Second Order Targeting using Nanocarriers of Phytoformulation

C.ESWARIPAVITHRA

(20PBT006)

Under the guidance of

Dr. R. Nirmaladevi

Assistant professor

A Thesis submitted in

Partial fulfilment of the

Degree of Master of Science in Biotechnology

Avinashilingam Institute for Home Science and Higher Education for Women

Coimbatore – 641043

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C.ESWARIPAVITHRA

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INTRODUTION

Cancer is the leading cause of death in the world. It is a group of diseases that can begin in any tissue or organ and spread to other parts of the body when abnormal cells grow uncontrollably and migrate from their original sites. These abnormal cells grow into a lump-or tumor. According to a World Health Organization survey conducted in 2022, cancer is estimated to be the first or second leading cause of death in 50-70 countries and the third or fourth leading cause of death in 20 countries (WHO, 2022).

Metastasis is the process by which cancer cells spread from their original site to secondary sites in the body, most commonly through the bloodstream or lymphatic system. Treatment cannot cure metastatic cancer in the majority of cases. As a result, metastasis is the leading cause of cancer mortality, accounting for more than 90% of all cancer deaths. (Jiramongkol *et al.*, 2020).

Leukemia may be a hematological clutter, caused by multiplying white blood cell-forming tissues coming about in a checked increment in circulating juvenile or abnormal hematopoietic cells with impeded separation, control, and modified cell death (apoptosis). The identified causes of leukemia are introduction to expansive dosages of ionizing radiation, certain synthetic agents, and disease with particular viruses (e.g. Epstein-Barr infection, human lymphotropic infection). Some forms of leukemia are more common in children. Leukemia is classified based on clinical behavior (intense or inveterate) and sort of the essential hematopoietic cell line affected (myeloid or lymphoid). The four vital demonstrative categoriesare: Acute Myeloid Leukemia (AML), Acute Lymphoblastic Leukemia (ALL),

Chronic Myeloid Leukemia (CML) and, Chronic Lymphocytic Leukemia (CLL) (Deliverska and Krasteva, 2013).

Acute Lymphoblastic Leukaemia is the most frequent type of cancer in children, accounting for around 25% of all malignancies in children under the age of 15. ALL is more common in patients with blastic transformation of T and B cells (Chennamadhavuni, 2021). In the vast majority of instances, ALL is only curable in affluent countries. Due to a lack of facilities to carry out modern treatment procedures, developing countries are still having trouble treating ALL (Abdelmabood *et al.*, 2020).

Free radicals are defined as atoms or molecules with at least one unpaired electron. They are very reactive because they contain unpaired electrons. They can give or receive electrons, making them either reductants or oxidants (Bo *et al*., 2019). They participate in a variety of cellular processes, including redox system disruption, DNA damage, and the activation of procarcinogens, all of which lead to cancer (Maddu, 2019). Free radicals are divided into two types: reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS are oxygen-derived free radicals, while RNS are nitrogen-derived free radicals. Hydroxyl radicals (•OH), peroxyl radicals (ROO•), superoxide anion ($O•_2$), alkoxyl radicals (RO•), singlet oxygen ($^1O_2$), and hydrogen peroxide are examples of ROS (Ramos and Muriel, 2019). Nitric oxide (NO•) is the major RNS involved in numerous free radical-related diseases. Excessive generation of these harmful free radicals in the body causes oxidative stress, which can result in a variety of ailments (Masuko *et al*., 2021).

Various anticancer drugs or agents have been used for cancer management in recent years, including hormone therapeutics, alkylating agents, biological response modifiers, antibiotics, platinum compounds, antimetabolites, and mitotic inhibitors. The main disadvantage of these anticancer agents is that they cause fatigue, immunosuppression, liver damage, severe nausea and vomiting, loss of appetite, abdominal pain, back pain, and weakness (Sagbo and Otang, 2021). The primary cause of these side effects is a lack of appropriate specificity and sensitivity.

As a result, an anticancer drug with few or no side effects is the live need of current situation (Schirrmacher, 2019).

Antioxidants are molecules that act as a protective shield for the body, preventing free radical damage. The actual mechanism of antioxidants is that they share their extra electrons with free radicals, preventing them from causing damage to cellular components (Jamshidi *et al.,* 2020). On the basis of their nature, antioxidants are divided into two major categories: enzymic antioxidants and non-enzymic antioxidants. Catalse, glutathione peroxidase, superoxide dismutase, and glutathione reductase are examples of enzymic antioxidants. Flavonoids, ascorbic acid, carotene, and tocopherol are the most important non-enzymatic antioxidants. To maintain a healthy homeostasis, the body must always maintain a balance of free radicals and antioxidants (Anju *et al.*, 2019). Natural antioxidants are abundant in plant parts such as barks, roots, leaves, nuts, seeds, vegetables, and fruits. These phytochemicals or secondary metabolites act as natural antioxidants (Lee *et al.*, 2020).

Plants are used in ancient ayurveda and Indian traditional medicine to treat a variety of ailments. Because of the abundance of phytochemicals and their derivatives, the plants have medicinal properties. One of these phytochemical's important properties is that they can act as anticancer agents. These phytochemicals are biologically active compounds found in nature that have significant anticancer potential. The main benefit of these phytochemicals is that they have very few or no side effects. As a result, purifying these phytochemicals from plants and using them as anticancer drugs will be the most effective, side-effect-free cancer treatment (Choudhari *et al.*, 2020).

Since the last century, nanotechnology has grown in popularity among scientists. Scientists have brought about numerous revolutions in the field of nanotechnology. Nanoparticles are materials with a diameter of less than 100 nm. The main advantage of these nanoparticles is their small size, which influences the physiochemical properties of a substance. As a result, different sized nanoparticles differ in shape, size, and colour (Ibrahim *et al*., 2019).

INTRODUTION

Nanoparticles can be synthesised in a variety of shapes, sizes, and dimensions, resulting in a wide range of physiochemical properties. Nanoparticles can be customised in a variety of shapes depending on the application, such as wires, rods, sheets, and particles (Modi *et al*., 2022). Based on the chemical composition used for the preparation of nanoparticles they are divided into four types: i) Organic nanomaterials include nanoconjugates, hydrogels, polymerosomes, dendrimers and micelles; ii) Inorganic nanomaterials include ceramic nanomaterials, metal oxides and metals; iii) Nanostructures Composite; iv) Carbon based nanomaterials which include graphene, carbon nanotubes, carbon nanofibres and fullerenes (Gaur *et al.,* 2021 and Sawy *et al.,* 2021).

Metal nanoparticles have attracted researchers attention due to their wide range of unique properties compared to bulk metals, and they have a wide range of applications in diagnostics, cell labelling, antimicrobial agents, drug administration, and cancer therapy (Krishnan *et al*., 2015). Silver nanoparticles (AgNPs) have received a lot of attention because of their unique physicochemical properties, such as chemical stability and electrical conductivity, as well as biological properties such as antibacterial, antifungal, anti-inflammatory, antiviral, antiangiogenesis, anticancer, and antiplatelet activities (Al-Sheddi *et al.*, 2018). Because of their ease of surface modification and production, robustly augmentable and adaptable visual features, and improved biocompatibility, AgNPs have gained the interest of cancer researchers (Pei *et al.,* 2019).

For the synthesis of silver nanoparticles, numerous and less expensive approaches are available, including chemical, physical, electrochemical, and sonochemical processes (Khandel *et al.*, 2018; Fahmy *et al*., 2019). However, the fundamental downside of these methods is that they are toxic to the environment, particularly when chemicals are used in the synthesis, and that silver nanoparticles generated using these methods would agglomerate readily due to their monodispersity (Gulati *et al.*, 2018). Among the various methods available for the synthesis of silver nanoparticles, the plant mediated green synthesis is found to be very effective, eco- friendly, easy to carryout and cost effective. The phytochemicals present in the plant

extracts acts as a capping and reducing agent that prevents them from agglomeration and this makes them very stable for a longer period (Bukhari *et al.,* 2019).

Liposomes are spherical vesicles that are composed of a lipid bilayer. Liposomes are primarily composed of cholesterol and phospholipids. They can encapsulate drugs that are both hydrophilic and hydrophobic (Beltrán *et al*., 2019). Liposomes are effective carriers because their phospholipid bilayer resembles the mammalian cell membrane, allowing for efficient cellular uptake. As a result, liposomes can reach cells at high concentrations, reducing unnecessary side effects and increasing efficacy (Gonda *et al.*, 2019).

Liposomes are classified into four types based on the number of phospholipid bilayers present. There are four types of vesicles: unilamellar, multilamellar, oligolamellar, and multivesicular vesicles. Liposomes can be prepared using a variety of techniques, including injection, microfluidic, heating, reverse phase evaporation, hydration methods, detergent depletion, thin film hydration, membrane extrusion, and free drying, all of which are well documented (Has and Sunthar, 2020). Liposomes with sizes ranging between 50 and 200 nm are preferred for drug delivery applications. The size of the liposomes has a significant impact on drug delivery to the targeted cells. Liposomes smaller than 200 nm have improved drug release, a longer circulation time, and increased accumulation at the target site. Liposomes can be administered via a variety of routes, including oral, ocular, and intravenous, to treat a wide range of diseases (Leitgeb *et al.*, 2020).

Pharmaceutical scientists and physicians are currently focused on improving the safety and efficacy of medication therapy. To this goal, medication targeting is the most effective strategy. This is important for some diseases, such as cancer treatments, which require a delicate balance between the destruction of diseased and healthy tissues, as well as immune system harm and quickly proliferating cells. Newly developed nanotechnological methods as active targeting carrier in the medical treatment have increased attention. Attaching different ligands to the drug or drug delivery system, such as antibodies, biological proteins, peptides, sugars, and vitamins,

or a specific ligand, causes the drug to meet and form a complex with cell receptors, causing the drug to accumulate in the target cells. In Second-order targeting or cellular targeting: When a drug delivery system releases the drug to a particular cell within an organ or a tissue (Derakhshandeh *et al*., 2019)

The plant kingdom is a source of drugs, and there is a growing awareness of the importance of medicinal plants. Plants with traditional medicinal value are being tested for their potential as drugs for the treatment of a variety of diseases. Plants are a rich source of natural antioxidants and antimicrobials, which could be used as a drug in modern biomedicine. Because of the presence of secondary metabolites such as phenols, flavonoids, and terpenoids, several plants have been reported to have significant free radical scavenging ability (Venkatesan *et al.,* 2017).

It is commonly referred to as Ashwagandha. It belongs to the Solanaceae family and is a subtropical bush native to the Mediterranean, Africa, and India. It contains Withanolides, Withaferins, anferine, isopellertierine, and sitoindosise. Due to its therapeutic properties, clears out and roots have been utilized within the Indian conventional framework of pharmaceutical and promoted all inclusive. Extricate of *Withania somnifera* balances different natural reactions. It has been utilized in different arrangements for its anti-stress, anti-ageing, anti-peroxidant, anti-tumor, cardiotonic, and immunomodulatory properties. The most abundant components of this plant are Withanolide A and Withaferin A. Withaferin A produces quick apoptosis within the cancer cells. Detailing of *Withania somnifera* appeared acceptance in cell cytotoxicity in different human cancer cell lines. *Withania somnifera* detailing too up directs populace of T cell populace in mice (bearing tumor) with expanded expression of IL-2 and IFN-gamma levels (Roy and Bharadvaja, 2017).

Terminalia bellirica (Gaertn.)Roxb. Extract (TBE) is obtained from the fruit of *T.bellirica* tree, which is found across Southeast Asia and is used as a folk medicine for diabetes, rheumatism, and hypertension in traditional Indian Ayurvedic Medicine. Multiple studies have suggested antibesity, hypoglycemic, hypolipidemic, and antihypertensive properties of the fruit. The major polyphenolic compounds of this fruit are reported to be gallic acid (GA), ellagic acid (EA), and gallate esters.GA has been shown to exert curative effects against obesity related atherosclerosis and insulin resistance via the activation of AMPK. In THP-1 macrophages, TBE suppressed inflammatory mediator erpression and ROS production, although little is known about TBE's anti-inflammatory and antioxidant properties, as well as the underlying processes in this process. This study examined protective effects of TBE and its major bioactive ingredients on inflammation and oxidative stress, as well as the underlying molecular mechanism, by utilizing LPS-stimulated macrophages and LPS-shock model mice (Gupta *et al.,2*020).

The current study is designed to investigate the following hypothesis:

- The null hypothesis (H0) states that *Withania somnifera* and *Terminalia bellirica* silver nanoparticles loaded liposomes lack anticancer properties and drug release efficacy.
- Alternate hypothesis (HA): *Withania somnifera* and *Terminalia bellirica* silver nanoparticles loaded liposomes have anticancer properties and drug release property.

As a result, the current study is designed with the following goals in mind: to test the null and alternate hypothesis mentioned above.

2

REVIEW OF LITERATURE

Cancer is one of the world's major health issues, resulting in millions of diagnoses each year and an increasing number of deaths as a result of this dreadful disease (Sharmila and Padma, 2013). Carcinogenesis is a complex and multistep process characterised by the accumulation of successive transformational events triggered by genetic mutations and epigenetic alterations that affect major cellular processes and pathways such as proliferation, differentiation, invasion, and survival. Cancer is characterised by massive deregulation of all components of the epigenetic machinery. These changes disrupt normal gene regulation and obstruct normal cellular processes such as cell cycle, DNA repair, cell growth, differentiation, and apoptosis (Schnekenburger *et al.*, 2014). Genetic changes can occur at many different levels, from the gain or loss of entire chromosomes to a single DNA nucleotide mutation. These changes have an impact on two broad categories of genes. Oncogenes can be either normal genes that are overexpressed or altered genes that have novel properties. Expression of these genes promotes the malignant phenotype of cancer cells in either case. Tumor suppressor genes are those that inhibit cancer cell division, survival, or other properties. Cancer-promoting genetic changes frequently disable tumour suppressor genes. Changes in many genes are typically required to transform a normal cell into a cancer cell (Knudson, 2001).

Cancer is a serious health issue that remains a leading cause of death worldwide. A growing understanding of the molecular mechanisms underlying cancer progression has resulted in the development of a plethora of anticancer drugs. However, the use of chemically synthesised drugs has not resulted in a significant increase in overall survival over the last few decades. As a

result, new strategies and novel chemoprevention agents are required to supplement existing cancer therapies and improve efficacy. Phytochemicals, which are naturally occurring plant compounds, are important resources for novel drugs as well as sources of cancer therapy. Several of these phytochemicals are naturally occurring biologically active compounds with antitumor activity. The testing of natural extracts (from dry/wet plant material) for potential anticancer biological activity is the first step in the development of effective and side-effect-free phytochemical-based anticancer therapy (Choudhari *et al.*, 2020).

The following headings are used to discuss the review of literature relevant to the study titled "Second Order Targeting using Nanocarriers of Phytoformulation".

2.1 Leukaemia

2.1.1 Types of Leukaemia

2.1.2 Treatment for Leukaemia

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2.4.2.1 Nitric oxide (NO·)

2.1 Leukaemia:

Leukemia is a group of malignant diseases originating from blood or bone marrow cells which includes Acute Myeloid Leukemia, Acute Lymphoblastic Leukemia, Chronic Myeloid Leukemia, and Chronic Lymphocytic Leukemia (Miladinia *et al.*, 2016).

2.1.1 Types of Leukaemia:

Leukemia is characterised by the creation of aberrant leukocytes as a result of either a primary or secondary event. They are characterised as acute or chronic based on the rate of proliferation, and myeloid or lymphoid based on the originating cell (Figure:1). Acute myeloid leukaemia (AML) and chronic myeloid leukaemia (CML), which affect the myeloid chain, and acute lymphoblastic leukaemia (ALL) and chronic lymphocytic leukaemia (CLL), which affect the lymphoid chain, are the most common subtypes. Other less common types of mature WBC cells include mature B-cell and T-cell leukemias, as well as NK cell-related leukemias (Chennamadhavuni *et al.*, 2021).

The following are the four major subtypes of leukaemia:

Acute lymphoblastic leukemia (ALL): Acute lymphoblastic leukaemia (ALL) is a heterogeneous haematological malignancy that primarily affects children (median age at diagnosis: 15 years); nonetheless, adult ALL accounts for 20% of all leukaemia cases (National Comprehensive Cancer Network, 2019). A significant number of immature lymphoid cells proliferate in the bone marrow, peripheral blood, and other organs in ALL. The rates of full remission and overall survival in children with ALL have improved dramatically, especially in the paediatric population, thanks to a better understanding of the biology of the disease and recent improvements in targeted therapy. Overall survival in children with ALL is 86–89% after five years, with the rate decreasing as they become older (overall survival in adults is 41%) (Heo *et al.,* 2019).

Acute myeloid leukemia (AML): Acute myeloid leukaemia (AML) is a genetically diverse cancer that is defined by the proliferation and accumulation of myeloid blast cells in the bone

marrow, peripheral blood, and lymphoid tissue. It is a lethal haematological cancer with significant humanistic implications (Joshi *et al.*, 2019).

Chronic lymphocytic leukemia (CLL): The most common type of leukaemia in adults, CLL is a lymphoproliferative disorder characterised by the expansion of monoclonal, mature CD5+ CD23 + B cells in the peripheral blood, secondary lymphoid tissues, and bone marrow. CLL is an incurable disease with a variable clinical course, and treatment decisions are still based on traditional parameters such as clinical stage and lymphocyte doubling time(Bosch and Dalla, 2019)

Chronic myeloid leukemia (CML): Chronic myeloid leukaemia (CML) is a clonal disorder of the hematopoietic stem cell compartment defined and driven by the BCR-ABL1 gene rearrangement and the tyrosine kinase it encodes. Clinically, it is accompanied by an increase in myelo-erythroid progenitors, which retain the ability to differentiate terminally into neutrophils (Krishnan *et al.,* 2021)

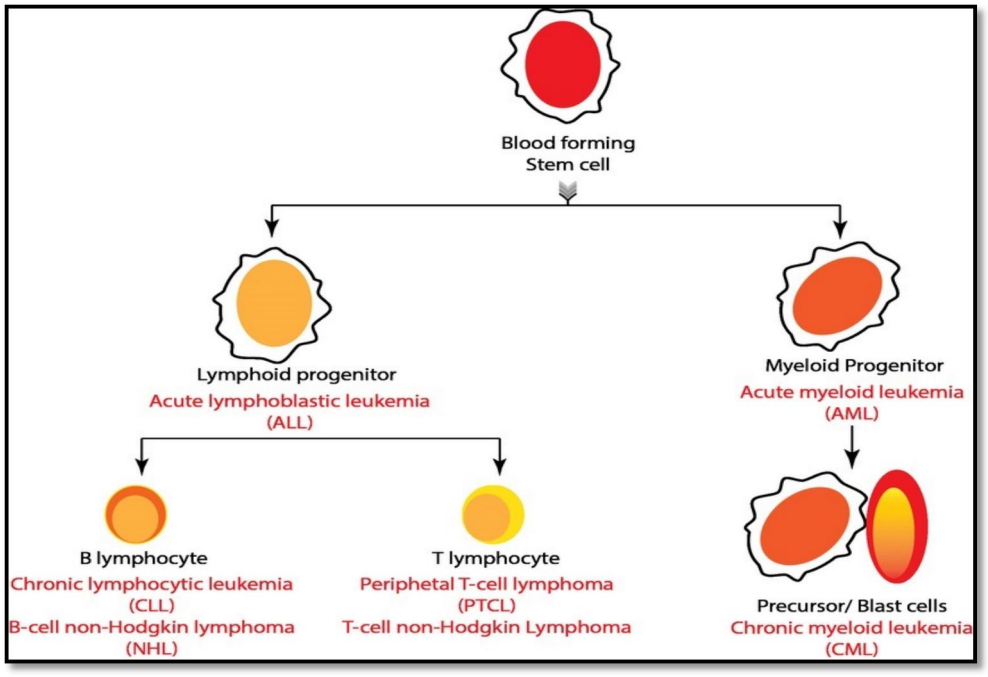


Figure. 1 Different types of Leukaemia based on its origin (Nighat *et al.*, 2020)

2.1.2 Treatment for Leukaemia

Chemotherapy, radiation therapy, and stem cell transplantation have long been the cornerstones of adult leukaemia treatment. Targeted therapies have also become part of the standard of care for some types of leukaemia over the last two decades. Different types of leukaemia necessitate different treatment regimens. Although significant progress has been made against some types of leukaemia, others continue to have relatively low survival rates. Furthermore, as the population ages, there is a greater need for less toxic treatment regimens. Chemotherapy, radiation therapy, and stem-cell transplant have been the standard treatments for childhood leukaemia. Despite significant advances in survival for children with certain types of leukaemia, some children do not respond to standard treatments or have a relapse of their disease. Others will have to live with the side effects of chemotherapy and radiation therapy for the rest of their lives, emphasising the need for less toxic treatments. For the treatment of childhood leukaemia, researchers are now focusing on targeted drugs and immunotherapies. Newer chemotherapy drugs are being tested as well. So, to avoid side effects, an anticancer drug with fewer side effects that targets only cancer cells is required (National Cancer Institute, 2021).

2.2 Free radicals:

A free radical is any molecule that can exist independently and has one or more unpaired electrons in an orbital. Free radicals can indiscriminately react with any molecules with which they come into contact. Once radicals are formed, they can form a covalent bond by reacting with another radical or a non-radical molecule. If no radicals are present, the radical may donate its unpaired electron to the other molecule or take one electron from it, thereby converting it to a free radical and initiating the chain reaction (Sisein, 2014). These electrically charged radicals are highly unstable and reactive in nature, capable of attacking proteins, nucleic acids, mitochondria, and enzymes, ultimately causing cell damage. Increased levels of reactive free radicals cause oxidative stress, which has negative consequences such as lipid peroxidation of

cellular membranes, changes in lipid protein interaction, enzyme inactivation, and DNA breakage (Sarma *et al.,* 2016).

2.3 Source of free radicals:

Endogenous and exogenous substances can both generate free radicals. They are constantly forming in cells and the environment (Kehrer and Klotz, 2015). Endogenous free radical production is caused by immune cell activation, inflammation, ischemia, infection, cancer, excessive exercise, mental stress, and ageing. Exogenous free radical production can be caused by environmental pollutants, heavy metals (Cd, Hg, Pb, Fe, and As), certain drugs (cyclosporine, tacrolimus, gentamycin, and bleomycin), chemical solvents, cooking (smoked meat, used oil, and fat), cigarette smoke, alcohol, and radiations. When exogenous compounds enter the body, they are degraded or metabolised, and free radicals are produced as byproducts (Pizzino *et al.,* 2017).The diverse source of free radicals is given in Figure.2

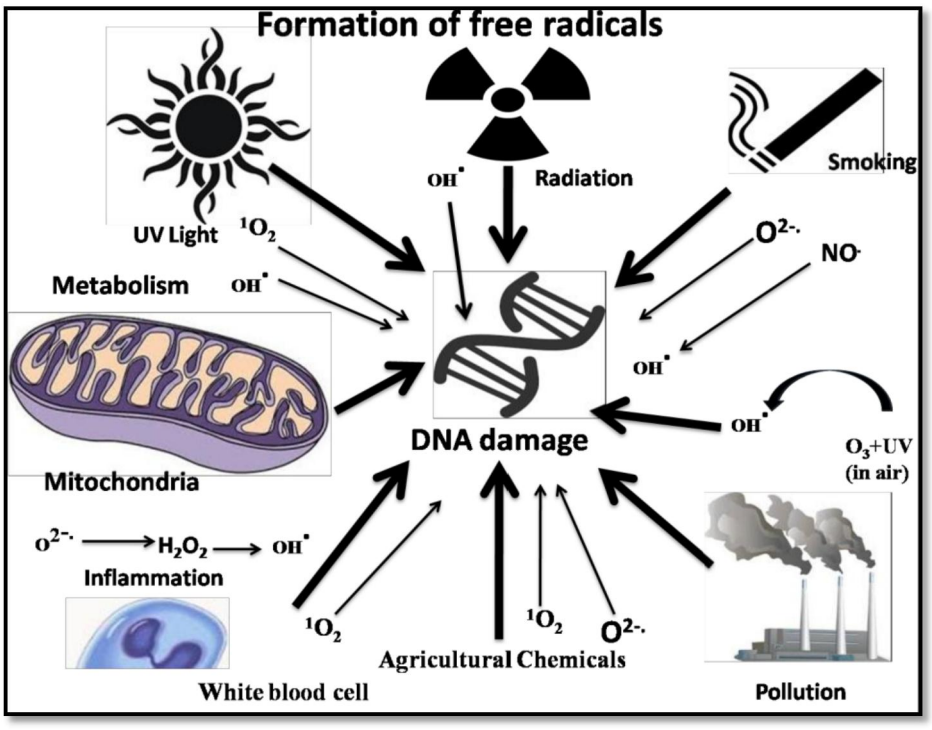


Figure. 2: Diverse source of free radicals (Pathak *et al.*, 2017)

2.4 Types of free radicals:

Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are two types of free radicals (RNS). ROS are oxygen-centered free radicals that include radicals like superoxide anion, hydroxyl radical, alkoxyl, and peroxyl, as well as non-radicals like hydrogen peroxide and singlet oxygen. Nitrogen-centered radicals such as nitric oxide, nitric dioxide, and peroxynirite are examples of RNS (El-Bahr, 2013).

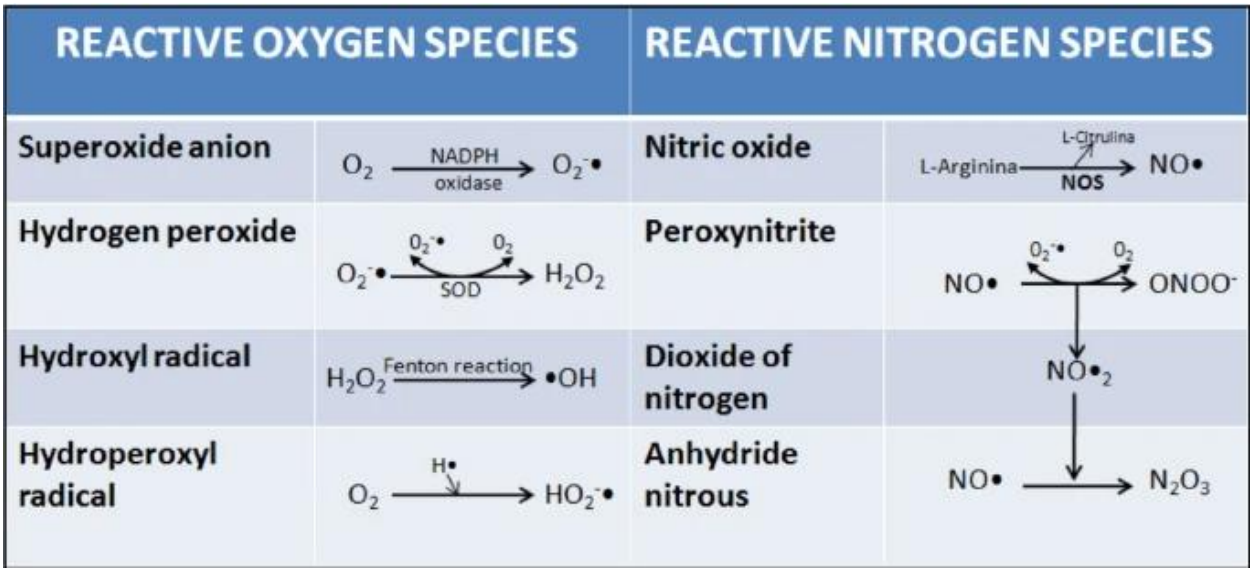


Figure 3. Reactive oxygen and nitrogen reactive species (Ríos-Arrabal *et al.*, 2013)

2.4.1 Reactive Oxygen Species

ROS play an important role in tumorigenesis by influencing a variety of biological processes such as cell proliferation, genomic instability, inflammation, apoptosis resistance, and metabolic reprogramming. ROS levels have been found to be elevated in a number of cancer cell lines. The mitochondria are the primary source of ROS in a tumour cell. ROS levels are frequently elevated in cancer; however, high levels of ROS can be harmful; thus, cells have evolved mechanisms to maintain a proper balance of ROS (Weinberg *et al.*, 2019).

2.4.1.1 Hydroxyl radical (HO•):

The neutral form of the hydroxide ion is the hydroxyl radical, which is a highly reactive free radical. It can cause more damage to cells than any other ROS by strongly reacting with both organic and inorganic molecules such as DNA, proteins, lipids, and carbohydrates. It is formed in a Fenton reaction, in which H_2O_2 reacts with metal ions (Fe^{+2} or Cu+), and is frequently complexed with proteins such as ferritin (an intracellular protein that stores iron) and ceruloplasmin (plasma copper carrying protein) or other molecules. An excess of O_2• releases free iron from ferritin under stress conditions, and the released free iron participates in the Fenton reaction to form OH•. It is also formed as a result of the Haber–Weiss reaction between superoxide radical and H_2O_2 (Phaniendra *et al.,* 2015).

2.4.1.2 Superoxide radical anion ($O_2^{\bullet-}$)

The one-electron reduction of molecular oxygen produces superoxide, which is an anion radical. Superoxide •O_2 is a harmful by-product of mitochondrial respiration (especially in Complexes I and III of the electron transport chain ETC) (Sousa *et al.,* 2018), where a small percentage of the electrons in the ETC chain escape, as well as several other enzymes that catalyse the electron transfer directly to molecular oxygen under strongly reducing conditions, such as those found in the mitochondrial matrix. It's also produced by the immune system to Figureht against invading microbes. The enzyme NADPH oxidase creates huge amounts of •O_2 in phagocytes for use in invading pathogen's oxygen-dependent killing mechanisms (Valenta *et al.*, 2020).

2.4.1.3 Peroxyl radicals (ROO•)

In living systems, it is derived from oxygen. The simplest form of peroxyl radical is the perhydroxyl radical (HOO•), which is generated when superoxide is protonated. The protonated form of O_2• makes up about 0.3 percent of total O_2• in the cytoplasm of a normal cell. It promotes tumour formation by initiating fatty acid peroxidation (Phaniendra *et al.,* 2015).

2.4.1.4 Hydrogen peroxide (H_2O_2) radical:

In vivo, hydrogen peroxide is produced by a dismutation reaction catalysed by the enzyme superoxide dismutase (SOD). It is not a free radical, but it can cause cell damage at relatively low concentrations (10 M), but at higher levels, it inactivates cellular energy producing enzymes such as glyceraldehyde-3-phosphate dehydrogenase. It is capable of easily penetrating biological membranes. H_2O_2 has no direct effect on DNA, but it can cause DNA damage by producing hydroxyl radicals (OH) in the presence of transition metal ions. Catalase, glutathione peroxidase, and peroxiredoxins are the major antioxidant enzymes that can eliminate H_2O_2 (Phaniendra *et.,al* 2015).

2.4.1.5 Singlet oxygen ($^1O_2$)

Singlet oxygen ($^1O_2$) is a molecular oxygen species with a higher energy state. It is a highly active intermediate in chemical and biochemical reactions. Singlet oxygen is not a free radical, but it can be formed during some free radical reactions and can cause free radicals to form. During phagocytosis, macrophages can produce it. Singlet oxygen has roughly the same properties as O_2• and has the highest affinity for protein Trp, His, Tyr, and Cys residues. It has now been demonstrated that $^1O_2$ can react with a wide range of biological molecules, including DNA, proteins, and lipids (Edge *et al.*, 2021)

2.4.2 Reactive Nitrogen Species

Aside from these ROS, nitrogen species (RNS) play an important role in oxidative damage and tissue dysfunction, as well as acting as molecular signals. Thus, nitrogen-containing species, now known as reactive nitrogen species (RNS), include nitric oxide (NO), which is relatively unreactive, and its derivative, peroxynitrite (ONOO), which is a dominant oxidant capable of causing damage to various biological molecules (Kapoor *et al.*, 2019). RNS (reactive oxygen and nitrogen species) are required for normal physiological processes and play critical roles in cell signalling, immunity, and tissue homeostasis. Excess radical species, on the other

hand, are implicated in the development and accelerated pathogenesis of a variety of diseases (Ferreira *et al.,* 2018).

2.4.2.1 Nitric oxide (NO·)

Nitric oxide synthase catalyses the enzymatic conversion of L-arginine to L-citrulline (NOS). In higher organisms, the NO radical (NO•) is formed by the oxidation of one of the terminal guanido-nitrogen atoms of L-arginine. NO can be converted to a variety of reactive nitrogen species depending on the microenvironment, including nitrosonium cation (NO+), nitroxyl anion (NO-), and peroxynitrite (ONOO-). At physiological concentrations, NO radical regulates mitochondrial respiration, causing reversible inhibition of respiration by altering the activity of the terminal enzyme of the mitochondrial respiratory chain, cytochrome C oxidase (Complex IV). To control vascular tone, NO radical also binds to soluble guanylate cyclase in the vascular endothelium (Aprioku, 2013).

2.4.2.2 Peroxy nitrite ($O=NOO^-$)

When superoxide anion and nitric oxide react, peroxynitrite anion (ONOO) is formed, which causes DNA fragmentation and lipid oxidation (Shastri *et al.,* 2016). The reaction of nitric oxide and superoxide in inflamed tissues results in the formation of an extremely reactive peroxynitrite (ONOO), which is a well-known oxidising and nitrating agent with high reactivity at physiological pH. The formed peroxynitrite can attack a wide range of biomolecules via direct oxidative reactions or indirect radical-mediated mechanisms, resulting in cellular responses such as cell signalling, oxidative injury, necrosis, or apoptosis. Cellular DNA is a common target for ONOO attack because it can react with deoxyribose, nucleobases, and induce single strand breaks (Rizwan *et al.*, 2019).

2.5 Oxidative stress mediated diseases:

Oxidative stress, defined as a relative excess of reactive oxygen species (ROS) in comparison to antioxidants, has been linked to neurodegenerative disease, cardiovascular disease, type 2 diabetes, and a variety of other pathologies (Sies, 2015). Oxidative stress plays a crucial

role in the development of age-related diseases including arthritis, diabetes, dementia, cancer, atherosclerosis, vascular diseases, obesity, osteoporosis, and metabolic syndromes (Liu *et al.,* 2017). ROS are generated within the biological system to modulate the cellular activities such as cell survival, stressor responses, and inflammation (He and Zuo, 2015). Elevation of ROS has been associated with the onset and progression of aging. Although ROS generation may not be an essential factor for aging (López-Otín *et al.,* 2013), they are more likely to exacerbate age-related diseases progression via oxidative damage and interaction with mitochondria (Dias *et al.*, 2013). Due to their reactivity, high concentrations of ROS can cause oxidative stress by disrupting the balance of antioxidant and prooxidant levels (Zuo *et al.,* 2015).

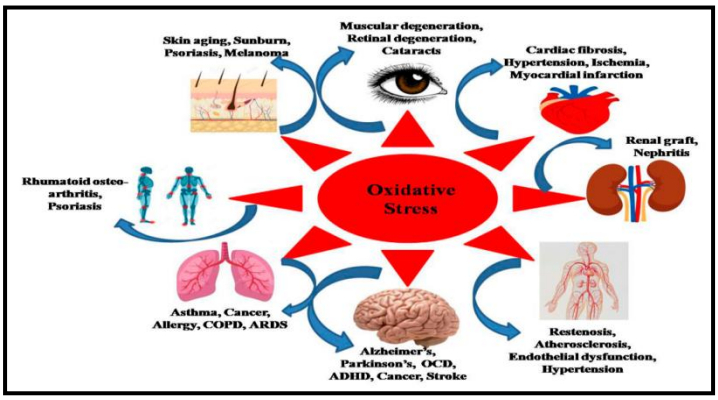


Figure. 4: Oxidative stress mediated diseases (Kumar *et al.,* 2020)

2.6 Role of ROS in cancer

Normal somatic cells require ROS for a number of cellular processes, such as immune defense mechanisms and obligate secondary signaling. In cancer cells, ROS levels are increased due to both environmental and internal mechanisms.In cancer cells ROS are usually considered oncogenic because they have been implicated in initiation, progression and metastasis of cancers however this is not clear cut, as ROS may also be crucial for tumor clearance. A clear mechanism by which ROS impact tumor development is by direct DNA damage during carcinogenic transformation such as catalyzing the modified DNA base 8-OHdG resulting in mutation. ROS catalysis of disulfide bond formation can impact a wide range of cellular proteins and lipid

modifications which result in unstable, short lived lipids that ultimately propagate reactive species by secondary messenger breakdown products. This may occur due to antioxidants decreasing ROS to a level supporting tumor proliferation and migration while minimizing some of the negative impacts of ROS in cancer cells, such as DNA damage. The obvious contradiction is a continuing area for resolution, and it is becoming more likely that ROS has both positive and negative roles in tumors (Yang *et al.,* 2018).

2.7 Role of RNS in cancer:

Reactive nitrogen species (RNS) also activate transcription factors (NF-KB, STAT-3) and cause cellular proliferation, genomic instability, angiogenesis, apoptosis resistance, invasion, and metastasis. Inflammatory mediators in the tumour microenvironment either inhibit or promote inflammation-induced cancer, depending on the stage of immune surveillance of the tumour, i.e. immunoediting, immunoprocessing, and immunoevasion. Immature myeloid progenitor cells give rise to myeloid derived suppressor cells. They are the primary immune suppressor cells in the tumour inflammatory microenvironment, where they activate the transcriptional factors NF-KB and STAT-3 to promote tumour progression (Shrihari, 2017). Infectious diseases and chronic inflammation are thought to account for approximately 25% of cancer-causing factors. Reactive nitrogen species (RNS) are produced by both inflammatory and epithelial cells during chronic inflammation. RNS cause DNA damage in inflammatory organs, which leads to cancer development. Chronic inflammation damages nucleic acids, proteins, and lipids through RNS generation, resulting in tissue damage. Tissue damage may activate progenitor/stem cells, allowing for tissue regeneration. RNS from inflammation damage stem cells, and the resulting mutations can accumulate, potentially generating cancer stem cells (Murata, 2018).

2.8 Antioxidants:

Antioxidants are a structurally diverse group of small organic molecules and large enzymes that form complex systems of overlapping activities that work synergistically to enhance cellular defence and combat oxidative stress caused by reactive oxygen species (ROS)

and reactive nitrogen species (RNS). Antioxidants can refer to either industrial chemicals added to products to combat oxidation or natural products found in foods, plants, and tissue (Moussa *et al.,* 2019). Antioxidants are divided into two categories: enzymic antioxidants and non-enzymic antioxidants. Superoxide dismutase, catalase, and glutathione peroxidase are the most important enzymatic antioxidants. Non-enzymatic antioxidants include vitamin C, E, and -carotene, as well as natural antioxidants like flavonoids, tannins, coumarins, phenolics, and terpenoids (Aziz *et al.,* 2019).

2.9 Role of medicinal plants in cancer therapy:

Medicinal plants have been used since the dawn of human civilization, as evidenced by ancient script and traditional herbal medicine recipes. Plants have been used as a source of food, shelter, and medicine by humans for nearly the same amount of time (Mohammad *et al.*, 2020). Cancer has been a constant battle around the world, and there has been a lot of progress in terms of cures and preventative medications. The condition is characterised by the inability to control or stop cells in the human body from proliferating indefinitely. As a result, malignant cells create tumours that have the ability to spread. Chemotherapy, radiation, and chemically generated medications are among the current treatments. Chemotherapy, for example, can put patients under a lot of stress and wreak havoc on their health. As a result, there is an emphasis on employing alternative cancer treatments and therapies. Herbal medicines have been used and continue to be used as the primary source of medical treatment in developing countries for many years. Plants have been used in medicine for centuries because of their natural antiseptic properties. As a result, research has focused on the potential properties and applications of terrestrial plant extracts for the development of potential nanomaterial-based drugs for diseases such as cancer. Many plant species are already being used to treat or prevent cancer development. Multiple researchers have identified plant species with anticancer properties, with a particular emphasis on those used in herbal medicine in developing countries (Greenwell and Rahman, 2015).

2.10 Nanotechnology

Nanotechnology is the study and development of technology at the atomic, molecular, or macromolecular levels in the range of approximately 1-100 nanometers in order to provide a fundamental understanding of phenomena and materials at the nanoscale. The nanometer scale is approximately one billionth of a metre. In comparison, the diameter of a human hair is approximately 10,000 nanometers. Because of their small size, nanotechnology is used to create structures, devices, and systems with novel properties and functions. Actually, the matter exhibits unusual physical and chemical properties due to an increase in surface area compared to volume as particles shrink in size, a phenomenon known as the quantum size effect. This means that the bulk properties of materials at the nanoscale can differ greatly from those at the larger scale. Using these material properties, scientists design and manufacture devices by manipulating shape and size at the nano scale, with a wide range of implications that could include medicine, electronics, military applications, computing, space science, and many more (Subedi, 2014). Nanotechnology is the use of nanoparticles that are very small in size but have a much larger surface area than their bulk form (Arasu *et al*., 2019; Roy, 2021; Savunthari *et al.,* 2021; and Kaur and Roy, 2021). Nanomaterials possess a wide range of properties, including chemical, optical, and thermal properties (Al-Dhabi and Valan, 2018).

2.10.1 Types of nanomaterials:

Nanoparticles are classified into four material-based categories, which are as follows:

Carbon Based Materials: These nanomaterials are composed mostly of carbon, most commonly taking the form of a hollow spheres, ellipsoids, or tubes. Spherical and ellipsoidal carbon nanomaterials are referred to as fullerenes, while cylindrical ones are called nanotubes. These particles have many potential applications, including improved films and coatings, stronger and lighter materials, and applications in electronics.

Metal Based Materials: These nanomaterials include quantum dots, nanogold, nanosilver and metal oxides, such as titanium dioxide. A quantum dot is a closely packed semiconductor crystal

comprised of hundreds or thousands of atoms, and whose size is on the order of a few nanometers to a few hundred nanometers. Changing the size of quantum dots changes their optical properties.

Dendrimers: These nanomaterials are nanosized polymers built from branched units. The surface of a dendrimer has numerous chain ends, which can be tailored to perform specific chemical functions. This property could also be useful for catalysis. Also, because three-dimensional dendrimers contain interior cavities into which other molecules could be placed, they may be useful for drug delivery.

Composites: Composites combine nanoparticles with other nanoparticles or with larger, bulk-type materials. Nanoparticles, such as nanosized clays, are already being added to products ranging from auto parts to packaging materials, to enhance mechanical, thermal, barrier, and flame-retardant properties (Savage *et al.*, 2007).

2.10.2 Silver nanoparticles:

Silver nanoparticles are nanostructures with diameters ranging from 1 to 100 nm that are largely employed for novel and enhanced biomedical applications such as medication delivery, wound dressings, tissue scaffolding, and protective coatings (Almatroudi, 2020). Due to their distinctive physical and chemical properties, silver nanoparticles (AgNPs) are increasingly used in a variety of industries, including medicine, food, health care, consumer goods, and industry. Optical, electrical, and thermal properties, as well as strong electrical conductivity and biological qualities, are among them. They have been used as antibacterial agents, in industrial, household, and healthcare-related products, in consumer products, medical device coatings, optical sensors, and cosmetics, in the pharmaceutical industry, the food industry, in diagnostics, orthopaedics, drug delivery, and as anticancer agents, and have ultimately enhanced the tumor-killing effects of anticancer drugs due to their peculiar properties. AgNPs have recently been found in a variety of textiles, keyboards, wound dressings, and biomedical devices (Zhang *et al.,* 2016).

2.10.3 Methods of silver nanoparticles synthesis

For the effective synthesis of silver nanoparticles, many approaches and methods have evolved, including physical, chemical (Chemical reduction method, Electrochemical method, Pyrolysis, and Irradiation-assisted chemical method), and biological techniques. While physical and chemical methods are more cost-effective commercially, biological methods are less harmful to the environment (Hulkoti and Taranath, 2017). The size, shape, structure, physical, chemical, and biological properties of nanoparticles are determined by the method of manufacture. The researchers have reported on a number of synthetic methods. The three most important approaches have primarily been presented. The most common and widely used method is chemical reduction, which involves reducing Ag+ species to Ag° using reducing agents such as NaBH4, LiAlH4, and others. Physical methods typically consume a lot of energy during the synthesis process. AgNPs have been synthesised biologically using fungi, plants, and bacteria, with no toxic reducing agents used (Arif and Uddin, 2021).

2.10.4 Green synthesis of silver nanoparticles using plant extracts

Nanoparticles can be synthesized using various approaches including chemical, physical, and biological approaches. Although chemical method of synthesis requires short period of time for synthesis of large quantity of nanoparticles, this method requires capping agents for size stabilization of the nanoparticles. Chemicals used for nanoparticles synthesis and stabilization are toxic and lead to non-eco-friendly byproducts. The need for environmental nontoxic synthetic protocols for nanoparticles synthesis leads to the developing interest in biological approaches which are free from the use of toxic chemicals as byproducts. Thus, there is an increasing demand for “green nanotechnology”. Plants provide a better platform for nanoparticle synthesis as they are free from toxic chemicals and provide natural capping agents. Moreover, use of plant extracts also reduces the cost of microorganisms isolation and culture media enhancing the cost competitive feasibility over nanoparticles synthesis by microorganisms (Krithiga *et al.*, 2015) The steps involved in the green synthesis of silver nanoparticles is elucidated in Figure. 5.

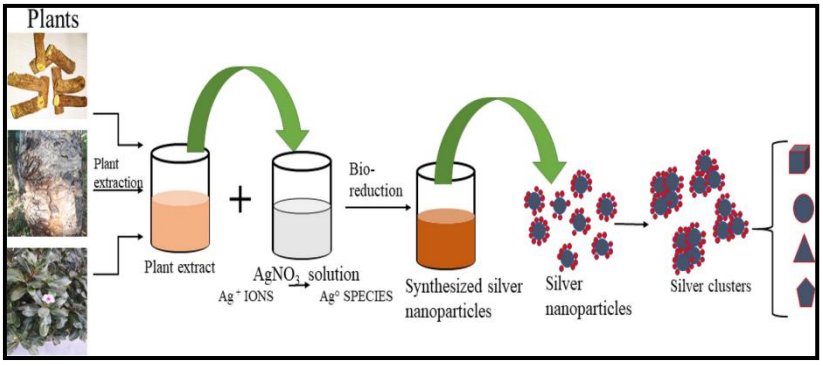


Figure. 5: Synthesis of AgNPs through Green synthesis method (Jain *et al.*, 2021)

2.10.5 Characterization of silver nanoparticles:

Different factors modulate the characteristics of AgNPs like shape, size, crystallinity, surface charge, surface coating, and biological activity. There are several technologies available to study the characters and properties of nanoparticles such as Ultra-violet visible spectroscopy (UV-vis), X-ray diffraction (XRD), Fourier Transform Infrared (FTIR) spectroscopy, scanning electron microscopy (SEM), Transmission electron microscope (TEM), Dynamic light scattering (DLS), Atomic Force Microscopy (AFM)

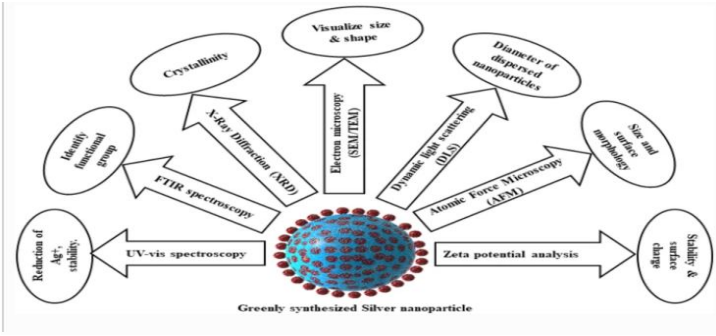


Figure. 6: AgNPs with their Characterization Methods (Jain *et al.*, 2021)

2.10.6 Therapeutic applications of silver nanoparticles:

Although AgNPs are used in a variety of applications including thin films, surface coatings, batteries, cosmetics, textile industry, food industry, energy harvesting and conductors,

medical applications have attracted great most attention due to increasing life threatening diseases worldwide and multidrug resistance challenges (Shanmuganathan *et al.,* 2019). Upon reaching nanoscale, silver particles have different physicochemical properties and create exceptional biological activities. This distinctiveness of silver nanoparticles widen their application in antibacterial, anti-fungal, anti-viral, anti-inflammatory, anti-angiogenic and anti-cancer therapy. Recent studies have indicated that AgNPs do not harm humans and kill viruses, bacteria and other eukaryotic microorganisms without any adverse effects in diluted concentrations (Sukriye and Cigdem, 2019). AgNPs are prominent and can prove to be the boon in the field of nanotechnology by which excellent, effective, efficient and very potent nanoproduct can be formulated to treat the giant disease like cancer (Karmous *et al.*, 2020)

2.10.7 Role of silver nanoparticles in cancer therapy

It is critical to develop or engineer a drug or gene delivery system that has an excellent ability to target tumour cells while sparing normal healthy cells for effective cancer therapy. It boosts therapeutic efficacy, protecting normal cells from the effects of cytotoxicity. It is possible to achieve this by delivering NPs in a well-organized manner into the tumour microenvironment (TME), thereby indirectly targeting cancer cells (Gavas *et al.*, 2021). Nanoparticulated silver's therapeutic promise is based on its unique manner of cell death in mammalian cells. Their method of action to promote cancer cell death is relatively dogmatic, regardless of their physical and chemical features, such as heterogeneity in size, shape, and capping substance. AgNPs are collected in endosomes after being taken up by endocytosis-related processes, and the organelles are then directed to lysosomal fusion. The lysosomal acidic environment causes an increase in the release of silver ions from AgNPs, and the reactive ions disrupt cellular homeostasis, leading to apoptotic cell death depending on the biological characteristic of the targeted cell (Cameron *et al.,* 2018). This is known as the "Trojan-horse" mechanism, and it implies that the cytotoxic property of AgNPs emerges only after they have been taken up by cells (Hsiao *et al.,* 2015). It is worth noting that the toxicity of AgNPs is caused by the oxidative stress of Ag nanoparticles rather than by free Ag+. It was discovered that AgNPs can induce oxidative stress

in cells by producing reactive oxygen species (ROS), resulting in cytotoxicity, apoptosis, and necrosis of cancer cells, which is a novel step in tumour treatment (Rui *et al.,* 2022).

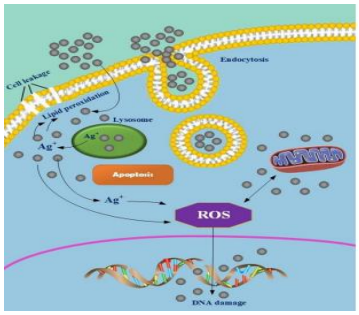


Figure.7. Schematic representation of the mechanism of anticancer effect of silver nanoparticles(Abdel-Fattah and Ali, 2018)

2.11 Liposomes:

Liposomes are small artificial vesicles of spherical shape that can be created from cholesterol and natural non-toxic phospholipids. Due to their size and hydrophobic and hydrophilic character(besides biocompatibility), liposomes are promising systems for drug delivery. Liposome properties differ considerably with lipid composition, surface charge, size, and the method of preparation.Furthermore, the choice of bilayer components determines the 'rigidity' or 'fluidity' and the charge of the bilayer. For instance, unsaturated phosphatidylcholine species from natural sources (egg or soybean phosphatidylcholine) give much more permeable and less stable bilayers, whereas the saturated phospholipids with long acyl chains dipalmitoylphos phatidylcholine) form a rigid, rather impermeable bilayer structure (Akbarzadeh *et al.,* 2013).

2.11.1 Types of liposomes:

Liposomes are classified as unilamellar, multilamellar, oligolamellar, or multivesicular vesicles based on the number of phospholipid bilayers, as illustrated in Figure. 8. Liposomes with a size of 50 to 200 nm are ideal for drug delivery applications. Liposome size is a critical factor in drug delivery into the body. Liposome size has a significant impact on the

pharmacokinetics of liposomes and drugs encapsulated in liposomes. Liposomes smaller than 200 nm have increased circulation and residence time in the blood, enhanced in vivo drug release from liposomes, and significant accumulation into tumour cells (Andra et al., 2022).

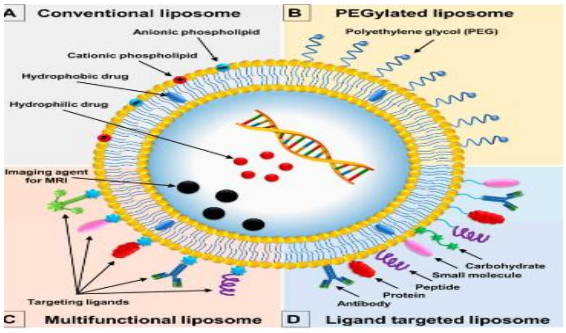


Figure.8 Classification of liposomes based on composition and application. (Le, 2019)

2.11.2 Methods of liposome preparation:

Liposome preparation can be done by conventional methods such as Bangham method [thin film hydration], ether/ethanol injection method, reverse phase evaporation method, detergent depletion method, heating method, microfluidic channel method, membrane extrusion method, homogenization and sonication method. Novel methods for liposomal-based drug delivery involve freeze drying, dual asymmetric centrifugation [DAC] and supercritical fluid [SCF] methods since a decade. Depo-foam liposome technique, lysolipid thermally sensitive liposome technique, non-PEGylated liposome technique and stealth liposome techniques are the innovative techniques used for the delivery of drugs in the recent past (Bulbake *et al*., 2017).

2.11.3 Thin Film Hydration Method [Bangham Method]

The Bangham method was the first widely used and simple method for preparing liposomes. This method dissolves lipids in an organic solvent (dichloromethane, chloroform, ethanol, or a chloroform–methanol mixture); the organic solvent is then removed by evaporation under vacuum at 45–60℃ to form a thin lipid film. Following that, the thin lipid film is hydrated in aqueous media by continuous agitation for up to 2 hours at 60–70℃, where it swells to form

round closed liposomes (Zhang, 2017). Finally, sonication is used to shrink the liposomes to nanoscale size.

2.11.4 Drug-Loading Methods

Passive or active drug loading into liposomes is possible. The passive drug-loading method entails encapsulating the drug agent during the liposome preparation process. Covalent, ionic, electrostatic, noncovalent, or steric interactions between drug molecules and lipids can encapsulate the drug within the inner aqueous space or embed it in the bilayer of liposomes. The main disadvantage of this method is its low encapsulating efficiency, which necessitates an additional step of free drug removal. The active drug loading method, also known as remote drug loading, entails loading the drug agent after empty liposomes have been produced. The transmembrane gradient of pH or ion concentration is the driving force that causes the drug to diffuse across the membrane and into the inner core of liposomes. The drug-entrapment process takes about 5 minutes to 30 minutes, and a high loading efficiency (above 90%) can be achieved (Liu *et al.,* 2022).

2.11.5 Administration Route of Liposomal Drugs

The use of nanoparticles, including liposomes, for drug delivery via oral administration has been highlighted as an effective strategy because nanoparticles increase drug bioavailability, improve drug interaction with cells, and prevent any changes in the molecular structure of the drug caused by enzymes and gastric juices in the gastrointestinal tract. Furthermore, they have the ability not only to increase the release of therapeutic molecules into the mucosal and epidermal layers, but also to protect drugs from unwanted changes during the first pass effect. Many liposomal drugs approved by the FDA or other authorities are administered primarily via intravenous injection. Subcutaneous (S.C.), intradermal (I.D.), intraperitoneal (I.P.), and intramuscular (I.M.) injections, on the other hand, are also used for the administration of liposomal drugs (Rommasi and Esfandiari, 2021).

2.11.6 Drug delivery of liposomes to cancer cells

Liposomes that selectively target tumour cells and deliver anticancer medicines to tumour locations have been produced using a variety of ways. The active and passive targeting and release of medications into the tumour location is made easier by the surface functionalization of liposomes (Riaz *et al.*, 2018)

Passive targeting

Passive targeting is a therapeutic targeting method that is primarily based on the pathophysiological properties of tumour tissues. Due to leaky tumour vasculature, liposomal medication formulations freely translocate over the endothelium of capillaries into the interstitial fluid. The pores in the tumour microvasculature that sit between the endothelial cells vary greatly in size. Endothelial cells that line normal capillaries have a gap size of 5 to 10 nm, whereas endothelial cells that line tumour capillaries have a gap size of 100 to 780 nm, allowing liposomes to engage in passive targeting. As a result, if liposomes are created with a size range suitable for extravasation into tumour tissues rather than normal tissues, optimal targeting can be achieved. The EPR effect is a mechanism that allows effective liposomal accumulation in tumours (Alavi and Hamidi, 2019) Blood capillaries in cancerous tissues have increased permeability and a limited fluid return to the lymphatic circulation as a result of the EPR effect. As a result of inadequate lymphatic drainage of extravasated molecules, liposomes up to 400 nm in size and their encapsulated drugs can preferentially accumulate within the microenvironment of solid tumours. As a result of the accumulation of liposomes in solid tumours, drug delivery improves because higher local drug concentrations are available. The EPR effect can be enhanced by creating liposomes with particle sizes ranging from 40 to 200 nm, which have been shown to have greater extravasation (Maeda *et al.*, 2013)

Active targetting

Various strategies have been used to design actively targeted liposomes in order to reduce off-target side effects even further. Active targeting entails directing drug payloads directly to the

target site. Actively targeted liposomes are typically created by conjugating targeting ligands such as peptides, monoclonal antibodies, and aptamers to the surface of liposomes. Ligands can be attached to liposomes in a variety of ways, including directly to lipids or at the terminal end of PEG chains. The postinsertion technique can be used to incorporate ligand-lipid-PEG conjugated micelles into preformed liposomes. Another widely used method is to incorporate ligands into the liposome formulation step (Latifa *et al.*, 2021)

2.12 Candidate plant- *Withania somnifera*

Withania somnifera (L.) Dunal, (Solanaceae), commonly known as Ashwagandha or winter cherry, is a well-known medicinal plant in Ayurvedic medicine. The principal active compounds include several withanolide-type compounds. Due to the nontoxic and high medicinal value of *W. somnifera* (WS), this plant is widely used all over the world. Roots, and less often leaves and fruits, have been used as phytomedicines in the form of decoction, infusions, ointment, powder, and syrup. Nowadays, this plant is cultivated as a crop to support the high demand of biomass and a sustainable quality for the needs of pharmaceutical industry (Marslin *et al.*, 2015).

Subkingdom:Tracheobionta

Division:Magnoliophyta

Class:Magnoliopsida

Subclass:Asteridae

Order:Solanales

Family:Solanaceae

Genus:*Withania*

Species:***Withania somnifera***

2.13 Candidate plant-*Terminalia bellirica*

Terminalia belerica Roxb. (Family Combretaceae) is a large deciduous tree with broadly elliptic leaves clustered at the ends of branches (Meena *et al.*, 2010). It is wildly distributed throughout the world especially Indian subcontinent, Srilanka, Pakistan, Nepal and South East Asia. *T. belerica* is used in traditional medicine due to the wide spectrum of pharmacological activities associated with the biologically active secondary metabolites present in this plant. Variety of phytochemicals are isolated from various parts of the plant which include alkaloid, coumarin, flavones, steroids, lignans, tannins, glycosides, terpenoid, saponin etc (Abraham *et al.*, 2014)

Kingdom:Plantae

Division:Magnoliophyta

Class:Magnoliopsida

Order:Myrtales

Family:Combretaceae

Genus:Terminalia

Species:belerica

3

METHODOLOGY

Nanoparticles (NPs) and Nano Structured Materials (NSMs) are the fields of active research subject and a growing techno-economic sector with applications covering broad areas of research. Because of their tunable physicochemical features such as melting point, wettability, electrical and thermal conductivity, catalytic activity, light absorption and scattering, NPs and NSMs have acquired significance in technological breakthroughs over their bulk counterparts (Jeevanandam *et al*., 2018). AgNPs are well-known for their broad-spectrum, high-efficiency anticancer and antibacterial properties (Xu *et al.*, 2020). AgNP aqueous solutions are unstable and soon agglomerate due to their strong reactivity. To keep the particles from aggregation, stabilising chemicals are utilised to separate them. Metal nanoparticles have been shown to be more biocompatible and stable when coated with liposomes. Liposomes are nanoparticulate systems that have been embraced by a number of researchers as the preferred method of drug administration, vaccine delivery, and therapeutic agent targeting. Liposomes have a number of advantages due to their unique composition and structure, including good biocompatibility, biodegradability, drug transportability, and easy handling (Espinoza *et al.*, 2020).

3.1 Layout of the study

The present study entitled "Second Order Targeting using Nanocarriers of Phytoformulation". The study was designed as four different phases. In phase I, hydroethanol leaf extract of *Withania somnifera* and dry fruit extract of *Terminalia bellirica* were prepared and evaluated using UV Spectrum for the presence of phytoconstituents. The phase II was designed to analyse the antioxidant activity of *Withania somnifera* and *Terminalia bellirica* extracts. In phase III, an attempt was made to synthesize and characterize silver nanoparticles and silver

nanoparticles loaded Liposomes from *Withania somnifera and Terminalia bellirica* extract. Fourth phase was designed to analyse the anticancer potential of silver nanoparticles and silver nanoparticles Loaded Liposomes of *Withania somnifera and Terminalia bellirica* against molt-3 cells.

Phase I

3.2 Evalution of hydroethanolic leaf extract of *Withania somnifera* and dry fruit extract of *Terminalia bellirica* .

3.2.1 Collection of plant species

Withania somnifera leaf and *Terminalia bellirica* fruit sample were collected in the form of dry powder from a homegrown shop in Seeranaicken Palayam, Coimbatore.

3.2.2 Extraction of the Plant Materials

The coarse powder (10g) was weighed and dissolved in 100ml of hydroethanol. The solvents used for the extraction of phytoconstituents were ethanol and water in different proportion like water:ethanol (20:80, 40:60, 60:40, 80:20 and 50:50) were kept overnight in a shaker incubator. Then filtered through the whatmann no.1 filter paper extracts obtained were then transferred to separate beaker and evaporated to dryness.

Phase II

3.3 Designed to analyse the antioxidant activity of *Withania somnifera* and *Terminalia bellirica*

3.3.1 DPPH Radical Scavenging Assay:

DPPH radical scavenging activity was assessed by the method of Brand-Williams *et al* (1995)

Principle:

DPPH radical is scavenged by antioxidants through the donation of a proton forms the reduced DPPH. The color change from purple to yellow after reduction can be quantified by its decrease in absorbance at wavelength 520m.

Reagents:

- DPPH – (2, 2-diphenyl-2-picryl hydrazyl hydrate) (0.1mM in methanol)
- Methanol
- Standard: Ascorbic acid

Procedure:

0.5ml of 0.1mM DPPH solution in methanol was mixed with varying concentrations of hydroethanolic plant extract (50, 100, 150, 200, 300, 400, 500µg/ml). A blank sample was prepared, and L- Ascorbic acid (50-500µg/ml) was used as a reference standard. As a control, 0.5ml methanol/0.5ml DPPH solution mixture was used. The reaction was carried out in triplicate, and the decrease in absorbance at 520nm after 30 minutes in the dark was measured using a UV-Visible spectrophotometer. The inhibition percentage was calculated using the formula below.

$$\textbf{Inhibition \%} = Ac\text{-}As/Ac \times 100$$

where Ac is the absorbance of the control and As is the absorbance of the sample.

Phase-III

3.4 Synthesis and Characterisation of Silver nanoparticles and Silver nanoparticles loaded Liposomes from *Withania somnifera* leaf extract and *Terminalia bellirica* dry fruit extracts

3.4.1 Sunlight mediated green synthesis of silver nanoparticles

10 ml of the leaf extract was added to 90 ml of freshly prepared 1 mM silver nitrate solution to attain a final volume of 100 ml. Then it is kept under direct sunlight for 20 mins. Change in color of the solution from brown to dark reddish brown indicates the reduction of Ag^{+}

to Ag^0. The solution was then subjected to centrifugation at 15,000 rpm for 45 min. The supernatant obtained after centrifugation was discarded and the pellet that has silver nanoparticles was purified 4 times by washing with deionized water for the removal of leaf extract residue and silver ions. After purification, the pellet was subjected to lyophilisation and stored in dark for further analysis (Mounil *et al.,* 2020 and Rautela *et al.*, 2019).

3.4.2 Characterisation of green synthesized *Tabebuia pallida* silver nanoparticles

3.4.2.1 UV-Visible spectroscopy

Preliminary characterization of the silver nanoparticles was done using UV-Visible spectroscopy. UV-Visible spectroscopy is mainly used to record the optical absorption spectra of the synthesized silver nanoparticles by dissolving it in DMSO by sonication. DMSO alone serves as a blank. The Nanoparticle solution was measured in UV-Visible nanospectrophotometer (Shimadzu Biospec nano) with an absorption range of 200-800nm.

3.4.2.2 X-Ray Diffraction

The crystalline nature of the synthesized silver nanoparticles were examined using X-ray Diffraction. X-ray diffraction study was carried out using X'pert Pro X-ray diffractometer. Cu K radiation (λ=1.54060Å) operated at 45 kV and 30 mA over the range 2θ=2-70º with scanning rate 2º/min.

3.4.2.3 FTIR Spectroscopy

The functional groups of the phytochemicals involved in the bioreduction of Ag ions and subsequent capping and stabilisation of biosynthesized silver nanoparticles were determined using FTIR analysis (Huq *et al.,* 2020). It was carried out by using Shimadzu 8400S Fourier-transform infrared spectrometer operated at a resolution of 4 cm^{-1} in the range of 400–4000 cm^{-1} (Femi-Adepoju *et al*., 2019)

3.4.3 Preparation of silver nanoparticles loaded liposomes

The silver nanoparticles encapsulated liposomes were prepared using thin-film hydration method coupled with sonication. Cholesterol and phosphatidylcholine (Lecithin) were used at a molar ratio of 2:1. The mixture of cholesterol and lecithin was dissolved in 10 ml of chloroform until the formation of a clear solution. Using a rotary evaporator the chloroform was evaporated at 40ºC and then the flask was kept in vacuum overnight for the removal of the organic solvent completely which results in the thin lipid film formation. This thin film was then hydrated with the 5ml of silver nanoparticles dissolved in DMSO by placing it in a rotary evaporator for 5 mins. Thus obtained silver nanoparticles loaded liposomes were subjected to sonication to reduce the size of the liposomes. Then the non loaded silver nanoparticles present in the supernatant were removed by centrifugation (Fathy *et al.,* 2019).

3.4.3.1 Encapsulation efficiency of silver nanoparticles loaded liposomes

The silver nanoparticle encapsulation efficiency was determined using the indirect spectrophotometric method. To determine the amount of silver nanoparticles encapsulated, the silver nanoparticles loaded liposomes were treated with chloroform and shaken well. This process releases the silver nanoparticles encapsulated in the liposomes which can be measured spectrophotometrically at 450 nm. The encapsulation efficiency can be calculated using the following formula (Nayyer *et al.*, 2019).

$$\text{Encapsulation Efficiency} = \frac{\text{Amount of Encapsulated Nanoparticle}}{\text{Amount of Encapsulated } + \text{Free Nanoparticle}} \times 100$$

Phase-IV

3.5 Designed to analyse Anticancer potential of Silver nanoparticles and Silver nanoparticles Loaded Liposomes of *Withania somnifera* and *Terminalia bellirica* against molt-3 cells and peripheral blood lymphocytes (Normal cells) were evaluated.

3.5.1 Culturing of Molt-3 cells

The Molt-3 cells were purchased from National Centre for Cell Science (NCCS), Pune, India. RPMI-1640 was the medium used for the culturing of Molt-3 cells supplemented with 0.5% penicillin streptomycin and 10% Fetal Bovine Serum. The flasks were incubated at 37ºC with 5% CO_2 in a CO_2 incubator.

3.5.2 Separation and Culturing of Peripheral Blood Lymphocytes (PBL)

Fresh blood was collected from a healthy individual under sterile condition by vein puncture. The blood was then heparinized to prevent it from clotting. The heparinized blood was then subjected to dilution using PBS in the ratio of 1:1. This diluted blood was added to a centrifuge tube containing lymphosep in the ration 1:2. The heparinized blood along with lymphosep was subjected to centrifugation at 4000 rpm for 30 mins at a temperature of 18-20ºC. After 30 mins of centrifugation the lymphocytes were separated as a grey coloured layer at the top of the centrifuge tube. The lymphocytes layer was then carefully separated without disturbing the remaining contents. Thus obtained lymphocytes were washed using phosphate buffered saline thrice. Then the cells were subjected to centrifugation to obtain in pellet form. These pellets were then resuspended in RPMI 1640 medium supplemented with 0.5% penicillin streptomycin, 10% Fetal Bovine Serum and phytohemagglutinin. The flasks were incubated at 37ºC with 5% CO_2 in a CO_2 incubator.

3.5.3 Treatment groups

The treatment groups setup include

- Cells alone
- Cells + Standard (Etoposide)
- Cells + hydroethanolic extract of *Withania somnifera* and *Terminalia bellirica*
- Cells + silver nanoparticles of *Withania somnifera* and *Terminalia bellirica*

- Cells + silver nanoparticles loaded liposomes of *Withania somnifera* and *Terminalia bellirica*

3.5.4 MTT Dye Reduction Assay

Cell viability was evaluated by the reduction of 3-(4,5- dimethylthiazol-2-yl)- 2,5-diphenyltetrazolium bromide (MTT) as described by Igarashi and Miyazawa (2001)

Principle:

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.

Reagent:

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

Procedure:

The treated Molt-3 cells and PBL were centrifuged and the medium was removed and then incubated with 50µl of MTT at 37ºC for 3 hours. After incubation, 200µl of PBS was added to all samples and the liquid was then carefully aspirated. Acid propanol of 200µl was added and left overnight in the dark. The absorbance was read at 650nm in a micro titer plate reader (Bio RAD, USA). The optical density of the control cells were fixed to be 100% viable and the per cent viability of the cells in the treatment groups were calculated using the formula

$$\text{Percentage viability} = \frac{\text{Control OD} - \text{Sample OD}}{\text{Control OD}} \times 100$$

The results obtained for all the parameters analysed are presented in the next chapter.

4

RESULTS AND DISCUSSION

People all over the world, particularly in Asian countries such as India, Japan, and China, as well as some African countries, used plants to treat a variety of diseases such as cancer, oxidative stress-mediated diseases, pain, depression, diarrhoea, thrombosis, and fever. Because of their low cost and widespread availability, the plants are widely used to treat a variety of diseases (Jahan and Ahmet, 2020). The medicinal properties of plants are due to the presence of various phytochemicals such as terpenoids, flavonoids, tannins, polyphenols, and lignins (Alam *et al.,* 2021).

Nanotechnology has been widely used in medicine in recent years due to the unique properties of nanomaterials, which include chemical, physical, and optical properties. Silver nanoparticles have anticancer and antimicrobial properties. Anticancer drugs are delivered to the tumour site by silver nanoparticles. The cancer cells absorb the silver nanoparticles, which causes them to die. With the help of silver nanoparticles, targeted drug delivery for cancer is now possible (Gomes *et al.,* 2021).

Liposomes are lipid vesicles that serve as a vehicle for drug delivery to a specific site. Their primary benefit is that they are biodegradable and biocompatible, and they can contain both hydrophilic and hydrophobic drugs. They are even capable of encapsulating nanoparticles. Liposomes act as a protective vesicle for the drug being encapsulated, shielding it from physiological degradation. They typically increase the shelf life of the encapsulated drug and provide controlled release. Nanoparticles can be used targeted drug delivery to avoid undesired side effects (Liu *et al.,* 2022).

In tune with the above cited literature, the present study focuses on "Second Order Targeting using Nanocarriers of Phytoformulation". The hydroethanol leaf extract of *Withania somnifera* and the dry fruit extract of *Terminalia bellirica* were evaluated, for their antioxidant activity of the extracts of *Withania somnifera* and *Terminalia bellirica.* As a result of their potent antioxidant capacity, an attempt was made to synthesise and characterise silver nanoparticles and silver nanoparticles loaded liposomes from *Withania somnifera* and *Terminalia bellirica* extracts, and also to investigate the anticancer potential of silver nanoparticles and silver nanoparticles loaded liposomes of *Withania somnifera* and *Terminalia bellrica* against molt-3 cells from various treatment groups.

The present study is divided into four phases and the results are discussed under the following headings.

Phase I

4.1 Extraction preparation

4.1.1.*Withania somnifera* leaf and *Terminalia bellirica* fruit hydroethanolic extract preparation

Phase II

4.2 Antioxidant activity- DPPH Radical Scavenging Assay

Phase-III

4.3 Synthesis and Characterisation of Silver nanoparticles and Silver nanoparticles loaded Liposomes of *Withania somnifera* leaf extract and *Terminalia bellirica* dry fruit extracts

4.3.1 Sunlight mediated green synthesis of silver nanoparticles

4.3.2 Characterisation of green synthesized *Withania somnifera* and *Terminalia bellirica* silver nanoparticles

4.3.2.1 UV-Visible spectroscopy

4.3.2.2. X-ray diffraction

4.3.2.3. FTIR Analysis

4.3.3. Synthesis of silver nanoparticles loaded liposomes

4.3.3.1 Encapsulation efficiency of silver nanoparticles loaded liposomes

4.3.3.2. Field Emission Scanning Electron Microscope (FESEM)

Phase-IV

4.4 Designed to analyse Anticancer potential of Silver nanoparticles and Silver nanoparticles Loaded Liposomes of *Withania somnifera* and *Terminalia bellirica* against Molt-3 cells and Peripheral Blood Lymphocytes (Normal cells).

4.4.1 MTT Dye Reduction Assay

Phase I

4.1 Extraction preparation

4.1.1. *Withania somnifera* leaf and *Terminalia bellirica* fruit hydroethanolic extract preparation

The hydroethanol leaf extract of *Withania somnifera* and the dry fruit extract of *Terminalia bellirica* were prepared. The coarse powder (10g) weighed, then dissolved in 100ml of hydroethanol. The solvents used for phytoconstituent extraction were ethanol and water in appropriate proportions such as water 20: ethanol 80, water 40: ethanol 60, water 60: ethanol 40, water 80 : ethanol 20, and water 50: ethanol 50 and were kept overnight in a shaker incubator. Then filtered through the whatmann no.1 filter paper extracts and the filtrate obtained was then transferred to beaker and evaporated to dryness. Equal proportion of *Withania somnifera* and *Terminalia bellirica* were mixed to form the phytoformulation which was used for all the assays performed in the present study.

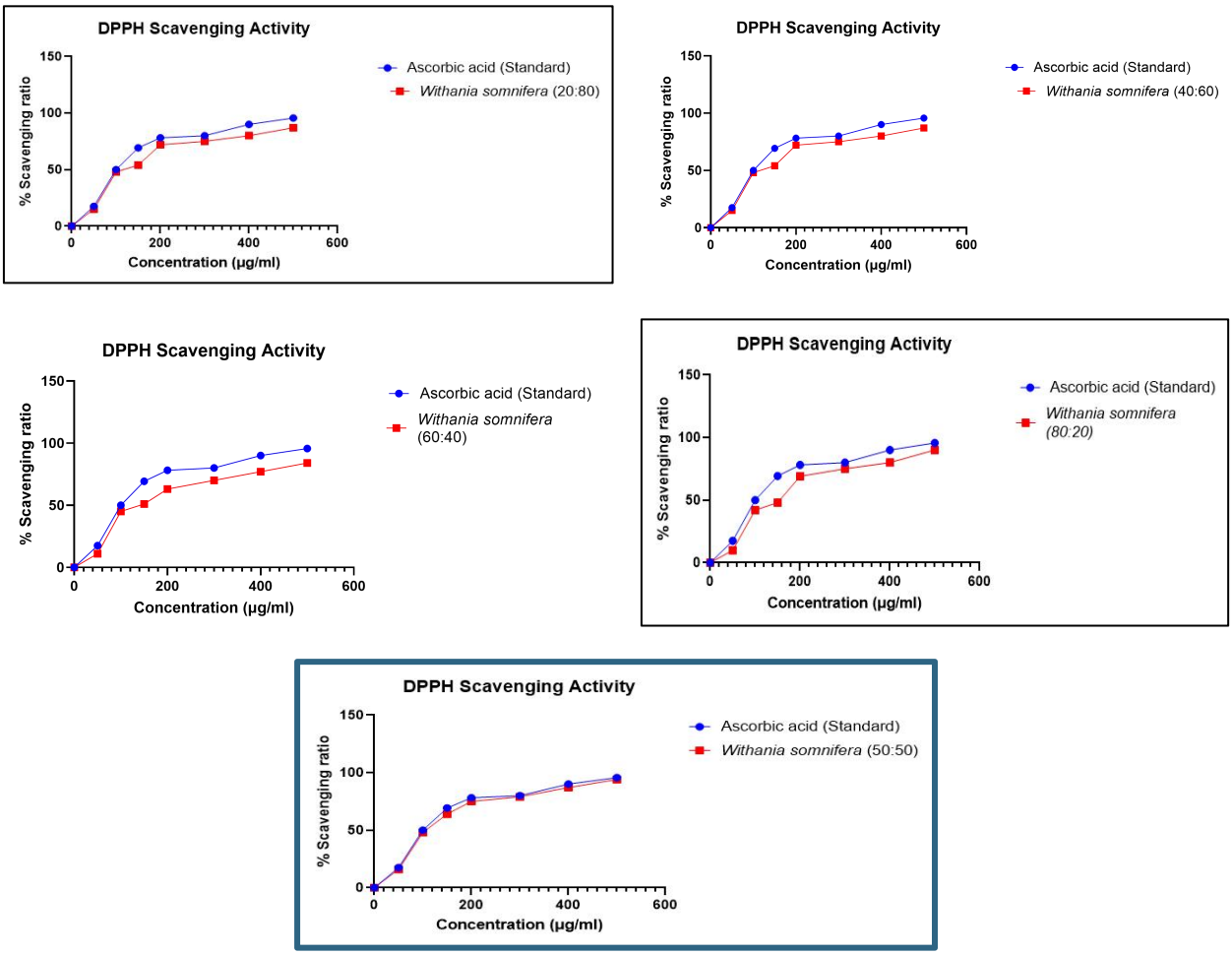
Phase II

4.2 Antioxidant activity- DPPH Radical Scavenging Assay

The stable free radical 2,2-diphenyl-1-picrylhydrazyl is used to assess the antioxidant capacity of plant samples. The antioxidants in the samples convert the purple DPPH to a yellow solution that can be measured at 517nm. The DPPH assay is widely used because it is dependable, quick, time consuming, simple, and inexpensive (Lalhminghlui and Ganesh, 2018).

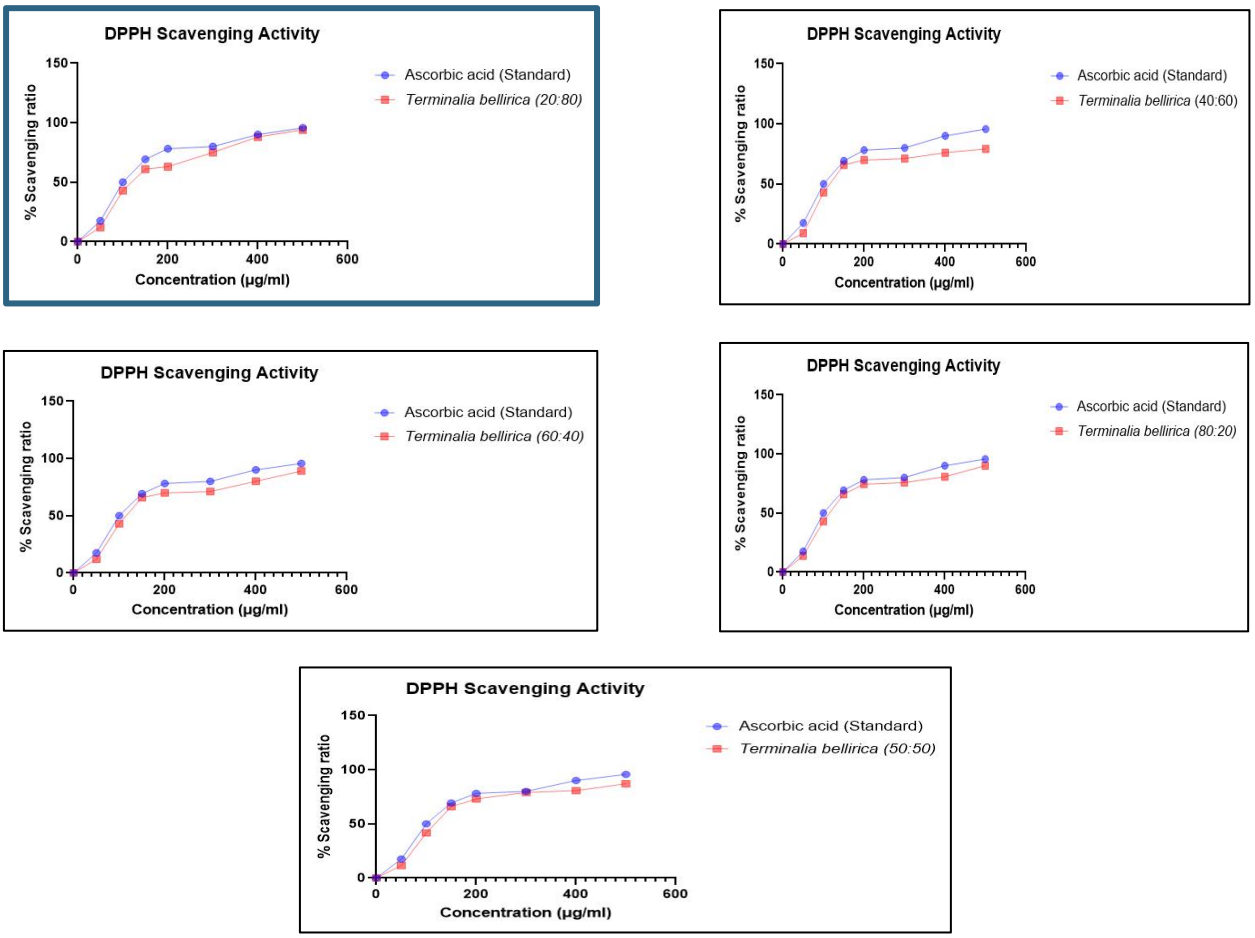
The assay was carried out using different concentrations (50, 100, 150, 200, 300, 400, and 500 µg/ml) of hydroethanol leaf extract of *Withania somnifera* and dry fruit extract of *Terminalia bellirica*, with Ascorbic acid as the standard. The results revealed that all of the tested samples and the standard could scavenge DPPH radicals in a dose-dependent manner. The samples were able to scavenge the DPPH radical even at low concentrations, and the scavenging ability was found to increase with increasing concentration, demonstrating the samples dose-dependent activity. The hydroethanol leaf extract of *Withania somnifera* and the dry fruit extract of *Terminalia bellirica* in various proportions such as water 20%: ethanol 80%, water 40%: ethanol 60%, water 60%: ethanol 40%, water 80 %: ethanol 20%, and water 50%: ethanol 50% when tested for antioxidant activity revealed that among the various proportions of the extracts tested, the *Withania somnifera* (50:50) and *Terminalia bellrica* (20:80) were found to possess high DPPH scavenging activity followed by other proportions. The results of the DPPH radical scavenging ability clearly indicate that all the samples tested shared its electron to the DPPH radical thereby exhibiting scavenging ability.

Figure.9:DPPH Radical scavenging assay of the hydroethanolic extracts of *Withania somnifera*



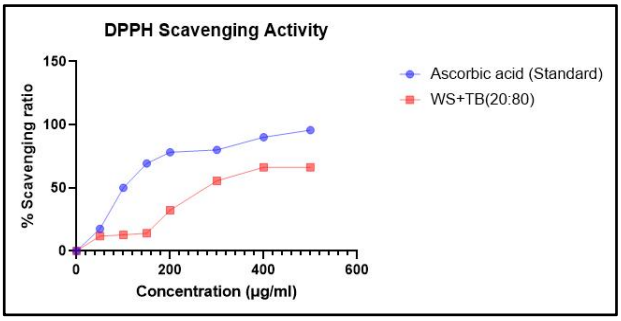
Among the five different proportions of hydroethanolic extract of *Withania somnifera* used for the DPPH radical scavenging assay, the extract with 50% of water and 50% of ethanol exhibited a good scavenging potential against DPPH as given in Figure. 9

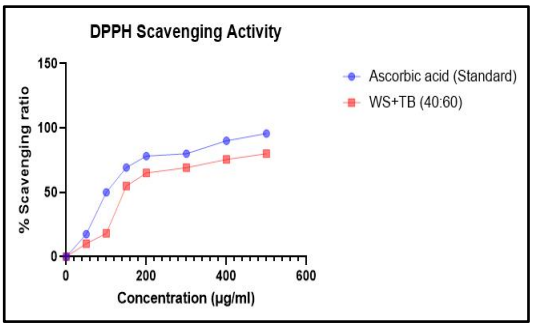
Figure.10:DPPH Radical scavenging assay of the hydroethanolic extracts of *Terminalia bellirica*

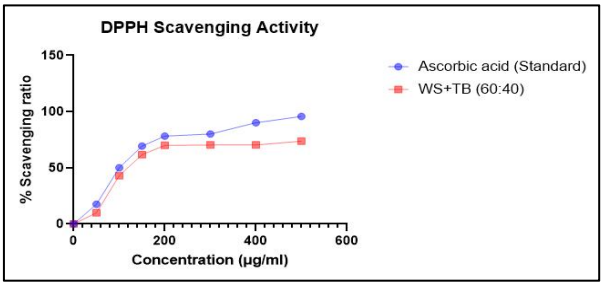


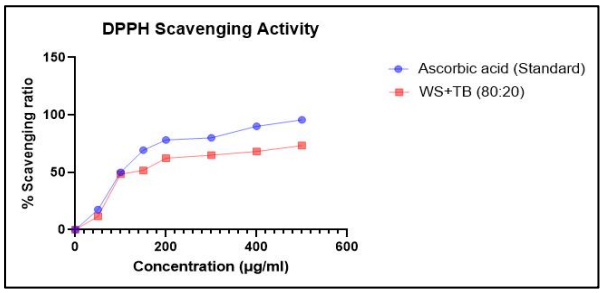
Among the five different proportions of hydroethanolic extract of *Terminalia bellirica* used for the DPPH radical scavenging assay, the extract with 20% of water and 80% of ethanol exhibited a good scavenging potential against DPPH as given in Figure. 10

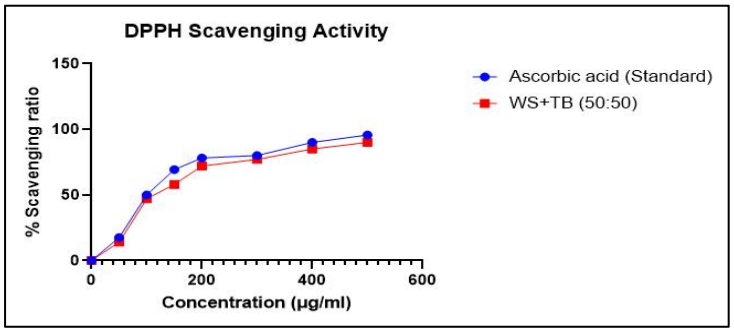
Figure.11:DPPH Radical scavenging assay for the formulation of *Withania somnifera* and *Terminalia bellirica*











The present study aims to prepare a phyto formulation using the hydro ethanolic extracts of *Withania somnifera* and *Terminalia bellirica*, for which the hydro ethanolic extract with maximum scavenging potential against DPPH was chosen. The selected extracts that is *Withania somnifera* (50:50) and *Terminalia bellirica* (20:80) were added in equal volume to get the phytoformulation and the obtained formulation was further subjected for nanoparticle synthesis using silver.

The phytochemical constituents that act as antioxidants can help detoxify free radicals such as reactive oxygen species that may be produced within the human body as a result of oxidation processes. Oxidative stress occurs when the human body is unable to manage oxidant agents due to a faulty antioxidant system. These conditions resulted in a variety of metabolic and other healthcare issues. In line with our observations, Hussian *et al.* (2022) prepared hydroalcoholic extracts from *Withania somnifera* stem bark, root bark, and leaves and carried out DPPH radical scavenging assay and observed that *Withania somnifera* root and leaves possess high antioxidant potential against DPPH radical when compared to the extracts of other parts.

Kuna *et al.* (2022) reported the antioxidant activity of the UNIM formulation (Poly herbal unani formulation) is measured using DPPH scavenging activity. According to the findings, the hydro ethanol extract of UNIM 302 had the highest scavenging activity, with an IC50 value of 229g/ml, aqueous extract of UNIM 301 demonstrated the lowest scavenging activity, with an IC50 value of 412g/ml. However, the study found that UNIM 302 had better antioxidant properties than UNIM 301 when compared to a positive control standard of ascorbic acid. Jamkhande *et al.* (2014) Elucidated that the extracts had antibacterial activity were also examined for antioxidant activity using the DPPH method; DPPH is a relatively stable free radical readily reducible by antioxidants. The DPPH method is routinely used as a valid, accurate and sensitive procedure to evaluate the radical scavenging activity of antioxidants.

In accordance with the above supporting studies, it is confirmed that all the tested samples of *Withania somnifera* and *Terminalia bellirica* phytoformulation donated an electron to the DPPH radical thereby exhibiting scavenging potential. The phenolic compounds, flavonoids and tocopherol present in the leaf extract might be involved in the scavenging action.

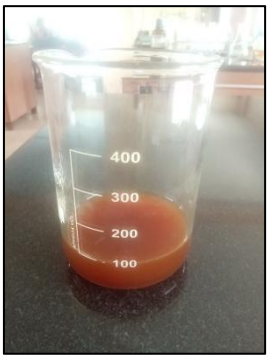
Phase-III

4.3 Synthesis and Characterisation of Silver nanoparticles and Silver nanoparticles loaded Liposomes from *Withania somnifera* leaf extract and *Terminalia bellirica* dry fruit extracts

4.3.1 Sunlight mediated green synthesis of silver nanoparticles

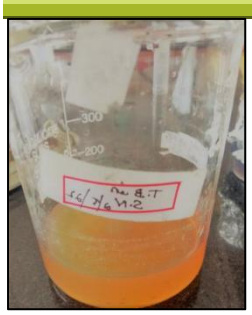
Silver nanoparticles synthesis was done using 2 different combinations of various solvents like water, ethanol, water 50: ethanol 50 of *Withania somnifera* leaf extract and water 20: ethanol 80 of *Terminalia bellirica* dry fruit extract by mixing with the 1mM aqueous solution of silver nitrate. On exposure to direct sunlight the silver ions were reduced to AgNPs which were confirmed by the colour transformation of the solution from green, the natural colour of the extract to dark reddish brown from this colour change, and as reported by Das *et al*. (2017) it is clearly evident that *Withania somnifera and Terminalia bellirica extracts* acts as an effective reducing agent that reduces silver ions to silver nanoparticles.

Before exposure to sunlight (*Withania somnifera*)

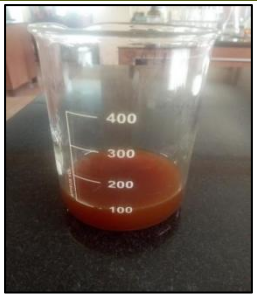


After exposure to sunlight for 30 mins (*Withania somnifera*)

Plate 1: *Withania somnifera*



Before exposure to sunlight *(Terminalia bellirica)*



After exposure to sunlight for 30 mins (*Terminalia bellirica)*

Plate 2: Terminalia bellirica

Vinh *et al.* (2020) made are attempt to synthesise silver nanoparticles from Pomelo peel extract and observed a colour change from yellow to reddish brown, indicating the ability to reduce silver. Mounil *et al.* (2020) reported similar results for the synthesis of silver nanoparticles using *Azadirachta indica* leaf extract. In line with our findings, Fucangfahkun *et al.* (2020) synthesised AgNP from Durian rind extract and observed a colour shift from pale yellow to dark brown and reported that the colour change is caused by electron oscillations on the surface of the nanoparticles, a phenomenon known as Surface Plasmon Resonance. The change in solution colour from pink to dark red indicates the synthesis of silver nanoparticles using *Vaccinium arctostaphylos* fruit aqueous extract (Khodadadi *et al.,* 2021). Vivek *et al.* (2021) synthesised silver nanoparticles from *Ocimum tenuiflorum* aqueous extract and reported a colour change from light yellow to brownish yellow due to silver ion reduction to AgNP's. It is clear that various phytochemicals found in plant extracts play a role in the reduction process.

According to the findings of the previous studies, *Withania somnifera* and *Terminalia bellirica,* the candidate plants of the current study, have the ability to reduce silver ions to silver nanoparticles, which is confirmed by the colour transformation of the solution from green to dark reddish brown. As observed from the supporting articles cited, the presence of various secondary

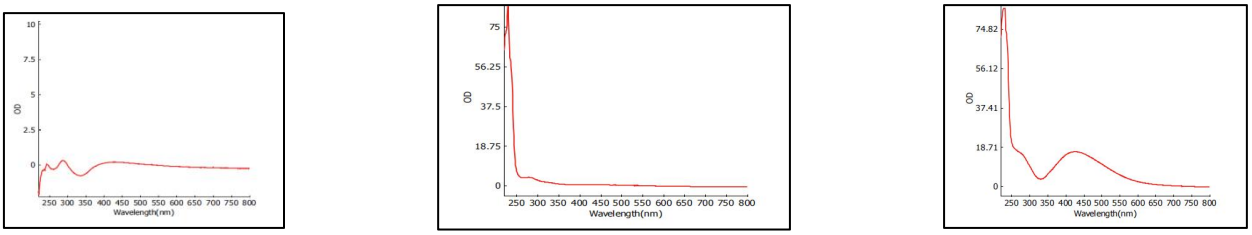
metabolites present in plant extracts of various solvents contributes to this reducing ability. This colour change is caused by the solution's surface Plasmon resonance excitation.

4.3.2 Characterisation of green synthesized *Withania somnifera* and *Terminalia bellirica* silver nanoparticles

The AgNPs were characterised using UV-Vis spectroscopy, Fourier transform infrared spectroscopy (FTIR) analysis, Field Emission Scanning Electron Microscope (FESEM) and X-ray diffraction (XRD).

4.3.2.1 UV-Visible spectroscopy

UV-Vis spectroscopy is a technique used to monitor the production of nanoparticles during the early stages of synthesis. When nanoparticles are generated from their respective salts, a prominent and well-defined peak appears in the visible spectrum. According to earlier study, the UV-Vis absorption band between 200 and 800 nm is the optimum for characterisation of nanoparticles. The conduction and valence bands of silver nanoparticles are usually closer together, making electron transportation easier and resulting in a surface Plasmon resonance peak. The absorption of silver nanoparticles is affected by particle size, dielectric medium, and nearby compounds (Almatroudi and Ahmed, 2020).



a b c

a-Hydroethanolic extract of *Withania somnifera*; b-Silver nitrate of *Withania somnifera;* c-Silver nitrate of *Terminalia bellirica*

Figure.12:UV -Vis spectral analysis of the synthesized silver nanoparticles

The UV-Vis spectra of silver nitrate extracted from *Withania somnifera* and *Terminalia bellirica*, as well as synthesised silver nanoparticles, were measured from 200 to 800nm. The green synthesised *Withania somnifera* silver nanoparticles had a strong, well-defined single spectrum at 250 nm, which is typical of AgNPs. The green synthesised *Terminalia bellirica* silver nanoparticles had a strong, well-defined single spectrum at 250-300 nm, which is one of the characteristics of AgNps. Single absorption spectra, according to Mie's theory, indicate that the silver nanoparticles formed will be spherical in shape. In contrast, anisotropic particles in nature exhibit two or more absorption peaks based on their shape (François *et al.,* 2016). The synthesised AgNPs in this study exhibited a single Surface Plasmon Resonance band (Figure. 12b), confirming their spherical shape.

Silver nanoparticles synthesised by Savan and Sumitra (2021) from *Mangifera indica* seed aqueous extract exhibited the typical Silver Plasmon Absorption maximum at 450 nm. Sedighe *et al.* (2021) observed an absorption peak at 443 nm for AgNPs prepared from *Vaccinium arctostaphylos* leaves. Our findings are consistent with those of Dogiparthi *et al.* (2021), who discovered a maximum absorption band at 440 nm for silver nanoparticles synthesised with *Micrargeria wightii* extract. Almasoud *et al.* (2020) green synthesised silver nanoparticles using *Ficus carica* and *Salvia rosmarinus* leaf extracts and observed a characteristic UV-Vis peak at 450 nm, which is consistent with our findings. Tomato fruit extract silver nanoparticles exhibited a peak at 450 nm (Kishore *et al.*, 2020).

In agreement with the previous reports, the current study confirms the formation of spherical shaped silver nanoparticles of *Withania somnifera* leaf extract, as evidenced by the single peak at 250 nm in UV-Vis spectroscopy, and dry fruit extract of *Terminalia bellirica*, as evidenced by the single peak at 250-300 nm in UV-Vis spectroscopy. The characterization of these synthesised silver nanoparticles was further investigated.

4.3.2.2. X-ray diffraction

XRD is a common technique used in the characterization of silver nanoparticles. It is used to determine the crystalline structure, size, and phase nature of synthesised AgNPs (Mourdikoudis *et al.,* 2021). Figure 13 depicts the XRD pattern of the prepared silver AgNPs. The X-ray diffraction pattern of synthesised silver nanoparticles shows several major diffraction peaks at 10.49° and 22.91°, corresponding to the (248) and (370) planes, respectively. These diffraction patterns indicate that the synthesised nanoparticles have an Face Centered Cubic Crystalline (FCCC) structure. The high intensity of these diffraction peaks indicates that the AgNPs are crystalline.The particle size was calculated using the Scherrer equation and the 2θ values of the diffraction pattern. Silver nanoparticles were 240 nm in diameter at 10.4937° and 400 nm in diameter at 22.9105° (XRD Crystallite (grain) Size Calculator (Scherrer Equation) - InstaNANO, 2022). The mean size was found to be 32.18 nm which is almost similar to the average size obtained using FE-SEM analysis.

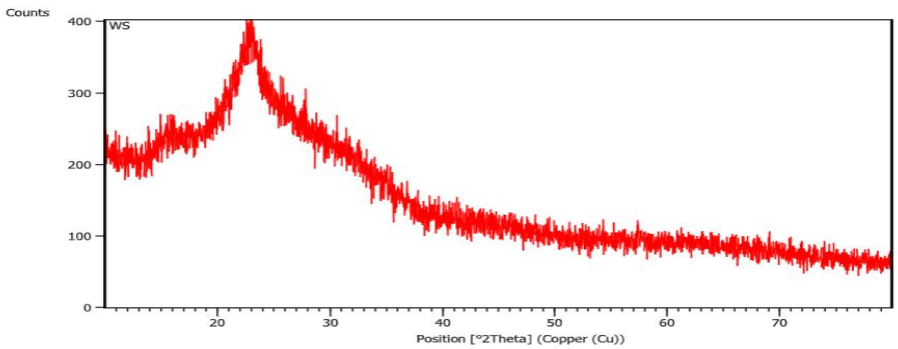


Figure.13:X-ray diffraction spectrum of AgNPs synthesized using *Withania somnifera*

S.No	Pos. [°2Th.]	Height [cts]
1	10.4937	9.18
2	22.9105	68.95

Table 1:X-ray diffraction of AgNPs synthesized using *Withania somnifera*

The XRD pattern of the prepared silver AgNPs is shown in the Figure. 14. The X ray diffraction pattern of synthesized silver nanoparticles reveals various major diffraction peaks at 10.47°, 21.60°, 27.88°, 32.37°, 38.24°, 46.35°, 54.97°, 57.68°, 64.57°, 67.59°, 74.55°, 76.77° and 77.41° which corresponds the (128), (144), (361), (763), (565), (388), (120), (121), (148), (66), (60), (115) and (168) planes respectively. These diffraction patterns indicate that the synthesised nanoparticles have an Face Centered Cubic Crystalline (FCCC) structure. The high intensity of these diffraction peaks indicates that the AgNPs are crystalline.The particle size was calculated using the Scherrer equation and the 2θ values of the diffraction pattern. Silver nanoparticles were 128 nm in diameter at 10.47°, 144 nm in diameter at 21.60°, 361 nm in diameter at 27.88°, 763 nm in diameter at 32.37, 565 nm in diameter at 38.24°, 388 nm in diameter at 46.35°, 120 nm in diameter at 54.97°, 121 nm in diameter at 57.68°, 148 nm in diameter at 64.57°, 66 nm in diameter at 67.59°, 60 nm in diameter at 74.55°, 115 nm in diameter at 76.77° and 168 nm in diameter at 77.41° (XRD Crystallite (grain) Size Calculator (Scherrer Equation) - InstaNANO, 2022). The mean size was found to be 50.03 nm which is almost similar to the average size obtained using FE-SEM analysis.

The XRD spectrum of AgNPs prepared with *Plantago lanceolata* extract revealed different diffraction peaks at 2θ values of 23.52° and 27.83°. 38.23°, 44.25°, 46:23°, 54.80°, and 57.50° are the coordinates. The intensity of these peaks is very high, indicating that the synthesised AgNPs are crystalline. The nature of these peaks reveals that the synthesised AgNPs have an FCCC structure. The size of the silver nanoparticles synthesised was determined to be 30±4 nm (Shah *et al.,* 2021).

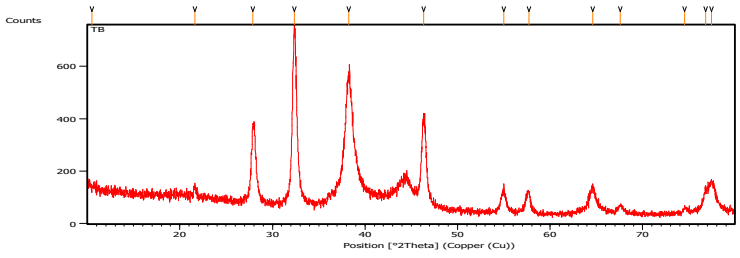


Figure.14:X-ray diffraction spectrum of AgNPs synthesized using *Terminalia bellirica*

S.No	Pos. [°2Th.]	Height [cts]
1	10.4753	20.21
2	21.6092	42.16
3	27.8836	289.68
4	32.3778	675.21
5	38.2450	415.57
6	46.3543	297.10
7	54.9753	81.14
8	57.6857	85.24
9	64.5762	90.91
10	67.5935	26.30
11	74.5513	19.44
12	76.7706	79.99
13	77.4151	114.02

Table 2:X-ray diffraction of AgNPs synthesized using *Terminalia bellirica*

(Hemlata *et al*., 2020) obtained comparable results when they synthesised AgNPs from *Cucumis prophetarum* extract and subjected them to XRD analysis. According to the XRD analysis, the synthesised AgNPs are FCCC in nature, with diffraction patterns at 2θ values of 32.18°, 38.04°, 46.13", 54.63°, and 77.08", which correspond to the (111), (200), (120), (202), and (311) planes, respectively. The XRD pattern of the green synthesised AgNPs using *Salvia officinalis* aqueous leaf extract coincides with the various peaks at 2θ values 27.82°, 38.08°, indicating the FCCC structure of the nanoparticles (Okaiyeto *et al.,* 2021). The *Abelmoschus esculentus* (L.) pulp extract X-Ray diffraction pattern revealed sharp 20 peaks at 27.2°, 31.63°, 45.66, 54.24°, and 57.04°, indicating the FCCC nature of the nanoparticles (Masud *et al.*, 2020).

As a result of characterization, green synthesised *Withania somnifera* silver nanoparticles with a mean size of about 32.18 nm and green synthesised *Terminalia bellirica* silver nanoparticles with a mean size of about 50.03 nm was confirmed. The size of the nanoparticles has a significant impact on the X-ray diffraction pattern. The various phytoconstituents found in plant extracts acted as a capping agent and stabilised the silver nanoparticles, resulting in the AgNP's crystalline nature (Rasheed *et al*., 2018). Thus, based on the findings, it can be concluded that the green synthesised *Withania somnifera* leaf extracts and dry fruit of *Terminalia bellirica* resulted in the formation of spherical nanoparticles containing 13.31 and 100% silver with a face-centered cubic crystalline nature. Because *Withania somnifera* leaf extracts and *Terminalia bellirica* dry fruit could provide a good support for nanoparticle synthesis. Further to achieve targeted delivery of the green synthesized plant extracts, it is necessary to prepare a liposome carrier for the synthesised nanoparticles.

4.3.2.3. FTIR Analysis

FTIR is a common technique used by researchers and industrialists to analyse the composition and structure of molecules. It is a quick and dependable tool for determining the functional group of substances. FTIR is an infrared reflectance and absorption spectroscopy. This technique is widely used by researchers to characterise materials (Fahelelbom *et al.*, 2022).

The FTIR spectrum of green synthesized AgNP of *Withania somnifera* produces various peaks which correspond to the following functional groups: peaks at 424.34 cm^{-1} and 455.20 cm^{-1} represent halogen compound [Iodo-compound] (C-I). Various peaks at 601.79 cm^{-1}, and 671.23 cm^{-1} corresponds to C-Br stretching of Halo Compounds. An FTIR spectrum at 702.09 cm^{-1} , 948.98 cm^{-1} and 1070.10 cm^{-1} indicates C-C bending of Alkenes. A peak at 1165.00 cm^{-1} corresponds to CO-O-CO stretching of Sulfoxide. Another peak at 1319.31cm^{-1} indicates C-O stretching of Aromatic ester. A peak at 1435.04 cm^{-1} indicates O-H bending of Carboxylic Acid functional group. Another peak at 1666.50 cm^{-1} C-C stretching of Alkene groups. Peaks at 3001.24 cm^{-1} represent C-H stretching of Alkane and Alkene respectively. A peak at 3394.72 cm^{-1} represents N-H streching of Aliphatic Primary Amines.

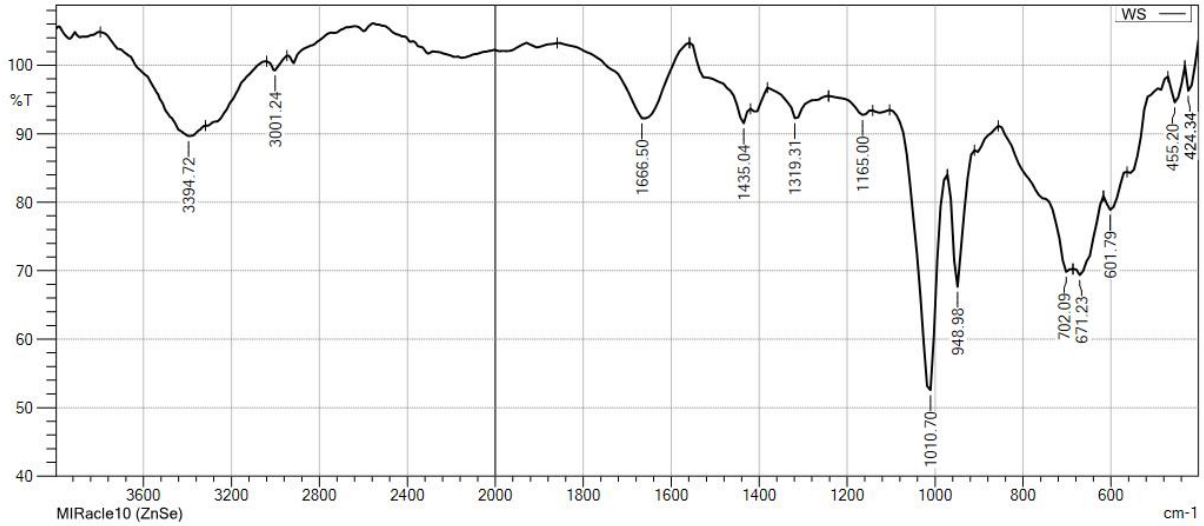


Figure.15: FTIR spectral analysis of green synthesized *Withania somnifera* silver nanoparticles

The FTIR spectrum of green synthesized AgNP of *Terminalia bellirica* produces various peaks which correspond to the following functional groups:peaks at 416.62cm^{-1}, 439.77cm^{-1} , 470.63cm^{-1}, 501.49cm^{-1}, 555.50cm^{-1} and 594.08cm^{-1} represent halogen compound [Iodo-compound] (C-I). Various peaks at 648.08 cm^{-1} and 678.94 cm^{-1} corresponds to C-Br stretching of Halo Compounds. An FTIR spectrum at 725.23 cm^{-1}, 756.10 cm^{-1}, 871.82 cm^{-1}, 948.98 cm^{-1} and 1033.85 cm^{-1} indicates C-C bending of Alkenes. A peak at 1203.58 cm^{-1} represent C-O stretching of Vinyl Ether. Another peak at 1327.03cm^{-1} indicates C-O stretching of Aromatic Ester. A peak at 1458.18 cm^{-1}indicates O-H bending of Carboxylic acid functional group. Another peak at 1591.91 cm^{-1} and 1612.49 cm^{-1} C-C stretching of Alkene groups. An FTIR band at 1705.07 cm^{-1} indicates C=O stretching of Aldehyde group. Peak at 2306.86 cm^{-1} represents C-N stretching of Ether. Peaks at 2846.93 cm^{-1} and 2916.37 cm^{-1} corresponds to C-H stretching of Alkane group. A peak at 3718.76 cm^{-1} represents N-H stretching of Aliphatic Primary Amines.

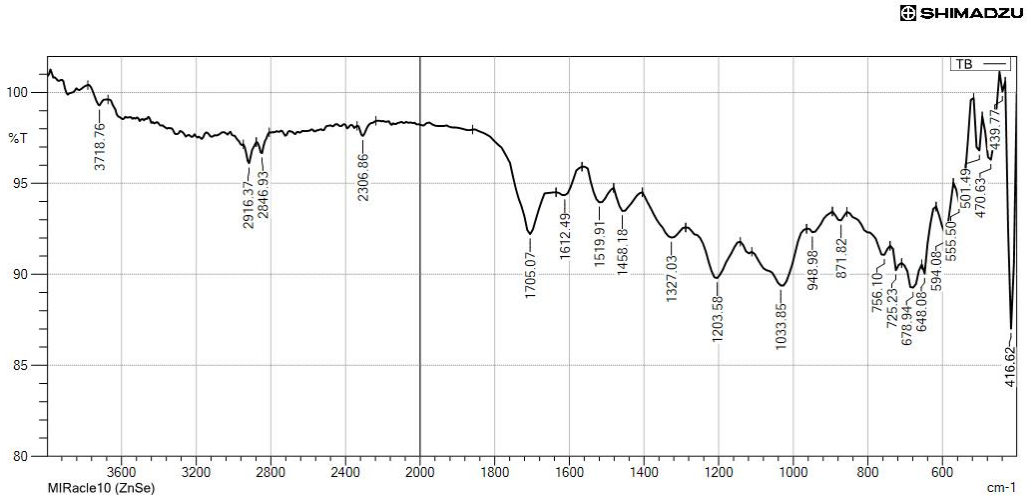


Figure.16: FTIR spectral analysis of green synthesized *Terminalia bellirica* silver nanoparticles

In line with our findings, Vinodhini *et al*. (2022) synthesised silver nanoparticles from *Tabernaemontana divaricate, Basella alba, and Allium fistulosum* extracts and analysed their functional groups using FTIR analysis, claiming that various functional groups found in plant extracts act as capping agents and are involved in the production of silver nanoparticles. Chandrasekharan *et al.* (2022) synthesised silver nanoparticles from *Gmelina arborea* extract and discovered that various functional groups such as alcoholic groups, hydroxyl flavonoids, carbonyl or carboxylic groups, phenolic and amino groups are used in the reduction process. These functional groups serve as a capping agent during the formation of silver nanoparticles and are also involved in the stabilisation of the nanoparticles that are synthesised. Sumaiya *et al.* (2022) performed green synthesis of AgNPs using *Alhagi graecorum* leaf extract and reported that the plant extract is complex in nature with a number of functional groups present and that when the plant extract is allowed to reduce the silver nitrate to silver nanoparticles, there is a drastic reduction in the functional groups, indicating the purity of the synthesised nanoparticles.

According to the FTIR results, the different functional groups present in the green synthesised AgNP of *Withania somnifera* and *Terminalia bellirica* are used in the reduction of

silver nitrate to silver nanoparticles and also act as reducing agents, improving the stability of the synthesised nanoparticles.

4.3.3. Synthesis of silver nanoparticles loaded liposomes

The AgNPs loaded liposomes were prepared using thin film hydration method coupled with sonication using triglyceride lipid lecithin and cholesterol in the molar ratio 2:1. Chloroform was used as the dissolving solvent for lipids.

4.3.3.1 Encapsulation efficiency of silver nanoparticles loaded liposomes

The amount of drug encapsulated in a drug delivery system is critical because it plays a direct role in the system's therapeutic effect. Encapsulation efficiency is defined as the percentage of drug or nanoparticles successfully entrapped by the lipid vesicle. Many factors influence encapsulation efficiency, including incubation time, lipid composition, lipid-to-drug ratio, and aqueous phase pH. The formula is used to calculate the encapsulation efficiency.

$$\text{Encapsulation Efficiency} = \frac{\text{Amount of Encapsulated Nanoparticle}}{\text{Amount of Encapsulated } + \text{Free Nanoparticle}} \times 100$$

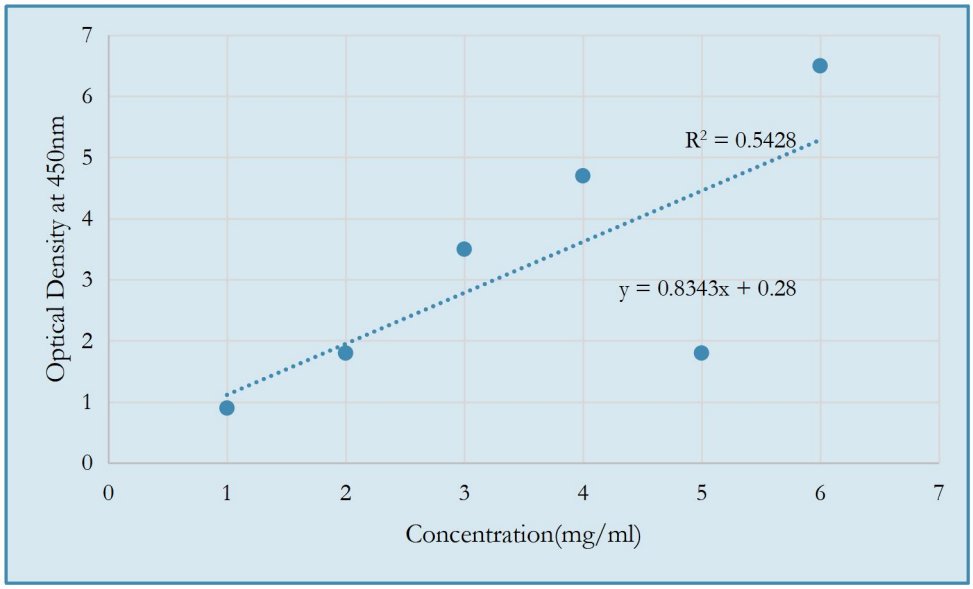


Figure.17:Encapsulation efficiency of silver nanoparticles loaded liposome

According to Figure. 17, the amount of encapsulated nanoparticle was 3.499 mg, and the amount of encapsulated + free nanoparticle was 5 mg (amount of nanoparticle present in the liposome initially). Using these values in the above formula, the encapsulation efficiency was calculated to be 69.98%. This high encapsulation efficiency is primarily due to the silver nanoparticle's low aqueous solubility, which allowed them to incorporate into the liposome's hydrophobic region. Furthermore, due to the appropriate ratio of lecithin, cholesterol, and silver nanoparticles, there is sufficient space in both lecithin and cholesterol for the binding of silver nanoparticles, which increased the encapsulation efficiency (Nayyer *et al.* 2019)

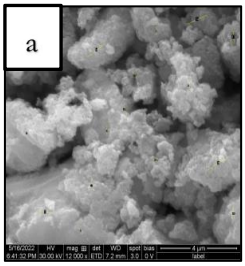
In line with our findings, Hardiansyah *et al.* (2017) loaded curcumin into both PEGylated magnetic liposomes and PEGylated liposomes and discovered that PEGylated liposomes have a higher curcumin encapsulation efficiency (78.06±0.57%) than PEGylated magnetic liposomes (76.15±1.6%). Curcumin was encapsulated in the lipophilic part of the liposome's lipid bilayer, and Najlah *et al*. (2019) created a disulfiram-encapsulated PEGylated liposome with an encapsulation efficiency of more than 80%. Ng *et al*. (2018) made similar observations when they prepared curcumin and salbutamol loaded liposomes and discovered that the encapsulation efficiency was 81.1% and 88.6%, respectively. This high encapsulation efficiency was achieved through the use of a suitable lipid-to-drug ratio.

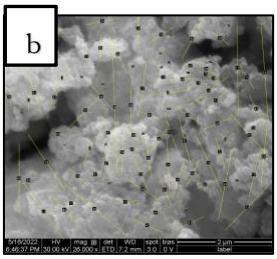
The current study's findings suggest that the high encapsulation of silver nanoparticles of *Withania somnifera* and *Terminalia bellirica* into liposomes might be due to the suitable ratio of lipids and nanoparticles, an adequate incubation period, and a suitable lipid composition.

4.3.3.2. Field Emission Scanning Electron Microscope

FESEM is used to examine morphology, size, aggregation, and the distribution of nanoparticles. The image is produced as a result of electron reflection by the samples. It generates high-resolution images of nanoparticles (Gupta *et al.,* 2019). According to the FESEM analysis of silver nanoparticles *Withania somnifera* and *Terminalia bellirica*, the size of the

nanoparticles ranges from 10.47-52.77 nm and 17.88-52.83 nm, respectively and are colloidal and spherical in shape (Figure.18a and Figure 18b).

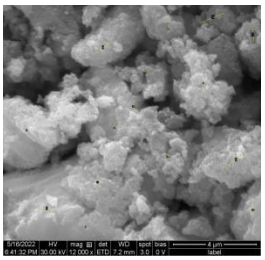


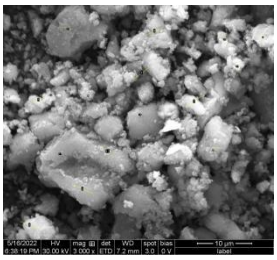


a)FESEM image of AgNPs of *Withania somnifera*

b)FESEM image of AgNPs of Terminalia bellirica

Figure.18: FESEM analysis of silver nanoparticles of *Withania sominifera and Terminalia bellirica*





a)FESEM image of AgNPs of *Withania somnifera* loaded liposome

b)FESEM image of AgNPs of *Terminalia bellirica* loaded liposome

Figure.19: FESEM analysis of silver nanoparticles of *Withania sominifera and Terminalia bellirica* loaded liposome

The size of the silver nanoparticles of *Withania somnifera* loaded liposomes and silver nanoparticles of *Terminalia bellirica* loaded liposomes were found to be ranging between 20.00-90.62 nm and 11.31-87.72 nm and are also spherical in shape (Figure.19a and Figure.19b).

The FESEM analysis of silver nanoparticles from *Azadirachta indica* reveals the formation of spherical AgNPs with an average particle diameter of approximately 33.20±3.79 nm (Chinnasamy *et al.,* 2021). Santhoshkumar *et al.* (2021) observed similar results when they synthesised silver nanoparticles from aqueous leaf extracts of *Piper colubrinum*, performed SEM analysis, and concluded that the synthesised AgNPs range in size from 10 to 50 nm and are spherical. Shah *et al.* (2021) reported the presence of spherical silver nanoparticles with an average size of about 30±4 nm that were synthesised using *Plantago lanceolata* extract. The FESEM micrographs of the turmeric extract loaded nanoliposomes revealed that the synthesized liposomes are uniformly spherical in shape with the size ranges between 74 to 101 nm (Karimi *et al.*, 2019). In another study carried out by Lujan *et al.* (2019) observed uniformly distributed spherical miRNA loaded liposomes with homogenous size.

The current study's findings are consistent with the previously mentioned literatures. According to the findings of this study, spherical shaped silver nanoparticles of varying sizes can be seen in the FESEM micrographs.

Phase-IV

4.4 Designed to analyse Anticancer potential of Silver nanoparticles and Silver nanoparticles Loaded Liposomes of *Withania somnifera* and *Terminalia bellirica* against molt-3 cells and peripheral blood lymphocytes (Normal cells) were evaluated.

4.4.1 MTT Dye Reduction Assay

MTT is a tetrazolium dye containing quaternary tetrazole, a positively charged ring structure containing four nitrogen surrounded by three aromatic rings, a thiazolyl ring, and two phenyl moieties: When the MTT dye is reduced by disrupting its tetrazole ring, a violet coloured formazan molecule is formed that is insoluble in water. Because MTT is positively charged, it easily penetrates the cell membrane as well as the inner mitochondrial membrane of living cells and is reduced to formazan. This assay is primarily used to assess the metabolic activity of cells.

MTT assays were used to determine the cytotoxic effect of *Withania somnifera* and *Terminalia bellirica* hydroethanolic extract, silver nanoparticles of *Withania somnifera* and *Terminalia bellirica*, and silver nanoparticles loaded liposomes all the there at these concentrations (20, 40, 60, 80, and 100 µg/ml). The cells alone were considered as the control in Figure.20, and the viability of the control group cells was set to 100. The current study found that all treatment groups exhibited dose-dependent cytotoxic potential against Molt-3 cells. Among the various treatment groups tested, silver nanoparticles loaded liposomes were found to have a high cytotoxic effect against Molt-3 cells, with a 1C50 value of 32.59 µg/ml, followed by silver nanoparticles (IC50-35.75 µg/ml) and the hydroethanolic extract (IC50-74.28 µg/ml). This observation led to the conclusion that all treatment groups had a significant cytotoxic effect on Molt 3 cells.

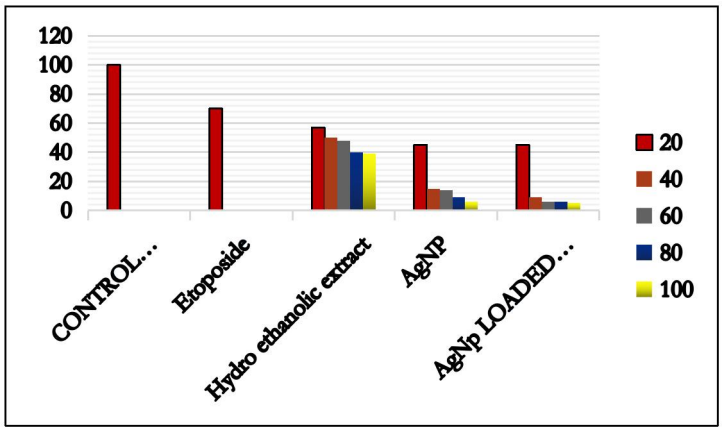


Figure.20: MTT Dye Reduction Assay(MOLT-3)

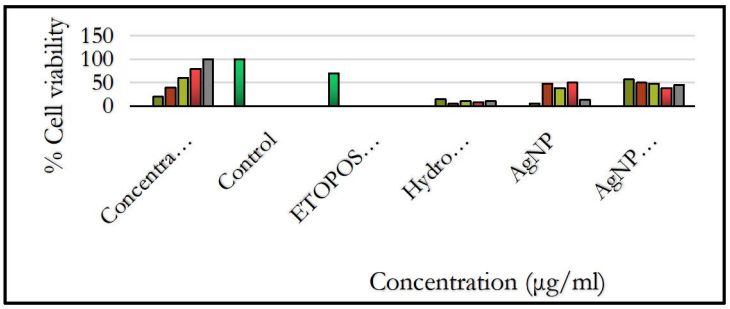


Figure.21: MTT Dye Reduction Assay(PBL)

MTT assay was performed to test the cytotoxic effects of various treatment groups against Peripheral Blood Lymphocytes (Figure. 21), which is the normal counterpart of the Molt-3 cells, to determine whether these treatment groups affect the normal cells. The findings revealed that all treatment groups had very little or no cytotoxicity to normal cells. The cytotoxicity against PBL IC50 values are as follows:hydroethanolic extract: 18.64 µg/ml, Silver nanoparticles: 40.77 µg/ml, and silver nanoparticles loaded liposomes: 42.64 µg/ml. Since the IC50 values were high, it is clear that the various treatment groups exhibited very little or no cytotoxicity to the normal PBL.The treatment groups were cytotoxic to PBL in the following order: hydroethanolic extract > silver particles > silver nanoparticles loaded liposomes. It is clear from this that the silver nanoparticles and silver nanoparticles loaded liposomes exerted a targeted drug delivery that caused toxicity only to Molt-3 cells and did not cause significant cytotoxicity to peripheral blood lymphocytes.

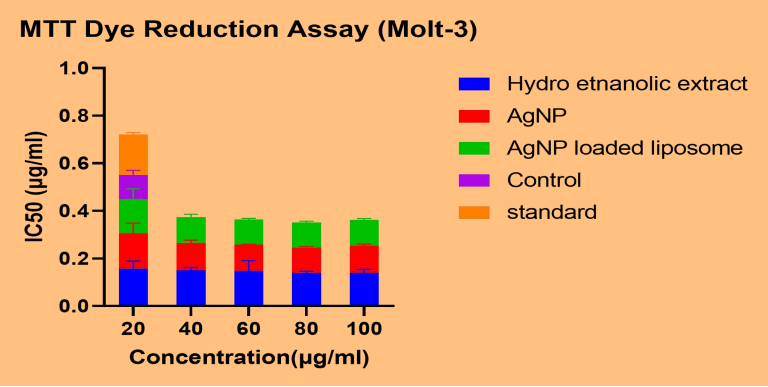


Figure.22: IC50 values for MTT Dye Reduction Assay(MOLT-3)

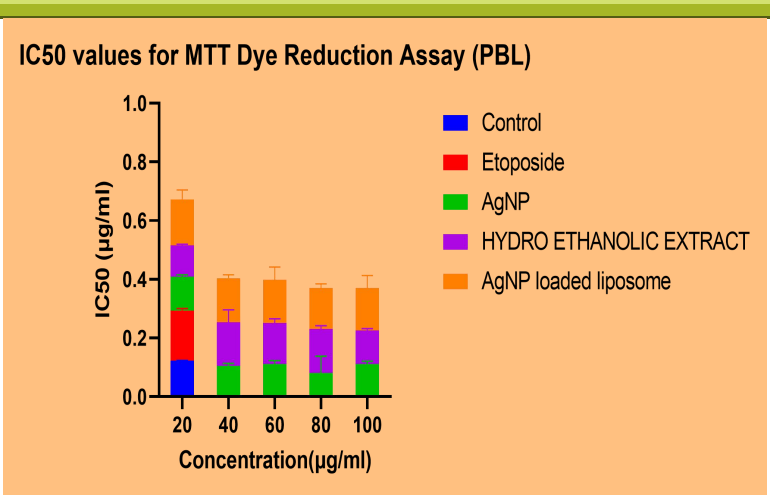


Figure.23: IC50 values for MTT Dye Reduction Assay(PBL)

qKarakaş *et al.* (2017) The MTT assay is a popular cell viability/cytotoxicity test. The MTT assay was used to test three different plant methanol extracts *(Hypericum adenotrichum, Salvia kronenburgii, and Pelargonium quercetorum)* in breast cancer cell lines (MCF-7 and MDA-MB-231) and the results were compared to the ATP assay, which is a much more sensitive and reliable assay due to its interference-free feature. In a study similar to ours, Niksic *et al.* (2021) extracted essential oil from *Thymus garis* (L) and performed an MTT dye reduction assay on three different cell lines (MOL-T 4, H460 and MCF-7). They discovered that the essential oil's cytotoxic activity was concentration dependent. Among the various cell lines studied, essential oil has a high cytotoxic effect on MCF-7 cells, followed by MOLT-4 and H460 cell lines. This is due to the presence of various phytochemicals found in essential oils. Ikram *et al.* (2020) used *Fagonia indica* extract to perform green synthesis of AgNPs and discovered a dose-dependent inhibition of breast cancer cell growth. The possible mechanism for this cytotoxic effect is that nanoparticles increase cancer cell membrane permeability, activate caspases, and generate ROS. Zarrabi *et al.* (2021) created Curcumin-loaded liposomes and stealth liposomes and tested their cytotoxicity against normal cells and cancer cells using the MTT dye reduction assay (MCF7). According to the findings, both curcumin-loaded liposomes and stealth iposomes have no significant cytotoxicity to normal cells, whereas free drug has a significant cytotoxic

effect. Stealth liposomes were found to be highly effective against MCF 7 cells, indicating the liposome's targeted drug delivery nature.

According to the cited literatures and the findings of the current study, silver nanoparticles and silver nanoparticles loaded liposomes of *Withania somnifera* and *Terminalia bellirica* exerted cytotoxicity towards Molt-3 leukemic cells and showing a differential response towards PBL. Normal counterpart of leukemic cells. The observed results indicate that the crude hydro ethanolic extract of *Withania somnifera* and *Terminalia bellirica* showed lesser cytotoxicity into Molt-3 when compared to green synthesized plant extracts using Ag and AgNP of *Withania somnifera* and *Terminalia bellirica* loaded liposomes indicating that the enhanced cytotoxic efficacy evoked might be due to targeted delivery of the plant extracts (*Withania somnifera* and *Teminalia bellirica*) to the leukemic cells. Further the differential response obtained in Molt-3 leukemic cells and its AgNPs of *Withania somnifera* and *Terminalia bellirica* loaded liposomes exerted targeted delivery when compared the standard chemo therapeutic drug etoposide and the crude hydroethanolic extracts of *Withania somnifera* and *Terminalia bellirica*. Further analysis has to be carried out to confirm their second order delivery achieved by the plant extracts to leukemic cells.

5

SUMMARY AND CONCULSION

Cancer is a genetic disease characterised by abnormal cell growth, development, and division. It can start in one part of the body and spread to the rest. It is the world's second leading cause of death. So far, over a hundred different types of cancer have been identified, including leukaemia, a cancer that forms tissues in the blood. It is a type of cancer that affects white blood cells. Leukaemia is classified into two types based on its origin: myeloid and lymphoid leukaemia. They are also classified into acute and chronic leukaemia based on their development time. Acute Lymphoblastic Leukaemia (ALL), Chronic Lymphocytic Leukaemia (CLL), Acute Myeloid Leukaemia (AML), and Chronic Myeloid Leukaemia (CML) are the four most common types of leukaemia that affect both children and adults. The average survival rate for leukaemia is only about 67%.

Chemotherapy, targeted therapy, radiation therapy, bone marrow transplantation, and immune therapy are some of the treatment strategies used for leukaemia. However, the most significant disadvantage of these treatment options is their side effects. When chemotherapy and radiation therapy are used for treatment, many side effects occur, including hair loss, pain, swelling, redness of the skin, nausea, and fatigue. These side effects are primarily caused by the fact that the treatment methods chosen will not only kill cancer cells but will also affect normal cells. As a result, a treatment strategy with few or no side effects is essential.

Silver nanoparticles have recently gained popularity among researchers as an alternative to chemotherapy due to their novel properties. Silver nanoparticles have apoptosis inducing and antiproliferative properties, making them a viable treatment option for cancer. Plant-mediated

green synthesised AgNPs have been found to be effective against various types of cancer. The phytochemicals found in plant extracts act as reducing agents and are also involved in the capping process. Thus, the anticancer potential is attributed to phytochemicals found on the surface of AgNPs.

When compared to larger particles, nanocarriers have a larger surface area. The nanocarriers can be modified to encapsulate a large amount of drug while increasing blood circulation. They can deliver targeted drugs into the tumour without harming normal cells. Liposomes are lipid vesicles composed of a lipid bilayer that can serve as an effective drug delivery carrier. They are less toxic and allow for controlled drug release and tumour targeting. Because they have both an aqueous and a hydrophobic membrane, they can encapsulate both lipophilic and hydrophilic drugs.

The present study entitled "Second Order Targetting using Nanocarriers of Phytoformulation". The study was designed as four different phases. In phase I, The hydroethanol leaf extract of *Withania somnifera* and dry fruit extract of *Terminalia bellirica* were prepared in a proportions and then added to make the phytoformulation. The phase II was designed to analyse the antioxidant activity of *Withania somnifera* and *Terminalia bellirica* extracts. In phase III, an attempt was made to synthesize and characterize silver nanoparticles and silver nanoparticles loaded Liposomes from *Withania somnifera and Terminalia bellirica* extract. Fourth phase was designed to analyse the anticancer potential of silver nanoparticles and silver nanoparticles Loaded Liposomes of *Withania somnifera and Terminalia bellirica* against molt-3 cells.

The salient findings of the study are summarised as follows: In phase I, preparation of the extracts was carried out, the hydroethanol leaf extract of Withania somnifera and dry fruit extract of Terminalia bellirica were prepared in a proportions and then added to make the phytoformulation. Equal proportion of *Withania somnifera* and *Terminalia bellirica* were added

to mixed to form the phytoformulation which was used for all the assays performed in the present study.

In phase II designed to analyse the antioxidant activity of *Withania somnifera* and *Terminalia bellirica* extracts was carried out, the radical scavenging activity of hydroethanolic extracts was assessed. From the study, an effective dose dependent radical scavenging potential was evidenced in the hydroethanolic extract and it was comparable with the standard ascorbic acid. It is confirmed that all the tested samples donated an electron to the DPPH radical thereby exhibiting scavenging potential. The present study aims to prepare a phyto formulation using the hydro ethanolic extracts of *Withania somnifera* and *Terminalia bellirica*, for which the hydro ethanolic extract with maximum scavenging potential against DPPH was chosen. The selected extracts that is *Withania somnifera* (50:50) and *Terminalia bellirica* (20:80) were added in equal volume to get the phytoformulation and the obtained formulation was further subjected for nanoparticle synthesis using silver.

In phase III, using hydroethanolic extract of Withania somnifera and Terminalia bellirica silver nanoparticles were produced under controlled conditions. The colour change from pale yellow to dark brown, which may be due to the excitation of surface plasmon resonance, proved the formation of silver nanoparticles. It was further confirmed based on the peaks observed in UV-Vis spectra of the green synthesized silver nanoparticles, exhibiting the plasmon resonance between 250-300 nm.

The X-ray diffraction pattern of synthesised silver nanoparticles *Withania somnifera* shows several major diffraction peaks at 10.49° and 22.91°, corresponding to the (248) and (370) planes, respectively. These diffraction patterns indicate the presence of face centered cubic crystalline structure of the synthesized nanoparticles. The average crystalline size was found to be 32.18 nm.

X ray diffraction pattern of synthesized silver nanoparticles *Terminalia bellirica* reveals various major diffraction peaks at 10.47°, 21.60°, 27.88°, 32.37°, 38.24°, 46.35°, 54.97°, 57.68°, 64.57°, 67.59°, 74.55°, 76.77° and 77.41° which corresponds the (128), (144), (361), (763),

(565), (388), (120), (121), (148), (66), (60), (115) and (168) planes respectively. These diffraction patterns indicate the presence of face centered cubic crystalline structure of the synthesized nanoparticles. The average crystalline size was found to be 50.03 nm.

FTIR analysis was carried out to Figureure out the various functional groups present in the *Withania somnifera* and *Terminalia belirica* extract that are involved in the bio reduction of silver ions to AgNPs and their subsequent coating and stabilization. The various band intensities at different regions of IR spectra for the *Withania somnifera* and *Terminalia belirica* extract and its silver nanoparticles were investigated. The absence of several fundamental peaks in the IR spectrum of silver nanoparticles validated the involvement of the functional groups of the phytoconstituents in the plant extract in the formation and subsequent capping of silver nanoparticles.

The silver nanoparticles loaded liposomes were synthesized using thin film hydration method coupled with sonication with the use of triglyceride lipid lecithin and cholesterol in the molar ratio 2:1. The encapsulation efficiency of the AgNPs loaded liposomes was found to be 69.98%. According to the FESEM analysis of *Withania somnifera* silver nanoparticles and *Terminalia bellirica*, the size of the nanoparticles ranges from 10.47-52.77 nm and 17.88-52.83 nm, respectively and are colloidal spherical in shape. The size of the silver nanoparticles of *Withania somnifera* loaded liposomes and silver nanoparticles of *Terminalia bellirica* loaded liposomes were found to be ranging between 20.00-90.62 nm and 11.31-87.72 nm and are also spherical in shape.

In phase IV, the cell viability and anticancer activity against Molt-3 cells of the hydroehanolic extract, AgNPs and AgNP loaded Liposomes were analyzed. Peripheral Blood Lamphocytes (PBL) was used as the normal counterpart for the Molt-3 cells. From MTT dye reduction assay , it was found that the liposomes are very effective against Molt-3 cells and does not affect the normal PBL cells much, which indicates that the various treatment groups are not cytotoxic to the normal cells. Normal counterpart of leukemic cells. The observed results indicate that the crude hydro ethanolic extract of *Withania somnifera* and *Terminalia bellirica* showed

lesser cytotoxicity into Molt-3 when compared to green synthesized plant extracts using Ag and AgNP of *Withania somnifera* and *Terminalia bellirica* loaded liposomes indicating that the enhanced cytotoxic efficacy evoked might be due to targeted delivery of the plant extracts (*Withania somnifera* and *Teminalia bellirica*) to the leukemic cells. Further the differential response obtained in Molt-3 leukemic cells and its AgNPs of *Withania somnifera* and *Terminalia bellirica* loaded liposomes exerted targeted delivery when compared the standard chemo therapeutic drug etoposide and the crude hydroethanolic extracts of *Withania somnifera* and *Terminalia bellirica*. Further analysis has to be carried out to confirm their second order delivery achieved by the plant extracts to leukemic cells.

Suggestions for the further research

The outcome of the present study paved a way for the number of future research. Some of them are given below

- ✓ In vivo studies can be carried out to analyze the anticancer efficiency of silver nanoparticles and silver nanoparticles loaded liposomes in animal models
- ✓ Clinical trial can be conducted using human volunteers for the pharmacological validation of the drug
- ✓ Mechanism of action of drugs on causing cancer cell death can studied.

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