoids. Thus, it can be concluded that, the leaf extract exhibit of A.muricata apoptosis inducing property against the oxidant induced damage only in leukemic cells, as the yeast/untransformed cells were readily protected by the leaf extract of *A. muricata* even in the presence of H_2O_2.

In the present study, phytochemical analysis was carried to out identify the predominant secondary metabolite in the ethanol extract of *Annona muricata* leaves. Further its antioxidant and anticancer property was analyzed by various methods. The results showed that alkaloid is one of the major phytoconstituents predominantly

present in the the *Annona muricata* leaf extract and it is able to scavenge free radicals effectively and also exhibited good cytotoxicity towards Molt-3 cell line while rendering protection against the normal/untransformed *S. cerevisiae* cells from oxidative damage. Thus, the ethanol extract of *Annona muricat*a leaves can serve as potential drug candidate for the free radical mediated diseases.

5.0 SUMMARY AND CONCLUSION

Cancer is one of the most fatal diseases in human population and one of the most frequent causes of death worldwide. Cancer is caused by excessive free radical damage, which ultimately causes damage to the DNA, protein and lipids. This process of DNA damage leads to mutations that cause normal cells to transform into a cancer cells. To prevent the cancer, synthetic and natural sources are used in alone or combination. Interest in the pharmacological effects of bioactive compounds on cancer treatments and prevention has increased dramatically over the past twenty years. The present study was carried out to identify the medicinal potential of *Annona muricata* leaf extract.

Preliminary phytochemical studies are helpful in finding out chemical constituents in the plant material that may well lead to their quantitative estimation. Thus, in the present study phytochemical screening for the candidate plant was carried out and the results of the preliminary phytochemical analysis of the ethanol extract of *Annona muricata*. Showed the presence of all the secondary metabolites including alkaloids, flavonoids, phenols, terpenoids, tannins, saponins and steroids thus showing that the leaf extract is rich in phytochemical constituents.

Quantitative phytochemical analysis was performed in order to determine the major phytoconstituents present in the ethanol extract of *Annona muricata* leaves. The concentration of the secondary metabolites present in the leaf extract was analyzed by calculating the dry weight of the alkaloids, flavonoids, phenols, terpenoids, tannins, saponins and steroids and it is found to be 0.65, 0.01, 0.15, 0.03, 0.39, 0.62 and 0.45g respectively. Among all the phytochemicals tested, alkaloids represent the predominant phytoconstituent of *Annona muricata* leaf.

The leaf extract showed the presence of one major peak and one minor peak with retention time of 2.80 and 3.12 while the retention time of standard quercetin is 2.88 and 7.38. This indicates the presence of compounds similar to quercetin or the derivatives of quercetin in the ethanol extract of *Annona muricata* leaves.

The results revealed that the ethanol extract of *A. muricata* leaves showed significant scavenging activity. The maximum scavenging activity is seen at the

concentration of 200µg/ml. The result demonstrates that leaf extract possess good antioxidant potential in a concentration dependent manner.

In H_2O_2 scavenging assay performed, the leaf extract was able to scavenge H_2O_2 to considerable extent. At lower concentration of 10µg, the leaf extract was able to scavenge H_2O_2 moderately. When the concentration is increased, the per cent scavenging is also increased in a concentration dependent manner.

In order to determine the cytotoxic effect of the leaf extract of *Annona muricata*, MTT assay was performed in leukemic cells (Molt-3 cell line) in comparison with the standard chemotherapeutic drug Imatinib at a concentration of 50µg/ml. The experiment was carried out using varying concentrations of leaf extracts ranging from 10, 25, 50, 100 and 200µg. The viability of the control cells were fixed as 100%. The results revealed that ethanol extract of *Annona muricata* leaves are able to induce cell death even at lower concentration of 10µg/ml. When Molt-3 cells are treated with increasing concentration of extract, the viability was found to be decreased in a dose dependent manner. The results showed that the leaf extract exhibited good cytotoxic effect towards the leukemic cell line. The data also revealed that 50% viability was observed at lower concentration (10µg/ml) while the viability was decreased to a greater extent of about 12% at the higher concentration of 200 µg/ml.

The anti-proliferative SRB assay was performed to assess growth inhibition by the *Annona muricata* leaf extract. The results demonstrated that the ethanol extract of *Annona muricata* leaves showed significant cytotoxic effect against Molt-3 cell line. When the cells were treated with different concentration of the leaf extract, cell death was observed in a dose dependent manner. It is evident that the leaf extract was able to induce cell death to a considerable extent. Upon treatment with leaf extract, the cytotoxic effect was observed even at lower concentration of 10µg/ml and was increased gradually at a higher concentration of 200 µg/ml which was comparable with the standard drug imatinib.

In the present study, the normal/ untransformed cells *Saccharomyces cerevisiae* are treated with varying concentrations of the extract and the results showed that the cell viability was found to increase with the increasing concentration.

Further, treatment with H_2O_2 caused a steep decrease in the cell survival rate and upon administration of the ethanol extract of *A. muricata* leaves there is an improvement in the cell viability indicating its protective property in normal/ untransformed cells. As the concentration increases, the cell viability also increased in a dose dependent fashion.

In the SRB assay, the normal/ untransformed yeast cells were subjected to oxidative stress by H_2O_2. Upon addition of the leaf extract, the oxidative damage induced by the H_2O_2 in the normal/ untransformed yeast cells was greatly reduced. The viability of the normal/ untransformed yeast cells is increased with the increasing concentration of the leaf extract.

To conclude, the present study was carried out to evaluate the efficacy of *A. muricata* leaves. The results showed that phytochemical analysis of the ethanol extract of *A. muricata* leaves sowed the presence of various alkaloids, flavonoids, phenols, terpenoids, tannins, saponins and steroids. *A. muricata* leaf extract exhibited considerable radical scavenging potential as well as good cytotoxicity towards Molt-3 cell line and also, able to render good protection against the normal/untransformed *S. cerevisiae* cells from oxidative damage. The anticancer activity of the *A. muricata* leaf extract might be due to the presence of various phytoconstituents and its mechanism to scavange the free radicals. Thus, *A. muricata* leaf extract might be considered as potential drug candidate for cancer.

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