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REVIEW OF LITERATURE

The present study aims to isolate and identify the flavonoid and acetogenin rich fractions from *Annona muricata* leaves and to investigate the antioxidant activity with the focus on the anticancer activity of the *Annona muricata* leaf extract and fractions. A brief overview of the vast literature applicable to the current study is reviewed in this chapter.

Oxidants

The free radical oxidants such as reactive oxygen species, reactive nitrogen species, and reactive sulfur species are produced inside cells through various metabolic processes (Ali *et al*., 2020). ROS can be generated by multiple endogenous and exogenous factors. Mitochondria are known to produce significant amounts of endogenous ROS that contribute to intracellular oxidative stress. At an ultra-structural level, mitochondria have a four- layer structure consisting of an outer mitochondrial membrane, intermembrane space, inner mitochondrial membrane, and matrix. The generation of ROS mainly occurs in the electron transport chain on the inner mitochondrial membrane during oxidative phosphorylation, a process that creates adenosine triphosphate (ATP) from oxygen and simple sugars. Five protein complexes contribute to the process: NADH: ubiquinone reductase (complex I), succinate: ubiquinone reductase (complex II), ubiquinol: cytochrome *c* reductase (complex III), cytochrome *c* oxidase (complex IV), and F_1F_0- ATP synthase (complex V). The 2 major sites for ROS generation are complexes I and III where large changes occur in the potential energy of electrons related to the reduction of oxygen. The leakage of electrons at complexes I and III leads to the generation of superoxide. Once generated, superoxide is quickly dismutated to hydrogen peroxide by superoxide dismutase 1 (SOD1) in the intermembrane space or by SOD2 in the matrix. Both superoxide and hydrogen peroxide in this process are recognized as ROS that are generated in mitochondria. Transition metals such as iron can also generate ROS non- enzymatically via Fenton reaction. The Fenton reaction involves Fe^{2+} reacting with hydrogen peroxide to yield a hydroxyl radical, which can

cause damage to DNA and other biomolecules. In addition, multiple external factors induce exogenous ROS, including air pollutants, tobacco smoke, radiation, foods, and drugs (Nakamura and Takada. 2021).

In living organisms, free radicals have long been regarded as harmful species. The famous free-radical theory of aging proposes that free radicals are important factor leading to aging. Free radicals can be produced from either endogenous or exogenous sources. In eukaryotic cells, 90% of the endogenous radicals are produced by mitochondria. Most radicals are generated by the escaping of mitochondrial electrons, which react with O_2 to give $O_2^{-\bullet}$. Then $O_2^{-\bullet}$ can be further converted to H_2O_2 and $^{\bullet}OH$ and small amount endogenous radicals generate in/at peroxisomes, plasma, and nuclear membranes as well as some oxidases. Exogenous radicals can attribute to various outer stimulus, such as ultraviolet radiation, air/water pollution, toxic chemicals, smoking, alcohol, drug abuse and psychological stress. The unwanted free radicals are responsible for aging, tissue damage, and various diseases, such as Parkinson's disease, Alzheimer's disease, diabetes mellitus and cardiovascular diseases. In particular, free radicals can increase the risk of cancer by activating original cancer gene transcription (Wang *et al*., 2021).

Free radicals

Free radicals are small diffusible molecules that are highly reactive because of the unpaired electron. Free radicals were initially thought to be oxygen centered radicals called reactive oxygen species but also include a subgroup of reactive nitrogen species and are all a product of normal cellular metabolism. Oxygen is necessary for energy production via the electron transport chain in living organisms, a mechanism by which energy (ATP) is released to enable the cell to carry out its normal physiological functions. This is attributed to its high redox potential which makes it a brilliant oxidizing agent capable of easily accepting electrons from reduced substrates. This contradictory effect of oxygen in living organisms necessitated the evolution of antioxidant system to protect against oxidant imbalance and combat reactive oxygen species. The mitochondria are the most vital source of ROS production. Just like free radicals, antioxidants can be endogenously produced and reduced glutathione and can also be introduced to the biological system exogenously, usually through diet. Antioxidants primarily function to balance out free radicals generated during metabolic processes including during mechanisms involved in protecting the gut from inflammation and injury (Ifeanyi, 2018).

Sources of free radicals

Free radicals are either derived from endogenous or exogenous sources. Endogenously, they are generated intracellularly; they act within the cell and are released into the surrounding area. Among the exogenous sources of free radicals, ionizing radiation plays a major role. Ionizing particles transfer energy to water and ionize the water molecule. This ionized water molecule produces free radicals upon dissociation. Endogenous sources comprise enzymes and transport molecules (e.g., xanthine oxidase, aldehyde oxidase), auto-oxidation reactions of biological molecules (e.g., thiols, flavins, catecholamines, ferredoxins, hemoglobin, as well as metal ions, Cu, Fe, Cd, As, Hg, Cr, Al, and Ni) are common sources. Exogenous sources consist of O_3 and NO, primarily from automobile exhaust, medications (especially anticancer agents), ionizing radiations, chemical pollutants (e.g., pesticides), viruses, bacteria, parasites, and smoking (both active and passive). Excessive alcohol and stress conditions may also cause the formation of free radicals (Sharma *et al*., 2018).

Formation of free radicals

Free radicals are generally formed

(a) Through the hemolytic cleavage of a covalent bond of a regular biomolecule

(b) Through the removal of an electron from a regular biomolecule

(c) Through the connection of an electron to a regular biomolecule

Increased production of free radicals is caused by excessive exposure to UV radiation, long-term stress conditions, intense physical exercise, improper diet and use of stimulants. Under physiological conditions, there is a balance between the generation and removal of free radicals from the body (Jakubczyk *et al*., 2020).

Classification of free radicals

Free radicals are classified into two major categories of compounds which includes the free radicals and non-reactive radicals. The free radicals include nitric oxide ($NO^{\bullet}$), superoxide ($O2^{\bullet}$), hydroxyl ($HO^{\bullet}$), peroxyl ($ROO^{\bullet}$), alkoxyl ($RO^{\bullet}$) and one form of singlet oxygen ($^1O_2$). These species are considered as free radicals since they contain at least one unpaired electron in the shells around the atomic nucleus which make them unstable and therefore can easily donate

or obtain another electron to attain stability. As such, they are highly reactive and capable of independent existence. On the other hand, the non-reactive radicals are a group of compounds which are not radicals but are extremely reactive or can be easily converted to reactive species. Examples of these substances include Hypochlorous acid (HCIO), Hydrogen peroxide (H_2O_2), organic peroxides, aldehydes, ozone (O_3) (Engwa, 2018).

1. Reactive Oxygen Species

Reactive oxygen species (ROS) is a collective term referring to unstable, reactive, partially reduced oxygen derivatives that are created as a by-product of normal metabolic processes. They include hydrogen peroxide (H_2O_2), superoxide anion (O_2^-), hypochlorous acid (HOCl), singlet oxygen ($^1O_2$) and hydroxyl radical (·OH), and act as second messengers in cell signaling, and are essential for various biological processes in normal and cancer cells (Yang *et al*., 2018).

ROS can be damaging to lipids, proteins and DNA, in recent years their role as important intracellular and extracellular signaling molecules has become evident. The mitochondria are the major source of ROS within a cell and play an essential role in regulation of proliferative, apoptotic and metabolic pathways. It is established that the hallmarks of cancer include metabolic reprogramming as well as a tumor promoting microenvironment. At the interface of both of these important biological events are ROS which are produced by cancer cells as well as cellular components in the microenvironment (Weinberg *et al*., 2019).

Reactive Oxygen Species refers to the highly reactive substances which contain oxygen radicals. Hypochlorous acid, peroxides, superoxide, singlet oxygen, alpha-oxygen, and hydroxyl radicals are the major examples of ROS. Generally, the reduction of oxygen (O_2) in molecular form produces superoxide ($\bullet O_2^-$) anion. ROS are produced during a variety of biochemical reactions within the cell organelles, such as endoplasmic reticulum, mitochondria, and peroxisome. Naturally, ROS are also formed as a byproduct of the normal metabolism of oxygen. The production of ROS can be induced by various factors such as heavy metals, tobacco, smoke, drugs, xenobiotics, pollutants, and radiation (Sahoo *et al*., 2022).

2. Reactive Nitrogen Species

Reactive Nitrogen Species are a group of important chemically reactive species and it includes nitric oxide (NO), nitroxyl (HNO), nitrogen dioxide (NO_2) and peroxynitrite ($ONOO^-$), which can damage cells via nitrosative stress. For example, NO can be endogenously generated by nitric oxide synthases, and failure of NO homeostasis can induce cancer, neurodegenerative diseases and stroke. $ONOO^-$, which is produced from NO with $O^{\cdot}{}_2^-$, was considered as ROS only after a report by Beckman in 1990. Due to its oxidizing and nitrating abilities, $ONOO^-$ also commonly oxidizes DNA, proteins and lipids, which can cause various cancers, inflammation and neurodegenerative diseases (Kwon *et al*., 2021).

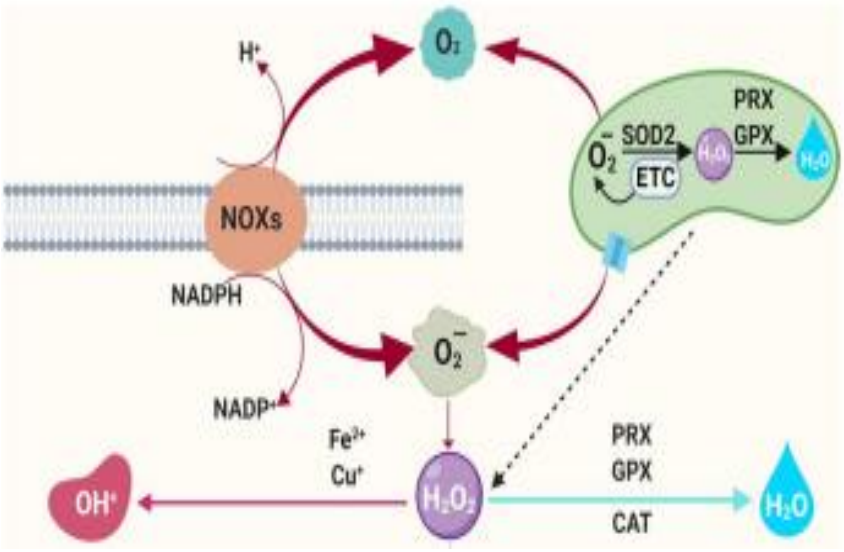
3. Reactive Sulfur Species

Reactive Sulfur Species is a newly classified broad group of sulfur containing reactive species that includes both radical and non-radical sulfur-based moieties. This broad group includes radical species such glutathionyl radical ($GSSG^{\cdot}$), as well as non-radical reactive sulfane species, reactive sulfur substances. Unlike other reactive oxygen and nitrogen species, RSS are capable of both oxidation and reduction. It has now become apparent that molecules with sulfur-containing functional groups can also be stressors in their own right, with pivotal roles in cellular function and homeostasis. A key distinction for RSS is that, unlike oxygen or nitrogen, sulfur not only forms a plethora of specific reactive species, but sulfur also targets itself, as sulfur containing molecules, that is, peptides, proteins, and enzymes, preferentially react with RSS. RSS are omnipresent and sometimes even considered as important as ROS and RNS which for decades have dominated the redox field (Ali *et al*., 2020).

Regulation of ROS generation

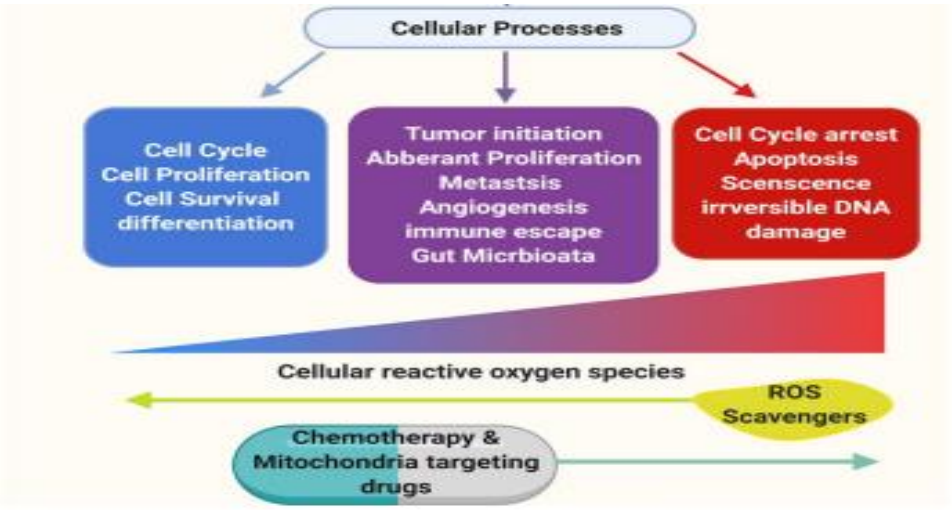
ROS balance is maintained by several enzymes that neutralize toxic oxidants. Superoxide dismutases (SODs) are responsible for the conversion of $O2^-$ into H_2O_2. To avoid cellular damage, catalase (CAT), glutathione peroxidase (GPXs), and peroxiredoxins (PRXs) convert H_2O_2 into water and oxygen.

Figure 1: Formation and regulation of ROS (Kirtonia *et al*., 2020)



ROS are generated during normal cellular functioning and homeostasis is maintained by antioxidants expressed by the cells. Low ROS is the basic need to maintain normal cellular proliferation, survival, and differentiation. Moderate to high ROS (tumor favoring ROS) is the signal for the increased cellular proliferation, survival, tumor initiation, immune escape to genomic instability, metastasis, invasion and angiogenesis. Extremely high ROS produced by chemotherapeutic agents is dangerous for the cells and leads to cell cycle arrest, apoptosis, senescence and unrepairable DNA damage (Kirtonia *et al*., 2020).

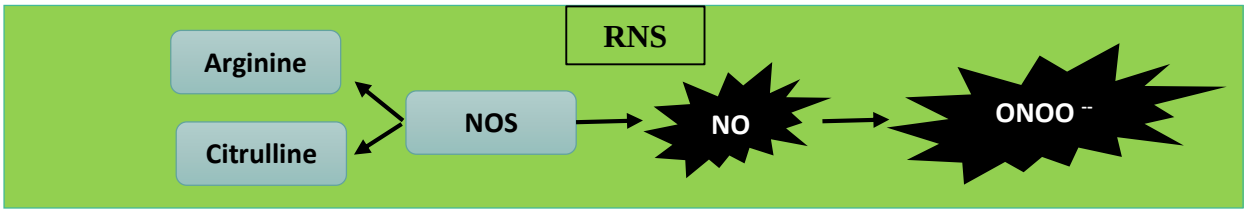
Figure 2: Effect of ROS in cellular system (Kirtonia *et al*., 2020)



Regulation of RNS generation

$NO^{\bullet}$ is produced from the metabolism of the amino acid, L-arginine. The enzymes catalyzing this process, known as nitric oxide synthases (NOS), convert L-arginine into L-citrulline and $NO^{\bullet}$ by a 5-electron oxidation of a guanidine nitrogen of L-arginine.

Figure 3: Formation of RNS



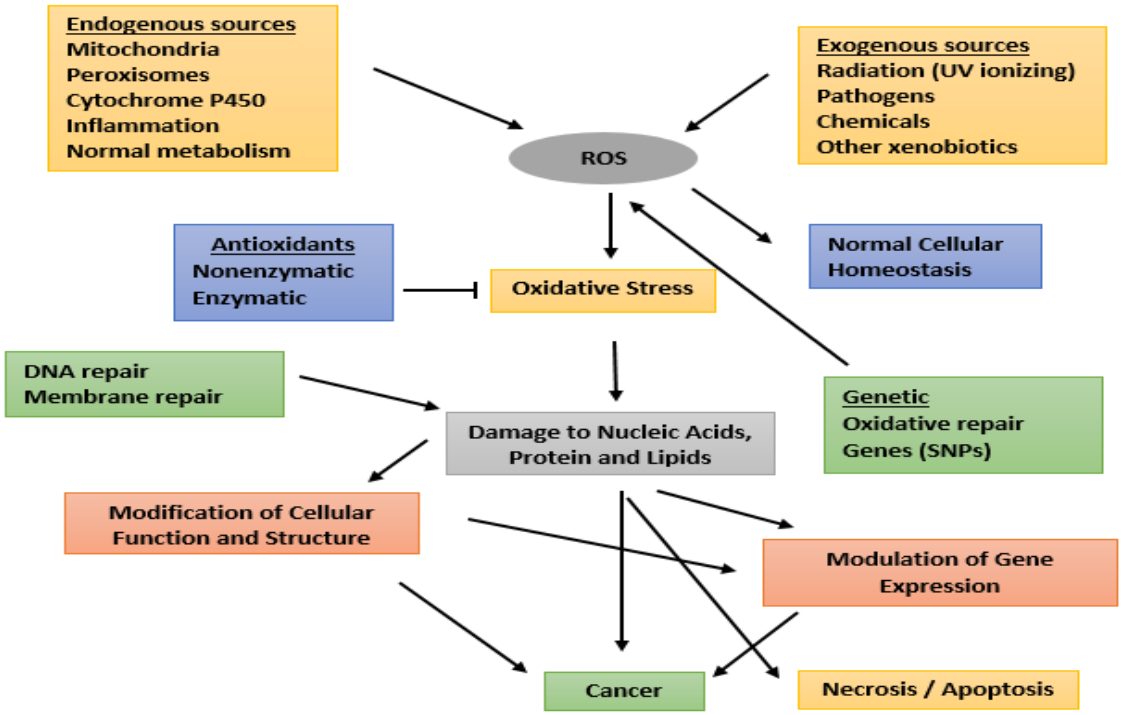
Three isoforms of nitric oxide synthase have been identified. Two isoforms, neuronal NOS (nNOS; type I NOS) and endothelial NOS (eNOS; type III NOS), are expressed constitutively and regulated by the interaction of Ca^{2+} with calmodulin. The other isoform, inducible-NOS (iNOS; type II NOS), is induced in response to infection, inflammation, or trauma and is not regulated by Ca^{2+} because it forms a complex with calmodulin at very low

concentrations of Ca^{2+}. $NO^{\bullet}$ generated by the NOS isoforms located in different cell types plays different roles. $NO^{\bullet}$ generated by nNOS in neurons serves in communication between nerve cells, whereas the free radical generated by iNOS in macrophages and smooth muscle cells contributes to their killing mechanism, and $NO^{\bullet}$ generated by eNOS in endothelium, brain, and heart relaxes blood vessels and maintains normal blood pressure. Some functions of $NO^{\bullet}$ in signaling and regulation of cell function are performed through cGMP-independent pathways including those involving mitochondria. At physiological concentrations, most mitochondrial effects of $NO^{\bullet}$ are exerted on the respiratory chain. First, $NO^{\bullet}$ competes with O_2 for the binding site at the binuclear center of cytochrome *c* oxidoreductase, leading to a reversible inhibition of cytochrome *c* oxidase activity. Secondly, $NO^{\bullet}$, reacting with respiratory Complex III, inhibits electron-transfer and enhances $O_2^{\bullet-}$ production. $NO^{\bullet}$ also gives rise to protein nitrosation, reacting reversibly with the nucleophilic centers in protein thiol residues, and mitochondria, treated with $NO^{\bullet}$ donors, exhibit *S*-nitrosation and inhibition of Complex I. Moreover, the reaction of $NO^{\bullet}$ with $O_2^{\bullet-}$, which is formed by the mitochondrial respiratory chain, leads to the switch from reversible inhibition of cellular respiration by $NO^{\bullet}$ to the pathological inhibition of mitochondrial function by ONOO (Bashan *et al*., 2009).

Oxidative stress

Oxidative stress is the cellular state in which levels of reactive oxygen species override the antioxidant defense mechanisms of the cell. Multiple studies have confirmed a strong relationship between oxidative stress and the formation or progression of several human pathologies including cancer. Epidemiological studies have linked chronic oxidative stress to cancer. Thus the participation of oxidative stress in the cancer process is well established. Both endogenous and exogenous agents have been shown to result in ROS induction in cancer. ROS from endogenous and exogenous sources, if not detoxified by antioxidants, can induce an increase in oxidative stress in the cell. The resulting oxidative stress, if severe enough can modify the structure and function of critical cellular macromolecules including DNA resulting in cell growth, mutation, and/or chromosome instability resulting in neoplasm formation (Klaunig *et al*., 2018).

Figure 4: Sources and effects of ROS (Klaunig *et al*., 2018)

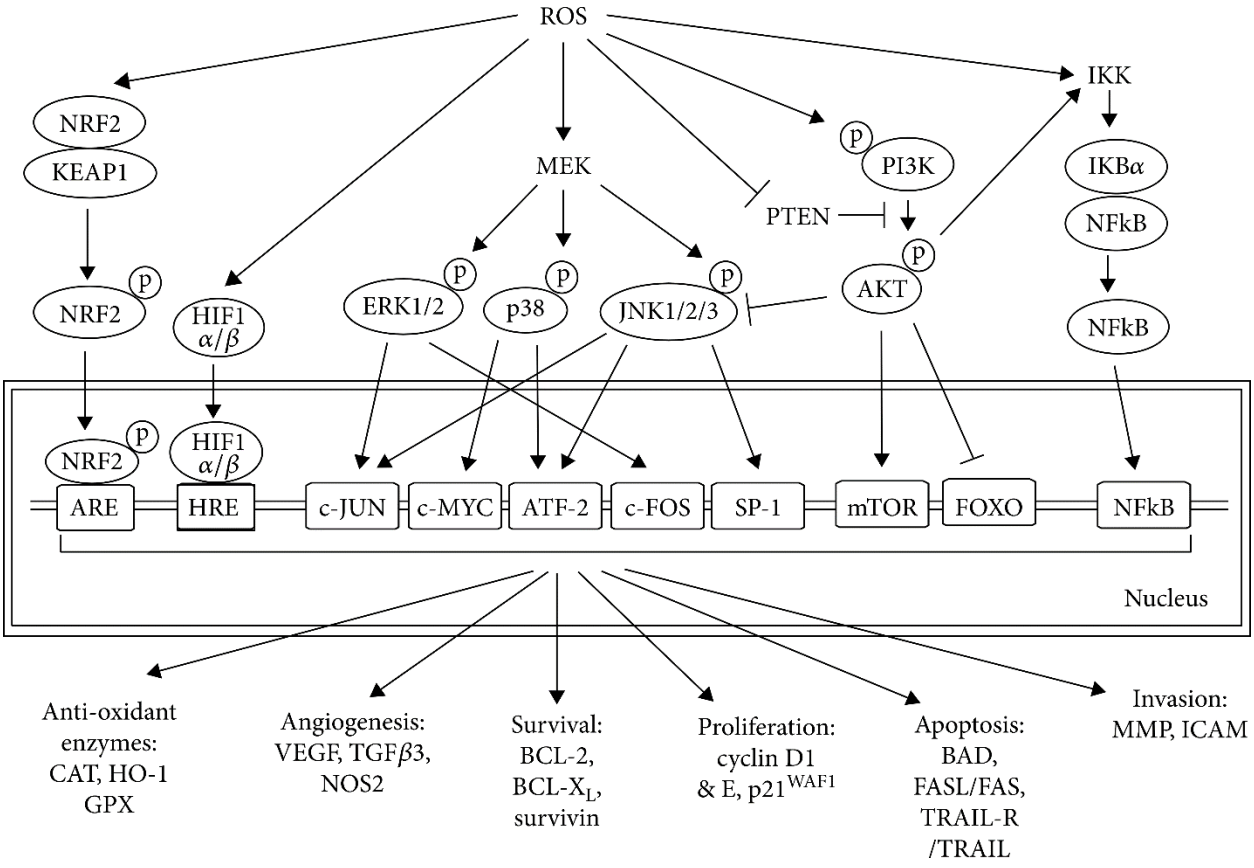


ROS signaling in cancer

The overproduction of ROS in cancer has been shown to induce a variety of biological effects including enhanced cell proliferation, DNA damage and genetic instability, adaptation, cellular injury and cell death, autophagy and resistance to drugs. The outcome is dependent on the genetic background of the cancer, the types of ROS involved ($O2^{\bullet-}$, H_2O_2) and the levels and duration of ROS exposure (Moloney and Cotter, 2018).

ROS play an important role in tumor initiation, promotion, and progression. At levels below the ROS threshold, ROS activate oncogenes such as Ras and c-Myc and induce p53-mediated DNA repair and survival in cancer cells. At levels above the ROS threshold, ROS trigger apoptotic signals. These cellular processes are controlled by ROS through its regulation of various signaling pathways, including the mitogen-activated protein kinase (MAPK)/extracellular-signal-regulated kinase (ERK), the phosphoinositide-3-kinase (PI3K)/protein kinase B (AKT), the inhibitor of kappa B (IκB) kinase (IKK)/nuclear factor κB (NFκB), and the protein kinase D (PKD) signaling pathways (Krishnan *et al*., 2019).

Figure 5: ROS-mediated intracellular cell signaling pathways (Krishnan *et al*., 2019)



Antioxidants

Antioxidants avert or remove oxidative stress related diseases by counteracting the deteriorating effect of ROS. Antioxidants nullify free radicals and play crucial role in conserving finest cellular functions. An antioxidant reduces the occurrence of different disorders like: aging, cancer, diabetes, inflammation, liver disease, cardiovascular disease, cataract, nephrotoxicity and neurodegenerative disorders. Dietary antioxidants are thought to have potential capacities to avert oxidative anxiety induced diseases (Neha *et al*., 2019).

Antioxidants are the agents that have the ability to prevent, retard or eradicate oxidative stress of a molecule. Antioxidants are organic or inorganic compounds, present naturally in plant sources or synthesized industrially. When added to a formulation even in minuscule quantities, they tend to give up their electron(s) to the free radicals, subsequently neutralizing them,

preventing the cells from potential damage and curing numerous diseases. The efficacy of exogenous antioxidant compounds, to safeguard the tissues from oxidative stress, is dependent upon the nature of the antioxidant, its physic-chemical and biopharmaceutical properties, and accessibility at the target site. Antioxidants are also useful as dietary supplements for sustaining health, disease prophylaxis or for reducing the adverse effects of chemo and radio-therapy (Khurana *et al*., 2018).

Types of Antioxidants

1. Antioxidants based on solubility

a) Water soluble antioxidants - water soluble antioxidants are also known as hydrophilic antioxidants. They react with the oxidants in the process of cell cytosol and the blood plasma. Some common water-soluble antioxidants are ascorbic acid, glutathione, lipoic acid, and uric acid.

b) Fat soluble antioxidants - Fat soluble antioxidants play a key role in keeping healthy body. These antioxidants protect cell membrane from damage and also from lipid peroxidation. These antioxidants can be synthesized from the cells and tissues or it can be obtained from the food. Some common fat-soluble antioxidants are carotenes, ubiquinol, vitamin A and E (Kumar *et al*., 2017).

2. Antioxidants based on occurrence

a) Natural antioxidants - Natural antioxidants are the innumerable ingredients of fruits and vegetables that have attracted much scientific attention. Natural antioxidants are created in all parts of plants and nutrients, because they live under continuous oxidation stress from free radicals and ROS. In this case, different tissues have produced antioxidant systems to manipulate free radicals, lipid oxidation catalysts, oxidation intermediates. These antioxidant mixtures consist of phenolic acids, carotenoids, tocopherols, flavonoids that can obstruct Fe^{+3} induced oxidation, eradicate free radicals and serve as metabolites. Spices and herbs are used in special foods for their flavour and in medicinal mixtures for their biological effects, which often involve large concentrations of phenolic compounds with strong hydrogen activity. Natural sources like fruits, vegetables and meats are noted to have all antioxidants. There are many natural antioxidants common to daily foods such as mostly vitamin A (carotenoids), vitamin

E (tocopherol), vitamin C (ascorbic acid) various polyphenols including flavonoids, lycopene of carotenoids), anthocyanins and coenzyme Q 10, recognized as Ubiquitin, a form of protein (Suleman *et al*., 2019).

Natural antioxidant system is sorted in two major groups, enzyme based and non-enzymes. Non-enzymatic antioxidants include direct acting antioxidants, which are extremely important in defense against oxidative stress. Most of them, including ascorbic and lipoic acid, polyphenols and carotenoids, are derived from dietary sources. The cell itself synthesizes some of these antioxidant molecules. Indirectly acting antioxidants mostly include chelating agents and bind to redox metals to prevent free radical generation. Enzymatic antioxidants are capable of stabilizing, or deactivating free radicals before they attack cellular components. They act by reducing the energy of the free radicals or by giving up some of their electrons for its use, thereby causing it to become stable. In addition, they may also interrupt with the oxidizing chain reaction to minimize the damage caused by free radicals. By reducing exposure to free radicals and increasing the intake of antioxidant enzyme rich foods or antioxidant enzyme supplements, our body's potential to reduce the risk of free radical related health problems is made more palpable. Antioxidant enzymes are, therefore, absolutely critical for maintaining optimal cellular and systemic health. The antioxidant enzymes–GPx, heme peroxidase, CAT, and SOD–metabolize oxidative toxic intermediates and require micronutrient cofactors such as selenium, iron, copper, zinc, and manganese for optimum catalytic activity. The antioxidant enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) serve as the primary line of defense in destroying free radicals.

Glutathione plays several roles in the body, importantly it improves the effectiveness of Vitamin C. SOD first reduces (adds an electron to) the radical superoxide ($O2^-$) to form hydrogen peroxide (H_2O_2) and oxygen (O_2). Catalase and GPx then work simultaneously with the protein glutathione to reduce hydrogen peroxide and ultimately produce water (H_2O). The oxidized glutathione is then reduced by another antioxidant enzyme glutathione reductase. Together, they repair oxidized DNA, degrade oxidized protein, and destroy oxidized lipids (fat-like substances that are a constituent of cell membranes). Various other enzymes act as a secondary antioxidant defense mechanism to protect from further damage (Atta *et al*., 2017).

b) Synthetic antioxidants - Synthetic antioxidants, such as butylated hydroxyanisole, butylated hydroxytoluene, propyl gallate and tertbutylhydroquinone, have been widely used in the food industry as antioxidants to control lipid oxidation and off-flavor development (Yang *et al*., 2018).

c) Antioxidants in body - Certain antioxidant enzymes are produced within the body. The most commonly recognized naturally occurring antioxidants are superoxide dismutase, catalase, and glutathione (GSH). SOD changes the structure of oxidants and breaks them down into hydrogen peroxide. CAT in turn, breaks down hydrogen peroxide into water and tiny oxygen particles or gases. GSH is a detoxifying agent, which binds with different toxins to change their form so that they are able to leave the body as waste (Kumar *et al*., 2017).

d) Miscellaneous antioxidants - In addition to enzymes, vitamins, and above antioxidants, there appear to be many other compounds that have antioxidant properties which are lycopene, selenium and zinc (Kumar *et al*., 2017).

Sources of antioxidants

The exogenous antioxidants are mainly derived from food and medicinal plants, such as fruits, vegetables, cereals, mushrooms, beverages, flowers, spices 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).

Antioxidant Defenses

Antioxidants act as a radical scavenger, hydrogen donor, electron donor, peroxide decomposer, singlet oxygen quencher, enzyme inhibitor, synergist, and metal-chelating agents. Both enzymatic and non-enzymatic antioxidants exist in the intracellular and extracellular environment to detoxify ROS. Endogenous antioxidants play a crucial role in maintaining optimal cellular functions and thus, systemic health and well-being. However, under conditions, which promote oxidative stress, endogenous antioxidants may not be sufficient and dietary

antioxidants may be required to maintain optimal cellular functions. Two principal mechanisms of action have been proposed for antioxidants. The first is a chain breaking mechanism by which the primary antioxidant donates an electron to the free radical present in the system. The second mechanism involves removal of ROS/RNS initiators (secondary antioxidants) by quenching chain initiating catalyst. Antioxidants may exert their effect on biological systems by different mechanisms, including electron donation, metal ion chelation, or by gene expression regulation. Many antioxidants have aromatic ring structures and are able to delocalize the unpaired electrons. Antioxidant mechanisms of polyphenolic compounds are based on hydrogen donation abilities and chelating metal ions. After donating a hydrogen atom, phenolic compounds become resonance-stabilized radicals, which do not easily participate in other radical reactions (Chahal *et al*., 2018).

Secondary metabolites

The medicinal plants are rich in secondary metabolites, a diverse group of chemicals, which include alkaloids, glycosides, steroids, flavonoids, and related metabolites, which have been extensively used in drug and pharmaceutical industry. Many of the plant secondary metabolites are constitutive, exist in healthy plants in their biologically active forms, but others occur as inactive precursors and are activated in response to tissue damage or pathogen attack. The beneficial medicinal effects of plant materials typically result from the combinations of secondary metabolite production such as alkaloids, steroids, tannins, flavonoids, resins, fatty acids. Out of the total number of secondary metabolites reported in the dictionary of natural products, 33,000 are terpenoids, 16,000 alkaloids, and 8,182 flavonoids. These being an integral part of the basic metabolism also have an ecological role and are often involved in plant protection against biotic or abiotic stresses. Some secondary metabolites such as flavonoids are also involved in cell pigmentation in flower and seed, which attract pollinators, seed dispersers and are also involved in plant reproduction. Moreover, plant secondary metabolites have pharmaceutical properties effective for human health (Jain *et al*., 2019).

Flavonoids

Flavonoids are a class of natural phenolic compounds that include a C_6-C_3-C_6 carbon framework (phenyl benzopyran). The basic flavonoid structure consists of a 2-phenyl-benzo-γ-pyrane nucleus comprising two benzene Rings A and B linked through a heterocyclic pyran or pyrone Ring C. Depending on the level of unsaturation and oxidation, flavonoids can be grouped into various subclasses, such as flavones, isoflavones, flavonols, flavanols (otherwise known as catechins, flavanones, flavanonols, chalcones and dihydrochalcones, aurones and anthocyanidins. Flavonoids are present in most of the plants, generally in all of their parts namely leaf, seed, root and fruit. As the most abundant secondary plant metabolites, their quantitative distribution varies from organ to organ or even plant to plant, depending on the environment. The flavonoids are known for their antioxidant, anti-inflammatory, antiallergic, anticancer, antiviral, and antifungal properties (Gorniak *et al*., 2019).

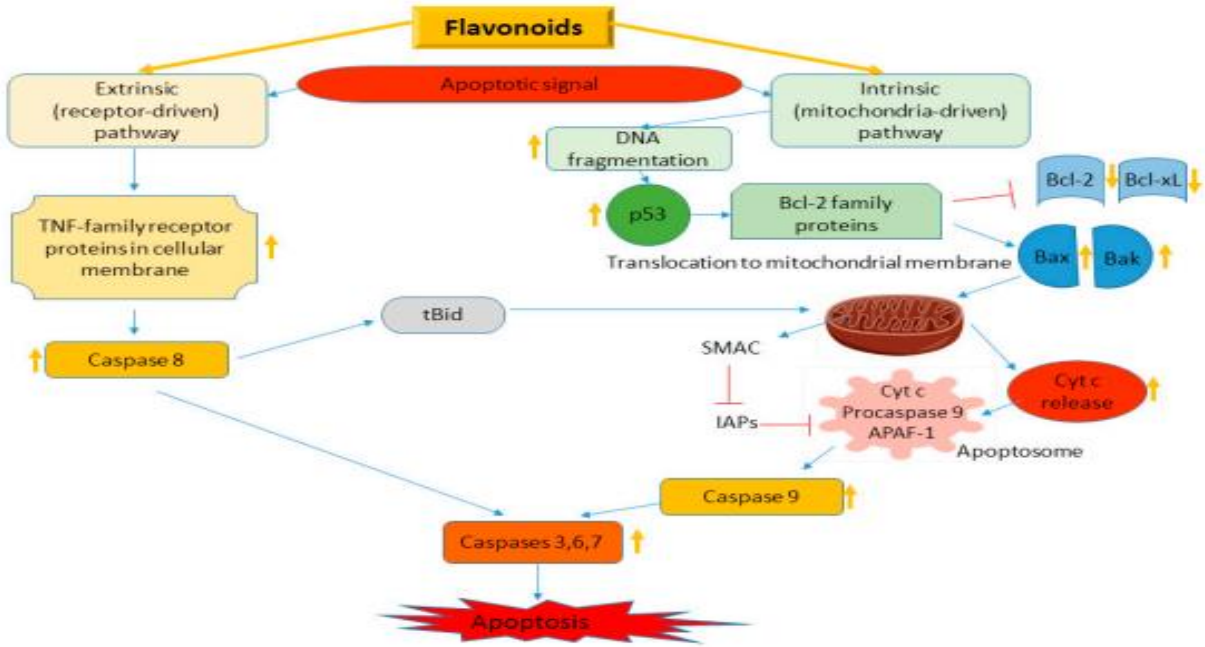
Flavonoids having several biochemical and antioxidant effects associated with various diseases such as cancer, Alzheimer's disease (AD), and atherosclerosis. Flavonoids are associated with a broad spectrum of health-promoting effects and are an indispensable component in a variety of nutraceutical, pharmaceutical, medicinal and cosmetic applications. This is because of their potent anti-oxidative, anti-inflammatory, antimutagenic, antimicrobial, anti-carcinogenic, vascular activities, free radical scavenging abilities, and other medicinal properties coupled with their capacity to modulate essential cellular enzyme functions (Karak, 2019).

Role of flavonoids in apoptosis

Cancer cells are resistant to apoptosis - a programmed cell death, usually induced by a series of signal transduction pathways and pro-apoptotic proteins - caspases and Bcl-2 family proteins. There are two main signaling cascades of apoptosis - extrinsic, related to tumor necrosis factor (TNF) superfamily with main signaling protein - caspase 8; and intrinsic mitochondrial pathway, where Bcl-2 family proteins launch the activation of caspases 9, 3 and 7. There is an overexpression of oncogenic genes leading to cellular proliferation and p53 suppression, and activated anti-apoptotic proteins of Bcl-2 family in cancer cells, whereas pro-apoptotic proteins and caspases could be downregulated. Flavonoids could target apoptotic signaling cascade stimulating the cell death pathways. Flavonoids acting as pro-oxidants could suppress proliferation of cancer cells by inhibition of epidermal growth factor receptor/mitogen activated

protein kinase (EGFR/MAPK), phosphatidylinositide 3-kinases (PI3K), protein kinase B (Akt) as well as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Kopustinskiene *et al*., 2020).

Figure 6: Flavonoid targets in extrinsic and intrinsic apoptosis pathways
(Kopustinskiene *et al*., 2020)



TNF—tumor necrosis factor, tBid—truncated Bid, Bcl-2—B-cell lymphoma protein 2, Bcl-xL—Bcl-2 homologue splice variants, Cyt c—cytochrome c, SMAC—second mitochondrial activator of caspases, IAPs—inhibitor of apoptosis proteins, APAF-1—apoptotic protease activating factor

Acetogenins

Among the 212 compounds that were isolated from different parts of the *Annona muricata* plant, acetogenins (AGEs) were reported to be the major phytochemicals, followed by alkaloids and phenols. These secondary metabolites were first isolated from multiple parts of plants belonging to the Annonaceae family in 1982 by Tempesta, and acetogenins were further determined to have toxicity towards the P-388 lymphocytic leukemia in mice (Yajid *et al*., 2018).

Annonaceous acetogenins (ACGs) are fatty-acid derived natural products found only in species of the Annonaceae family. More than 400 ACGs obtained mainly from roots, stems,

seeds and leaves have been described in the last few decades. These compounds contain a long aliphatic fatty acid chain with 35–37 carbon atoms, derived from the polyketide pathway, a terminal methyl-substituted α, β-unsaturated γ-lactone ring and either none, one, two or three tetrahydrofuran (THF) rings that can be adjacent or not adjacent to one another. Furthermore, several hydroxyl groups are present in different positions, frequently alongside the THF rings. Overall, the molecular structure comprises two functional moieties: the polar core and the terminal lactone (Gutierrez *et al*., 2020).

Role of acetogenins in apoptosis

Acetogenins are molecules with great potential for future cancer therapy. Their most prominent biological activity is inhibition of the mitochondrial Complex I due to their bis-THF structure. Indeed, it was previously reported that the mono-THF AAs bearing an alkyl chain that links the lactone moiety with the THF group are noncompetitive inhibitors of Complex I (i.e., NADH: ubiquinone oxidoreductase) in the respiratory chain, which leads to a blockade of phosphorylative oxidation and a subsequent decrease in ATP production. Such inhibition involves a large group of pathways that can induce cell death, including apoptosis and autophagy, or act in other metabolic networks as inhibitors of the lactate dehydrogenase A enzyme, as an antioxidant, or by arresting the cell cycle (Herrera *et al*., 2019). Fuentes *et al*. (2019) reported that acetogenins are able to cause the depolarization of mitochondrial membrane in whole cells, and able to induce cell death through late apoptosis and necrosis.

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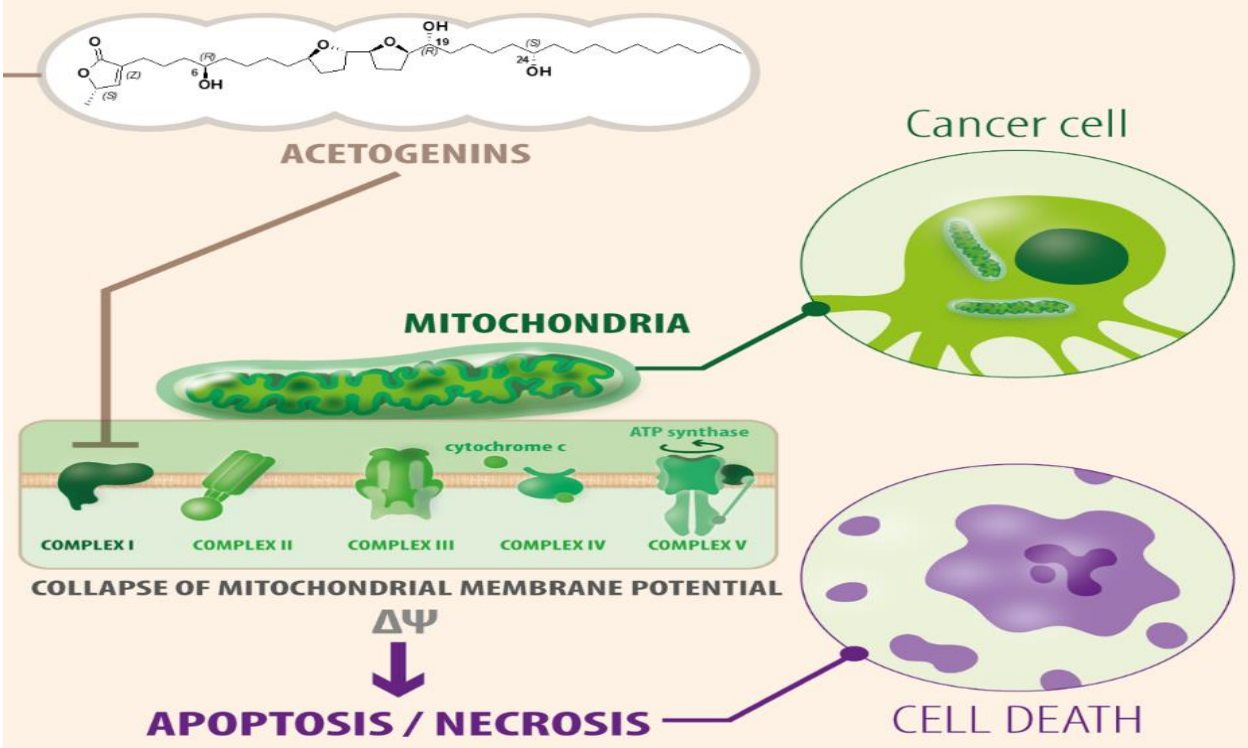
Figure 7: Role of Acetogenins in apoptosis (Fuentes *et al*., 2019)

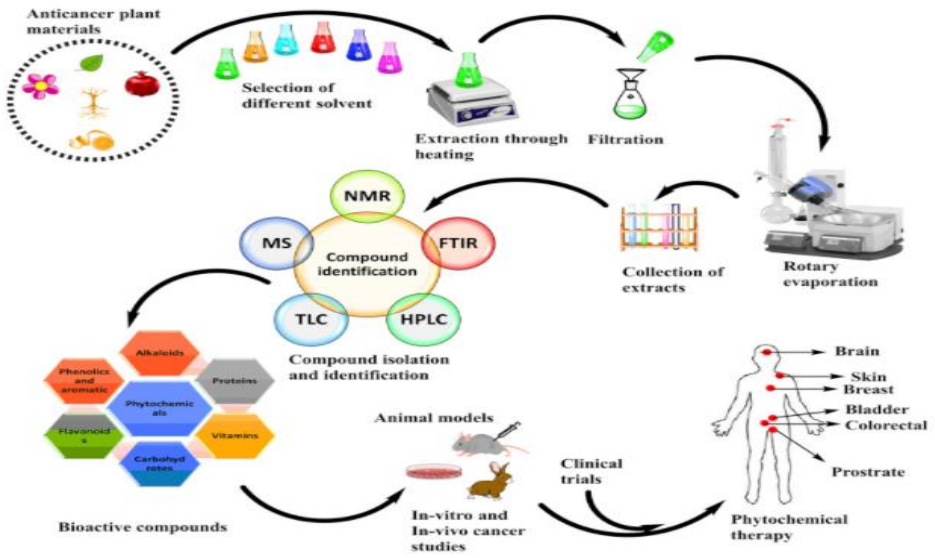


Different strategies for the development of anticancer phytochemicals

The potential of medicinal plants as therapeutic agents depends upon the quality and quantity of active phytochemicals in them, which vary with latitude, longitude, altitude, age, climate and season from species to species. Pharmacological functions and their level vary with plant parts. These bioactive phytomolecules can also be used in anticancer therapeutics but they still demand further research. The purification of active phytomolecules may involve various strategies such as combinatorial chemistry, isolation assays, and bioassay-guided fractionation. Bioassay guided fractionation with various analytical techniques could be used to separate various bioactive compounds from the mixture of compounds. The process begins with the natural extracts test (from dry/wet plant material) with confirmed biological activity. Then, suitable matrices are used for the fractionation of active extracts, tested for bioactivity and various analytical techniques such as TLC, HPLC, FTIR, Mass spectroscopy and NMR must be used for the separation of active fractions. Superdex, Sephadex, Silica or any other suitable matrix can be used for fractionation. There are so many dyeing agents used for the detection of

natural compounds in medicinal plants. These procedures could be change however purity, quality and quantity of the bioactive compounds should be high as much as possible and this can be achieved by using high quality of solvents, matrices and careful handling. After purification of these phytomolecules they must be examined for *in vitro* or *in vivo* anticancer effects. If a better anticancer property is achieved by the molecule, then other aspects like pharmacokinetics, pharmacodynamics, immunogenicity, metabolic fate, biosafety and side effects, drug interactions, dose concentration must be researched for future drug designing (Iqbal *et al*., 2017).

Figure 8: Scheme of anticancer phytochemical synthesis, optimization, characterization and prospective use as cancer therapeutic agent (Iqbal *et al*., 2017)



Cancer

Cancer is a disease characterized by uncontrolled cell growth and proliferation. Although overall survival term is increased slightly due to early detection, cancer-related mortality is the second biggest cause of death worldwide. The International Agency for Research on Cancer (IARC) reported that 14.1 million new cancer cases and 8.2 million deaths took place worldwide in 2012, and 21.7 million cancer incidences and 13 million deaths were predicted in 2030 (Kim *et al*., 2018).

Leukemia

Leukemia (blood cancer) begins in the bone marrow and causes the formation of a large number of abnormal cells. The most common types of leukemia known are Acute lymphoblastic leukemia (ALL), Acute myeloid leukemia (AML), Chronic lymphocytic leukemia (CLL) and Chronic myeloid leukemia (CML) (Jagadev *et al*., 2017). Globally, noncommunicable diseases (NCDs) accounted for 71% of total deaths. In India, NCDs were estimated to account for 63% of all deaths, and cancer was one of the leading causes (Mathur *et al*., 2020).

Acute Lymphoblastic Leukemia

Acute lymphoblastic leukemia (ALL) is a malignant transformation and proliferation of lymphoid progenitor cells in the bone marrow, blood and extramedullary sites. While 80% of ALL occurs in children, it represents a devastating disease when it occurs in adults. The incidence of ALL follows a bimodal distribution, with the first peak occurring in childhood and a second peak occurring around the age of 50 (Terwilliger *et al*., 2017). Leukemia accounts for 40–50% of childhood cancer burden in India with acute lymphoblastic leukemia (ALL) being the commonest type (Ganguly *et al*., 2021). The rate of new cases of leukemia was 14.3 per 100,000 men and women per year. The death rate was 6.1 per 100,000 men and women per year. These rates are age-adjusted and based on 2014–2018 cases and 2015–2019 deaths and estimated 61, 090 new cases and 23, 660 deaths by the year 2021 (National Cancer Institute).

T-cell Acute Lymphoblastic leukemia

T cell acute lymphoblastic leukemia (T-ALL) is an aggressive hematologic malignancy arising from early T cell progenitors. It accounts for approximately 15% of pediatric and 25% of adult ALL cases, and is twice as prevalent in males as in females. The World Health Organization (WHO) classifies T-ALL and T cell lymphoblastic lymphoma (T-LL) together despite differences in their clinical presentation. T-ALL typically presents with leukocytosis and extensive bone marrow involvement and frequently shows a mediastinal thymic mass and meningeal infiltration of the central nervous system. T-LL, on the other hand, accounts for 1–2% of non-Hodgkin lymphoma and frequently presents as a large mediastinal mass with limited bone marrow involvement. It is distinguished from T-ALL somewhat arbitrarily by the presence of less than 20% marrow blasts (Fattizzo *et al*., 2020 and Hefazi and Litzow, 2018).

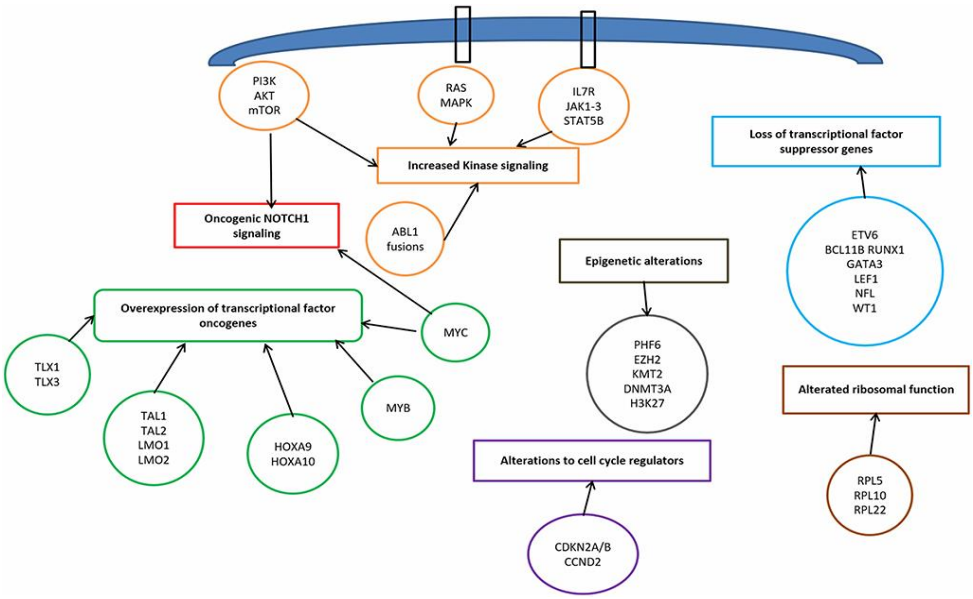
Types of T-cell Acute Lymphoblastic leukemia

T-ALL can be divided into subcategories according to different stages of intrathymic differentiation, including pro-T, pre-T, cortical-T, and medullary (mature)-T-ALL. Lymphoblasts in T-ALL are TdT-positive, and typically express cytoplasmic CD3, which is the only lineage-specific marker for lymphoblastic T cell disease. Other T cell markers including CD1a, CD2, CD4, CD5, CD7, and CD8 are variably expressed according to the degree of T cell differentiation (Hefazi and Litzow, 2018).

Molecular Pathways Involved in T-ALL Pathophysiology

T-ALL results from a multistep transformation process in which the accumulation of genetic alterations affects key oncogenic/tumor suppressors pathways, that are responsible for proliferation, survival and differentiation of T-cells. The molecular steps involved in T-ALL pathogenesis encompass: transcriptional deregulation of oncogenes/onco suppressors, NOTCH1 signaling, cell cycle deregulation, kinase signaling (including IL7R-JAKSTAT pathway, PI3K/AKT/mTOR pathway, RAS/MAPK signaling pathway, ABL1 signaling pathway), epigenetic deregulation, ribosomal dysfunction, and altered expression of oncogenic miRNAs or long non coding RNA (Fattizzo *et al*., 2020).

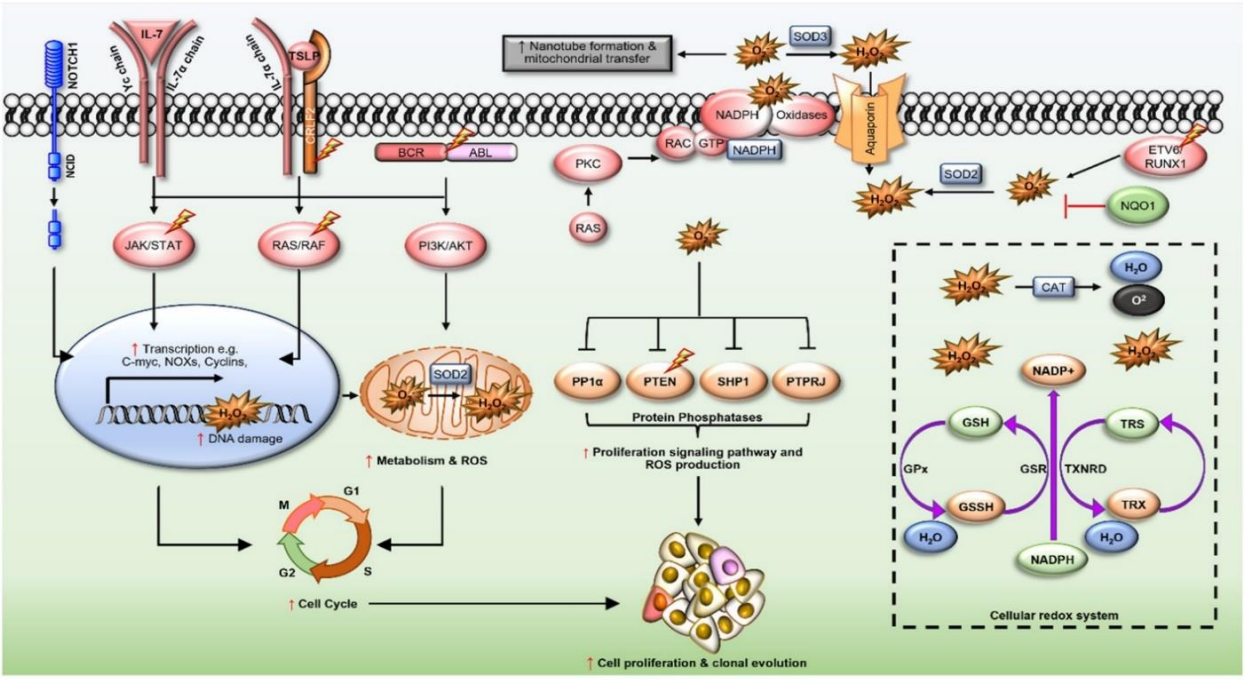
Figure: 9 Signaling pathways involved in T-cell acute lymphoblastic leukemia pathophysiology (Fattizzo *et al*., 2020)



ROS in Acute Lymphoblastic Leukemia

Reactive oxygen species are elevated in a range of cancers and are emerging as significant contributors to the leukaemogenesis of ALL. ROS modulate the function of signaling proteins through oxidation of cysteine residues, as well as promote genomic instability by damaging DNA, to promote chemotherapy resistance. Recurring somatic mutations to *CRLF2*, *JAK*, *NQO1*, *NOTCH1* and *RAS*, chromosomal translocations such as BCR/ABL and ETV6/RUNX1 as well as overexpression of CRLF2 and IL7 receptor drive excessive production of intracellular reactive oxygen species production (superoxide- $O_2^{\cdot -}$ and hydrogen peroxide- H_2O_2) in acute lymphoblastic leukemia (ALL). High-level ROS production drives redox signaling through oxidative post translational modifications that increase the activity of kinases and inactivate protein tyrosine phosphatases, and cause lipid peroxidation and genomic instability leading to leukemia progression and chemotherapy resistance (Mannan *et al*., 2021).

Figure: 10 Signaling pathways linked to reactive oxygen species production in acute lymphoblastic leukemia (Mannan *et al*., 2021)



Side effects of chemotherapy and radiotherapy

The side effects of chemotherapeutic agents and irradiation are closely related to the special properties of the substances, which are prone to accumulate in a particular anatomic region such as the cardiovascular system, kidneys, liver, or lungs, leading to cardiotoxicity, nephrotoxicity, hepatoxicity, or pulmonary fibrosis. As many anticancer drugs target DNA in proliferating cells, their side effects are primarily manifested in tissues with high-proliferative cells such as bone marrow and gastrointestinal tract, and result in gastrointestinal toxicity, myelosuppression, and immunosuppression. Further, chemotherapeutic drugs and irradiation are known to induce oxidative stress and inflammatory response, and they are closely associated with chromosomal instability and secondary tumor generation. Therefore, a better understanding of the mechanisms that underlie the induction of the side effects can facilitate the design of supportive or adjuvant therapies for combating them (Liu *et al*., 2020).

Plant secondary metabolites for cancer therapy

Secondary plant metabolites reveal numerous biological activities making them attractive as resource for drug development of human diseases. As the majority of cancer drugs clinically established during the past half century is derived from nature, cancer researchers worldwide try to identify novel natural products as lead compounds for cancer therapy. Natural products are considered as promising cancer therapeutics, either as single agents or in combination protocols, to enhance the antitumor activity of additional therapeutic modalities. Most natural compounds exert pleotropic effects and modulate various signal transduction pathways. A better understanding of the complex mechanisms of action of natural products is expected to open new perspectives in coming years for their use alone or in combination therapies in oncology. Two major strategies to identify novel drug candidates from nature are the bioactivity-guided fractionation of medicinal plant extracts to isolate cytotoxic chemicals and the identification of small molecules inhibiting specific targets in cancer cells (Fulda *et al*., 2020).

Humans have turned to natural products, obtained from plants, animals and aquatic life for treating diseases since time immemorial. Modern medicine is based on ancient wisdom transferred over generations. Drug development relies mainly on natural sources. Herbal medicines are making a comeback due to lower side effects, and positive results in the long term when compared to synthetic drugs. The current drug discovery process relies on identifying

traditional medicines followed by Bioactivity-guided fractionation to isolate significant lead molecules. Plants have a history of long-term use by humans and hence it can be presumed that the bioactive compounds obtained from plants will have low human toxicity. There exists a huge potential for discovering new antitumor drug leads by screening natural products either in the form of crude extracts purified phytochemicals (Mohan, 2020).

Role of phytotherapy in leukemia

Management in contemporary medicine includes chemotherapy (the use of anticancer drugs), radiation therapy (in which the high-energy radiation is used to kill cancer cells), and bone marrow transplant (in which transplantation of blood-forming stem cells is done after high doses of chemotherapy and radiation therapy as required) along with other supportive treatment as platelet transfusion to control bleeding. Conventional medical science manages the disease quite effectively by targeting the signaling pathways causing leukemogenesis, but with serious side effects as it also damages healthy cells, leading to low survival rate and rate of cure (Chauhan *et al*., 2022).

Annona muricata

Annona muricata is a small deciduous tropical evergreen fruit tree, belonging to the Annonaceae family and also known as soursop, graviola and guanabana, is widely grown and distributed in tropical and subtropical regions around the world, including India, Malaysia and Nigeria. A. *muricata* is an evergreen, terrestrial, erect tree reaching 5–8 m in height and features an open, roundish canopy with large, glossy, dark green leaves. The edible fruits of the tree are large, heart-shaped and green in color, and the diameter varies between 15 and 20 cm The aerial parts of *Annona muricata* have several functions: the fruits have been widely used as food confectionaries, while several preparations, especially decoctions of the bark, fruits, leaves, pericarp, seeds, and roots, have been extensively used in traditional medicine to treat multiple ailments including cancers by local communities (Rady *et al*., 2018).

Scientific Classification

Kingdom	- Plantae
Division	- Spermatophyta
Division	- Magnoliophyta

Class - Magnoliopsida
Subclass - Magnolidae
Order - Magnoliales
Family - Annonaceae
Genus - *Annona*
Species - *Annona muricata*
Common names - Soursop, Graviola, Guanabana

Phytochemistry of *Annona muricata*

Phytochemical studies have been extensively carried out on different parts of A. *muricata* and till date, 212 secondary metabolites have been isolated and identified, such as acetogenins, alkaloids, phenolic compounds, and megastigmanes. A. *muricata* leaves have been reported to be a rich source of annonaceous acetogenins, a unique group of derivatives of long chain fatty acids derived from the polyketide pathway that belong to the family of Annonaceae. Thorough investigations have been carried out on the leaves of A. *muricata* as the leaves are the most utilized parts used for a wide array of ethnomedicinal uses (Wahab *et al*., 2018).

Plant-derived compounds exhibiting anti-cancerous activity

In light of increasing resistance to chemo- and radiotherapy along with the toxicity of these traditional therapies, a new, non-toxic anticancer treatment is needed. Additionally, chemotherapeutic agents are toxic to both normal cells and tumor cells. The optimal therapy would be able to differentiate between the cell types. Plant-based or -derived compounds are typically non-toxic to normal cells. For over 5000 years, plants have been utilized as medicines and therapies; a quarter of all modern medicine is directly or indirectly derived from plants. *Annona muricata* is a fruit tree that has been used in both alternative and traditional medicine for a wide variety of ailments, particularly for its anticancer properties. It has been shown to inhibit BCL-2 proteins while increasing BAX and promoting apoptosis. The mechanism *Annona muricata* uses is still unknown, however, it is a potential anticancer treatment due to its nontoxicity towards healthy cells. This makes *Annona muricata* an exciting new possible therapy especially in comparison to the current treatments of chemo- and radiotherapy (Pfeffer and Singh, 2018).

Combination of drugs

Current chemotherapeutic agents can lead to many adverse effects and can be toxic to healthy cells. For this reason, the identification of new agents that can effectively eradicate tumorigenic cells without damaging normal cells is necessary. A possible solution could be the use of combinations of drugs. Indeed, the efficacy of treatment could be improved, as individual drugs can target different biological pathways. Moreover, combinations of drugs could also potentially reduce drug resistance (Cava and Castiglioni, 2020).

Synergistic potential of nutraceutical

Combinations of nutraceutical can trigger new mechanisms that are not activated by either compound alone, thus leading to synergistic benefits. The synergistic effect is characteristic of the enhancement of individual functions in the presence of other compounds. Different combinations of nutraceutical compounds are proven to show improved therapeutic effects in terms of potent anti-oxidant, anti-inflammatory, anti-obesity, anti-cancer, and cardio-protective effects, amongst others. Several nutraceutical compounds exhibit anti-cancer effects by acting against cell proliferation, metastasis, and apoptosis through different mechanisms. More than individual compounds, nutraceutical in combination play an effective role in treating cancer with their synergistic effects (Leena *et al*., 2020).

Mechanical Bases of Synergistic Effects

1. **Synergistic Multi-Target Effects -** A compound, a mixture or a plant extract may act not only, as expected, on a single target, but may impact on different targets; i.e., all functional or structural cell constituents, such as metabolites, receptors, enzymes, ion channels, transporters, nucleic acids, ribosomes, and proteins.
2. **Modulation of Pharmacokinetic or Physicochemical Effects -** Synergistic effects may also impact on the physicochemical properties, including solubility, of a compound or mixture, providing an improvement of the bioavailability.
3. **Interference with Resistance Mechanisms -** Synergistic effects may be observed on drug-resistant microorganisms (bacteria, fungi) or cancer cells, the presence of natural derivatives that may antagonize the development of drug resistance (supplied together with antibiotics or cancer drugs).

4. **Elimination or Neutralization Potential -** A compound or a plant extract may have the ability to remove or neutralize the toxic effect of a drug (synthetic or not), even reducing or nullifying its adverse effects and resulting in treatment amelioration (Pezzani *et al*., 2019).

Role of apoptosis in cancer therapy

Apoptosis is a process of the cell's natural mechanism for death which occurred in multicellular organisms to maintain tissue homeostasis and act as a defensive strategy to remove infected, damaged or mutated cells. Apoptosis also known as programmed cell death is a highly regulated energy-dependent process that occurs normally during development and aging. Apoptosis can be triggered through two major pathways, either mitochondrial- or death receptor-mediated pathways resulting from the intracellular (e.g., stress, DNA damage) and extracellular signals (death-inducing signals by other cells), respectively. This machinery mainly depends on caspases cascade for executing cell death, that eventually because of the proteolytic cleavage of thousands of target proteins within the cells that are essential for normal cellular function such as cytoskeletal and nuclear proteins. Consequently, the apoptotic cells undergo a series of morphological and biochemical alterations leading to recognition by macrophages and cell phagocytosis. Moreover, B-cell lymphoma-2 (Bcl-2) family of proteins has long been identified for their significant involvement in regulating the cellular program of apoptosis through mitochondrial outer membrane permeabilization, as the critical decision-point at which cells commit to death, representing their vital role in the protection against cancer (Farghadani and Naidu. 2021).

Role of Apoptosis in Leukemia

Programmed cell death, or apoptosis, is a process generally characterized by distinct morphological changes. It is mediated through energy-dependent biochemical mechanisms. Relapse in childhood ALL is associated *in vivo* with a lower Bax/Bcl-2 ratio, and a loss of spontaneous processing of caspase-3. Therefore, tumor-inhibiting effects can be exerted mainly through apoptosis. Mitochondria plays a key role in a number of cellular pathways, including the apoptosis pathway, reactive oxygen species production and cell death induction. Mitochondria is an important organelle, which regulates many cellular pathways in mammalian cells. The

dysfunction of apoptosis is common in cancer. Therefore, mitochondria is a target for anticancer treatment. During apoptosis, the mitochondrial membrane is depolarized, leading to a drop in the membrane potential. The loss of MMP increases the permeability of its outer membrane. Consequently, the leakage of mitochondrial membrane releases mitochondrial apoptosis factors, including cytochrome c, apoptosis–inducing factor and endonuclease G, leading to the activation of caspase-cascade. Regulating mitochondrial functions is a therapeutic strategy which stops oxidative phosphorylation and releases proapoptotic proteins, like cytochrome c, Bcl-2 family, Bak and Bax. Bcl-2 family proteins, such as *BCL-2, BCL-XL, BAX* and *BAK* are important proteins responsible for regulating pro- and anti-apoptotic effects. Among them, Bcl-2 is the most important anti-apoptotic protein and Bax is a pro-apoptotic protein. Remission failure of acute leukemia cases are closely related to high Bcl-2 / Bax ratios. This Bcl-2 / Bax ratio is an important parameter in ALL. When comparing ALL patients and healthy controls, polymorphism within the Bcl-2 promoter region is more reliable than that within the Bax promoter region for estimating the survival time of ALL patients (Huang *et al*., 2021).

Methods of Detection of Apoptosis

1. **Morphological Analysis** - Conditions such as shrinkages, membrane blebbing, at the initial stages of apoptosis can be evident, these changes can be monitored with varying forms of microscopy which is directly dependent on the nature of cells or tissues. Among these, are the Light Microscopy, Fluorescence Microscopy, Electron Microscopy, and Phase Contrast Microscopy. 2. **Biochemical Analysis** – **a) DNA fragmentation analysis -** This phenomenon is mostly examined by Agarose gel electrophoresis. DNA fragment cleaved into an inter base pairs of about 180-200, are observed as ladder upon electrophoresis. This method can be divided into Conventional gel electrophoresis, Pulse field gel electrophoresis, Field inversion gel electrophoresis and Single cell gel electrophoresis. Also, Terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) is another means of determining DNA fragments as an *in-situ* labelling of 3-OH ends and allows analysis of frozen and fixed sections. Other approaches require the labelling with E-coli polymerase. Lastly in cell cultures, inter-nucleosomal DNA fragmentation can be analyzed by enzyme-linked immunosorbent assay (ELISA). **b) Changes in the Organelles and Cell Membrane -** Annexin V. FITC assay allows the use of Annexin V accompanied by fluorescent labelling, example FITC and a fluorescence microscopy detection or

an augmentation with other method such as flow cytometry following the labelling process. Fluorescence microscopy can give false positives, it can therefore be replaced with the flow cytometry. This makes the flow cytometry a promising tool in the analysis of apoptosis, allowing the accessibility of results within a short time. Western Blotting also ascertains the presence of apoptosis related proteins e.g., the Bcl-2 family protein with other apoptosis related proteins, the state and translocation of some molecules such as the cytochrome from the mitochondria to the cytoplasm. **C) Caspase Activity** - This can be analyzed using either an immunology or immunohistochemical method (Obeng. 2020).

***In-silico* analysis**

Cancer develops, when cell growth exceeds cell death following a loss in control of the fundamental cellular checkpoints required to maintain healthy tissue turnover which results in tumour formation. In healthy cells, apoptosis is promoted by various stress signals, most prominently regulated by the B cell lymphoma 2 (Bcl-2) family of proteins but cancer cells are more resistant to apoptotic cell death. There are two major ways in which apoptosis can be induced. The first is the activation of the intrinsic apoptotic pathway (also known as the mitochondrial apoptotic pathway) and the second involves activation of the extrinsic pathway. The intrinsic apoptotic pathway is controlled by the Bcl-2 superfamily (such as Bcl-2, Bcl-XL, Mcl-1), initiates the release of pro-apoptotic proteins from the mitochondrial intra-membrane space including the release of cytochrome c which triggers caspase 9 followed by downstream activation of effector caspases 3, 6, and 7 (Rathore *et al*., 2017).

NOTCH1, a well-known oncogene in T-cell acute lymphoblastic leukemia (T-ALL), encodes a class I transmembrane protein functioning as a ligand-activated transcription factor and playing an important role in cell differentiation, proliferation, and apoptosis (Fabbri *et al*., 2017).

MDM2 is a well-known oncogene which affects expression of various genetic factors. It encoded oncoprotein *Mdm2* of 491 amino acids divided into four major domains (p53-binding, acidic, Zn-finger and ring finger). Acting as an E3-ubiquitin ligase, Mdm2 induced degradation of various proteins which affect the cellular transformation such as p53, p73, JMY, FOXO3a, HPIP, HDAC, $p21^{Waf1/CIP1}$ etc. Thus, Mdm2 found to be responsible for up and down regulation

proteins in cellular system and plays crucial role in several human cancers through variety of biological pathways (Nayak *et al*., 2017).

The transcription factor NF-κB is a key player in inflammation, cancer development, and progression. NF-κB stimulates cell proliferation, prevents apoptosis, and could promote tumour angiogenesis as well as metastasis. Extending the commonly accepted role of NF-κB in cancer formation and progression, different NF-κB subunits have been shown to be active and of particular importance in distinct types of cancer (Kaltschmidt *et al*., 2018)

Molecular docking is an established *in silico* structure-based method widely used in drug discovery. Docking enables the identification of novel compounds of therapeutic interest, predicting ligand-target interactions at a molecular level, or delineating structure-activity relationships (SAR), without knowing the chemical structure of other target modulators. Although it was originally developed to help understanding the mechanisms of molecular recognition between small and large molecules, uses and applications of docking in drug discovery have heavily changed over the last years (Pinzi and Rastelli, 2019).

Molecular docking in the pharmaceutical industry is a powerful *in silico* approach for discovering novel therapies for unmet medical needs predicting drug–target interactions. It not only provides binding affinity between drugs and targets at the atomic level, but also elucidates the fundamental pharmacological properties of specific drugs. Historically, natural products, which contain a wide range of compounds for drug discovery, have been considered in multiple clinical trials, especially as anticancer and antimicrobial agents. The advantage of natural products with respect to synthetic compounds is that they are also metabolites. Therefore, they are biologically active and can also be substrates for transporter systems. The use of *in silico* (computer-based) modelling in the search for lead compounds is a promising endeavour in drug discovery, since it often accelerates the process and cuts down costs. Virtual screening methods are useful because they narrow down the number of compounds to be actually tested in biological assays. *In silico* scoring methods are sufficiently able to discriminate between active and inactive metabolites (Cava and Castiglioni, 2020).

Effective and safe drugs exhibit a finely tuned combination of pharmacodynamics (PD) and pharmacokinetics (PK), including high potency, affinity and selectivity against the molecular

target, along with adequate absorption, distribution, metabolism, excretion and tolerable toxicity (ADMET) (Ferreira *et al*., 2019).

The layout of the study and the methods used to meet the objectives drawn for the study, are explained in the next chapter.