Chapter 1

General Introduction and review

Chapter 1

General Introduction

1.1. General Introduction

Natural products have played a significant role in drug development over the past century, with many drugs derived from natural sources. In fact, approximately 50% of the drugs approved by the U.S. Food and Drug Administration (FDA) between 1981 and 2010 were either natural products or derivatives of natural products (Majolo *et al*., 2019). Natural products are compounds that are produced by living organisms, including plants, animals, fungi, and microorganisms. These compounds have evolved over millions of years to perform specific functions in their native organisms, such as defense against predators or competition for resources (Zhang *et al.,* 2005). The diversity of natural products is vast, and they offer a vast array of chemical structures and biological activities that can be harnessed for drug discovery. For example, alkaloids such as morphine and quinine are derived from plants and have been used for centuries to treat pain and malaria, respectively (Wase *et al.,* 2008; Chopra *et al.,* 2021). More recently, the anti-cancer drug paclitaxel was derived from the Pacific yew tree, and the anti-malarial drug artemisinin was isolated from the sweet wormwood plant. Natural products offer several advantages for drug development. They often have unique chemical structures and complex molecular frameworks that are difficult to synthesize, making them valuable starting points for drug discovery (Li *et al.,* 2017; Grygorenko *et al.,* 2020). They also have a proven track record of safety, as many of these compounds have been used in traditional medicine for centuries. Moreover, natural products offer a vast reservoir of novel chemical structures and biological activities that can be explored for drug discovery. With the increasing availability of genomic and metagenomic data, scientists are discovering new natural products from previously unexplored sources, such as plants, marine microbes and soil bacteria (Milshteyn *et al.*, 2014).

Ethnomedicine is the study of traditional knowledge and practices related to the use of plants and other natural resources for healing purposes. It involves the use of plants, animals, minerals, and other natural substances in the prevention and treatment of diseases. Ehnomedicinal plants play a crucial role in the search for new natural products with pharmacological properties (Sheng *et al.*, 2001; Mustafa *et al.*, 2017). Many traditional medicines are derived from plant-based sources, and a significant number of modern drugs have their origins in natural products. Ethnobotanical studies have identified numerous plants with medicinal properties, including anti-inflammatory, antimicrobial, anticancer, antidiabetic, and antihypertensive properties, among others. These plants have been used in traditional medicine systems for centuries and are still used today in many parts of the world. The use of ethnomedicinal plants is an important source of new natural products for drug discovery and development (Antal *et al.*, 2021). By studying the traditional uses of these

plants, researchers can identify potential pharmacological properties and isolate the active compounds responsible for their medicinal effects (Süntar *et al.*, 2020). This knowledge can be used to develop new drugs that are more effective, safer, and more affordable than synthetic drugs. Hence, ethnomedicinal plants are a valuable source of new natural products with pharmacological properties.

Ethnomedicinal plants, which are plants used in traditional medicine by indigenous people, have been used for centuries to treat various ailments, including cancer. Many of these plants contain bioactive compounds that have potential anticancer properties, making them valuable sources of lead compounds for the development of new anticancer drugs (Shah *et al.*, 2013). One of the significant advantages of using ethnomedicinal plants for anticancer drug development is that they have been used in traditional medicine for centuries, indicating their safety and efficacy (Roy *et al.*, 2018). Furthermore, these plants have evolved over millions of years to produce bioactive compounds that have specific biological activities, making them a valuable source of natural products for drug discovery. Studies have shown that several ethnomedicinal plants have anticancer properties, and many of their bioactive compounds have been identified and characterized. For example, the Madagascar periwinkle (Catharanthus roseus) is a well-known source of two potent anticancer drugs, vincristine and vinblastine. Similarly, the bark of the Pacific yew tree (Taxus brevifolia) is the source of the anticancer drug paclitaxel (Chen *et al.*, 2006). Moreover, the use of ethnomedicinal plants in anticancer drug development can also provide economic benefits to local communities by promoting sustainable harvesting practices and the conservation of medicinal plant species (Pandey *et al.*, 2017).

The Darjeeling Himalayan region of West Bengal is known for its rich biodiversity and is home to a variety of medicinal plants that have been used for centuries in traditional medicine for various ailments, including cancer (Palit *et al.*, 2016). As this region has a diverse range of plants with many rare and endangered species and many of which have not been fully studied for their potential medicinal properties. Several factors make the Darjeeling Himalayan region a favorable location for the discovery of natural compounds with anticancer properties: 1) Rich biodiversity: The Darjeeling Himalayan region is known for its rich biodiversity, and it is estimated that around 50% of India's plant species are found in this region. This diversity increases the likelihood of discovering new natural compounds with anticancer properties (Tiwary *et al.*, 2015). 2) Traditional knowledge: The local communities in the Darjeeling Himalayan region have a long history of using medicinal plants for various ailments, including cancer. Therefore, there is a wealth of traditional knowledge that can be leveraged for the identification and screening of medicinal plants with potential anticancer activity (Dikshit *et al.*, 2016). 3) Unique environmental conditions: The unique environmental conditions in the Darjeeling Himalayan region, such as high altitude and varying climatic conditions, can lead to the production of unique natural compounds with anticancer properties (Badola *et al.*, 2013). 4) Sustainable harvesting: The use of medicinal plants from the Darjeeling Himalayan region can provide economic benefits to local communities, and sustainable harvesting practices can ensure the conservation of medicinal plant species (Uprety *et al*., 2016). Therefore, the biological evaluation of indigenous

medicinal plants from this region for their potential anticancer activity has gained significant attention. The region's unique biodiversity, climate, and traditional knowledge of medicinal plants make it an ideal location for the search for novel NPs for the discovery of natural compounds with anticancer properties.

The biological evaluation of medicinal plants involves the screening of crude extracts and isolated compounds for their biological activity against a specific disease target. In the case of cancer, the evaluation focuses on the inhibition of cancer cell proliferation. This evaluation is typically carried out using in vitro assays, such as cell viability assays and colony formation assays (Lahlou *et al.*, 2007; Adan *et al.*, 2016). Several studies have been conducted on the biological evaluation of indigenous medicinal plants from the Darjeeling Himalayan region for their potential anticancer activity. These studies involve screening crude extracts and isolated compounds from medicinal plants for their cytotoxicity against various cancer cell lines. The compounds that demonstrate promising cytotoxicity are further characterized and tested for their mechanism of action using molecular biology techniques (Tiwary *et al*., 2015; Ganie *et al.*, 2014).

Hence the present main goal of our present studies is to identify natural compounds that can selectively inhibit the proliferation of cancer cells without affecting normal cells. Such compounds have the potential to be developed into new anticancer drugs with improved efficacy and fewer side effects. In addition to evaluating the biological activity of natural compounds, studies on their molecular mechanism are also essential for drug development. Molecular mechanism studies aim to understand the molecular interactions between the natural compound and its target protein(s) in cancer cells. This understanding can guide the optimization of the natural compound for improved potency and selectivity. Furthermore, molecular mechanism studies can also identify potential synergistic effects between natural compounds and existing anticancer drugs, leading to the development of combination therapies with improved efficacy.

In summary, the biological evaluation of indigenous medicinal plants from the Darjeeling Himalayan region for their potential anticancer activity involves the screening of crude extracts and isolated compounds for their cytotoxicity against cancer cell lines. The compounds that demonstrate promising cytotoxicity are further characterized and tested for their mechanism of action using molecular biology techniques. This approach can lead to the discovery of novel natural compounds for drug development and improve the treatment of cancer.

1.2. Objectives

1. Retrival of traditional knowledge of ethnomedicinal plants from local people Darjeeling Himalayan region and literature.
2. Taxonomic identification and selection of ethnomedicinal plants from Darjeeling Hill region of West Bengal, India.
3. Extraction of plant materials and screening of crude extracts for *in vitro* biological activities.
4. Fractionation of active crude extracts for the isolation of pure compound(s) using preparative chromatographic methods.
5. Screening of pure compounds for anti-tumorigenic potential by *in vitro* enzymatic assays, cytotoxic activity on different cancer and normal animal cell lines and spectrometric analysis of interaction with DNA.
6. Structural elucidation of active compound(s) by spectroscopic (NMR, IR / UV) and spectrometric (MS) method.
7. Identification of molecular putative cellular target(s) of the selected active compound.

1.3. Review of Literature

1.3.1. Cancer

Cancer is a global health issue responsible for one in six deaths worldwide. In 2020, about there were an estimated 19.3 million new cancer cases and about 10 million cancer deaths globally (**Sung *et al.*, 2021**). Cancer is a generic term for a large group of diseases that can affect any part of the body. One defining feature of cancer is an uncontrolled division of abnormal cells in a part of the body of a cancer patient. The rapidly proliferating abnormal cells grow beyond their usual boundaries, and which can then invade adjoining parts of the body and spread to other organs; the process is referred to as metastasis. Metastasis is the primary cause of death from cancer. The two important factors mainly responsible to cause cancer are external factors, including tobacco, radiation, chemicals; and other infectious organisms, like some oncogenic viruses; and internal factors, including inherited genetic defects and mutations. All these factors are responsible to initiate and develop the normal cell to a malignant one through either working together or in sequential manner **(Fig. 1.1)** (**Ganesh *et al.*, 2021; Merriel *et al.*, 2021**).

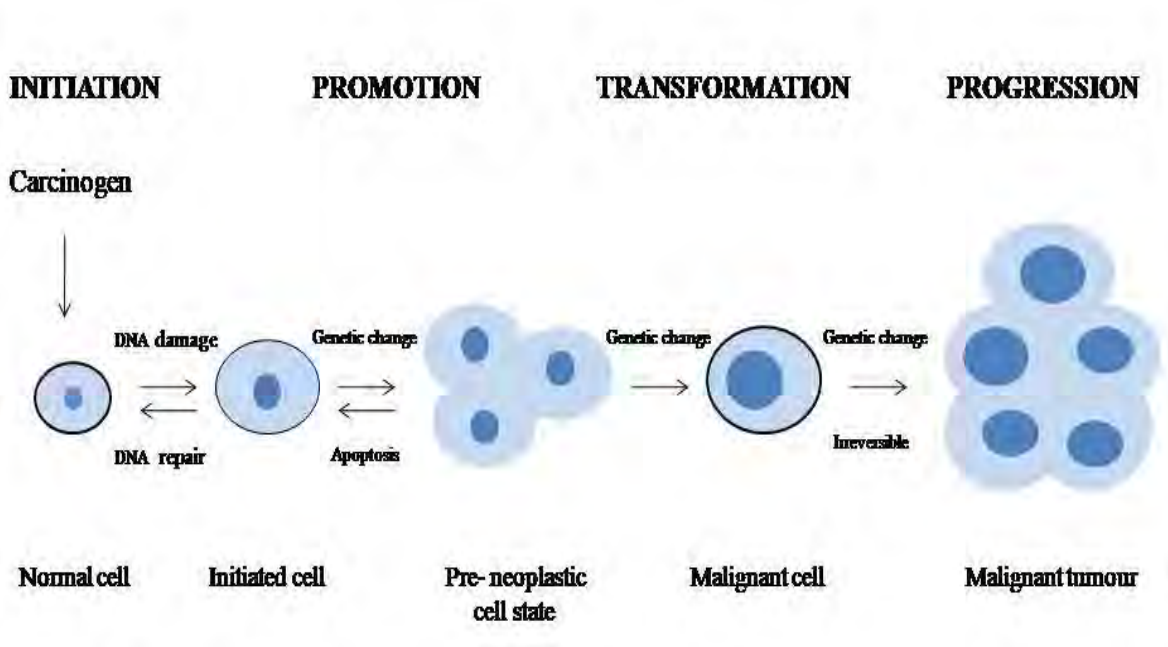

Fig.1.1: Mechanism of carcinogenesis: Multistep process involved in carcinogenesis that transforms a normal cell into a malignant tumor (Kotecha *et al.*, 2016).

1.3.1.1. Cell signaling pathways in cancer

Cancer is a disease in which cells acquire the characteristics to divide continuously in a rapid and uncontrolled manner. In early days the only means to treat the disease were surgery and chemotherapies. The discovery of oncogenes and tumor suppressor genes has revealed new therapeutic opportunities by targeting single or multiple proteins. While recent advances in DNA sequencing and multiomics analyses can provide a better understanding of the commonly involved processes and signaling pathways, which can be exploited for more accurate targeted therapies (Sanchez-Vega *et al.*, 2018). Although several important signaling pathways have been identified as frequently altered genetically in cancer, two pathways, namely the PI3K/AKT/mTOR and Ras/MAPK signal transduction pathways **(Fig. 1.2)**, are frequently activated or mutated in various forms of cancer (Aksamitiene *et al.*, 2012).

1.3.1.1.1. The PI3K/AKT signaling pathway

The phosphoinositide-3-kinase (PI3K)/AKT signaling pathwayplays an essential role in the regulation of different biological processes, such as cell proliferation, metabolism, cell growth, and cell migration (Vidal *et al.*, 2021). Disruption of PI3K/AKT signaling pathway or its abnormal upregulation are associated with different diseases, such as cancer, inflammation, immunodeficiency, and diabetes (Fruman *et al.*, 2017). Initial stimulation by one of the growth factors causes activation of a cell surface receptor and phosphorylationof PI3K. Activated PI3K generates phosphatidylinositol-3,4,5-triphosphate, which recruits PDK1 and Akt serine/threonine kinase at the plasma membrane. This results in the activation of Akt (Osaki *et al.*, 2004). Akt activates the mTOR pathway through activating multiple downstream effector proteins including the mTORC1 complex, the FOXO family of transcription factors, and cyclin D1, which supports cell growth and proliferation. Multiple tumor suppressor proteins have shown to limit the PI3K pathway activation, which includes the lipid and protein phosphatase PTEN (Papa *et al.*, 2019), the promyelocytic leukemia protein PML (Papa *et al.*, 2012), and the tuberous sclerosis complex TSC (Crino *et al.*, 2006).

1.3.1.1.2. The Ras/MAPK pathway

The Ras-Raf-MEK-ERK signaling pathway is responsible for regulating cell proliferation, differentiation, and survival. The signaling pathway begins with activation of RTKs, such as the EGFR family, that leads to activation of the GTP-binding protein Ras, which switch from its inactive Ras–GDP form to the active Ras–GTP form (Vetter *et al.*, 2001; Mitin *et al.*, 2005). By transmitting the signals received by the receptor, Ras/Raf/MEK/ERK cascade reaction activates transcription factors and regulates gene expression. Briefly, the process involves (i) the activation of Ras protein by receptor tyrosine kinases (Knight *et al.*, 2014). The activated Ras recruits and activates the protein

kinase Raf (Cseh *et al*., 2014), (ii) Raf serine/threonine protein kinase phosphorylates MEK, another protein kinase in the pathway which further phosphorylates ERK, which can directly and indirectly activate many transcription factors; (Knight *et al.*, 2014; Rauen *et al.*, 2013) and (iii) the activation of these transcription factors by ERK leads to the expression of

different genes encoding proteins that regulate cell proliferation and survival (Zhang *et al.*, 2009; Santarpia *et al.*, 2012; Rauen *et al.*, 2013).

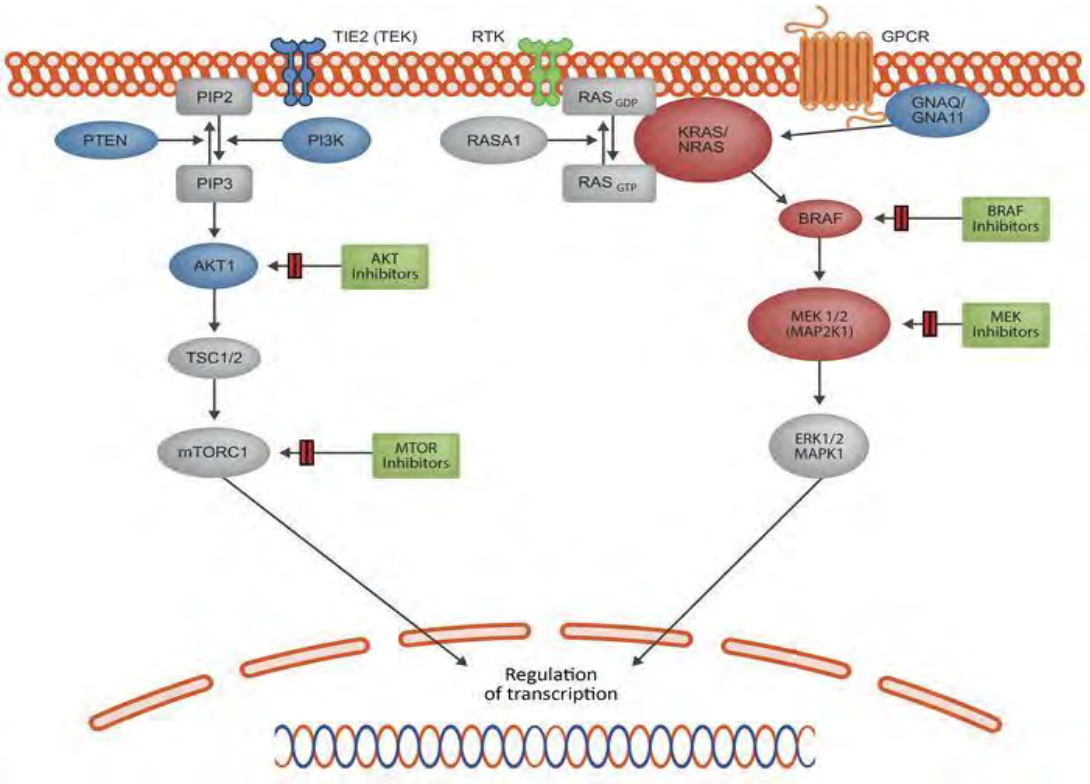

Fig.1.2: Schematic summary of signaling pathways PI3K/AKT/MTOR and RAS/RAF/MEK/ERK leads tothe different gene expression through complex transcriptional regulation which deal to cellular growth, apoptosis, and differentiation. (adapted from Al-Olabi *et al.*, 2018).

1.3.2. Apoptosis

Human body needs to maintain constant number of cells during their complete lifecycle for normal tissue development and homeostasis (Kerr *et al,* 1972). This steady-state cell number is achieved through regulation of the process of cell proliferation and cell death. The imbalance between the cell growth and cell death leads to the overabundance of cells which is term as neoplasm or tumours (Bortner *et al*., 2014). Apoptosis is an organized cell death which is necessary for normal tissue development as well as the process helps to remove potentially harmful cells and thus blocking tumor growth (Plati *et al*., 2008). Apoptosis play a crucial role of tumor formation and also critically determines treatment response. The strategies used to kill tumor cells using anticancer agents in clinical oncology, such as chemotherapy, γ-irradiation, suicide gene therapy or immunotherapy, have involved activation of intrinsic and extrinsic apoptosis signal transduction pathways in cancer cells **(Fig. 1.3)** (Fulda, *et al.*, 2006). The characteristics features of apoptosis includes chromatin condensation, the cleavage of DNA, cell rounding up, reduction in cellular volume (pyknosis) and membrane blebbing (Schwartzman *et al*., 1993; Wong *et al*., 2011).

` Apoptosis is a tightly regulated energy-requiring process that is stimulated by various cellular stresses, such as chemotherapy and oxidative stress (Fulda *et al,* 2010). In general, two major cellular pathways trigger apoptotic cell death. The first is receptor-mediated pathway which involves a sequential activation of Caspase-8 and Caspase-3 for cleaving the target proteins leading to apoptosis. The second pathway is directly or indirectly activated by intrinsic death stimuli, such as reactive oxygen species (ROS), DNA-damaging reagents, resulting in the release of cytochrome-c and the activation of Caspase-9 (Hamsa *et al*., 2011).

1.3.2.1. Extrinsic Pathway of Apoptosis

The extrinsic pathway of apoptosis begins when external stimulus i.e death ligands bind to death receptor. In most of the cases the external stimuli for apoptosis happens to be cytokines. The well studied cytokines which act as extracellular messengers to induce extrinsic pathway of apoptosis are TNF and Fas ligand (FasL) and their respective death receptors are type1 TNF receptor (TNFR1) and a related protein called Fas (CD95) (Hengartner *et al.*, 2000). The binding of cytokines to their respective death receptor results in the recruitment of many apoptosis related adapter protein factors, such as TNF receptor-associated death domain (TRADD) and Fas-associated death domain (FADD), as well as cysteine proteases, like caspase 8 (Schneider *et al*., 2000). These activated death domains form multi-protein complex of ligand-receptor-adaptor protein which is also known as the death-inducing signaling complex (DISC) (O'Brien *et al*., 2008). The complex then leads to the assembly and activation of pro-caspase 8. The activated caspase 8 is an initiator caspase in the extrinsic pathway of apoptosis which activates the downstream caspases also called executioner caspases (caspase 3) that carry out the self-destruction of the cell i.e. apoptosis (Karp *et al.*, 2008).

1.3.2.2. Intrinsic Pathway of Apoptosis

The intrinsic pathway of apoptosis initiates within the cell. The internal signals, such as DNA damage, hypoxia, very high concentrations of cytosolic Ca^{2+} and severe oxidative stress are some of the apoptogenic factors which are responsible for the initiation of the intrinsic mitochondrial pathway (Chipuk and Green *et al.*, 2006; Karp *et al.*, 2008). The increased mitochondrial membrane perturbation and release of cytochrome c into the cytoplasm play a key role in the consequence of this pathway (Danial *et al.*, 2004). Intrinsic pathway is tightly controlled by the B-cell lymphoma 2 (Bcl-2) protein family, named after the bcl2 gene found in follicular lymphoma, identified as one gene responsible in cell death either by activating pro-apoptotic or inhibiting anti-apoptotic pathway. The Bcl-2 protein family is comprised of both anti-apoptotic proteins (e.g. Bcl-2, Bcl-XL, Bcl-W, Bfl-1 and Mcl-1) and pro-apoptotic proteins (e.g. Bax, Bak, Bad, Bcl-Xs, Bid, Bik, Bim and Hrk) (Gogvadze *et al,* 2009; Reed *et al.,* 1997).The pathway is influenced by Bcl family members that are bound to the mitochondrial membrane, including both anti-apoptotic regulatory proteins which regulate apoptosis by blocking the mitochondrial release of cytochrome-c, while pro-apoptotic regulatory proteins act by promoting such release (Reed *et al.*, 1997). The increased permeability of the outer mitochondrial membrane due to the pro-apoptotic molecules leads

to efflux of cytochrome c, which further binds to the adaptor Apaf-1 and the initiator caspase-9 in the cytosol to form the apoptosome complex, which in turn activates the effector caspases like caspase 3 (Kroemer *et al.*, 2007). Additionally, the mitochondrion also releases other apoptotic factors such as Smac/DIABLO. They promote apoptosis indirectly by blocking the effects of a group of anti-apoptotic proteins called inhibitor of apoptosis proteins (IAPs) (Kroemer *et al.*, 2007, LaCasse *et al.*, 2008).

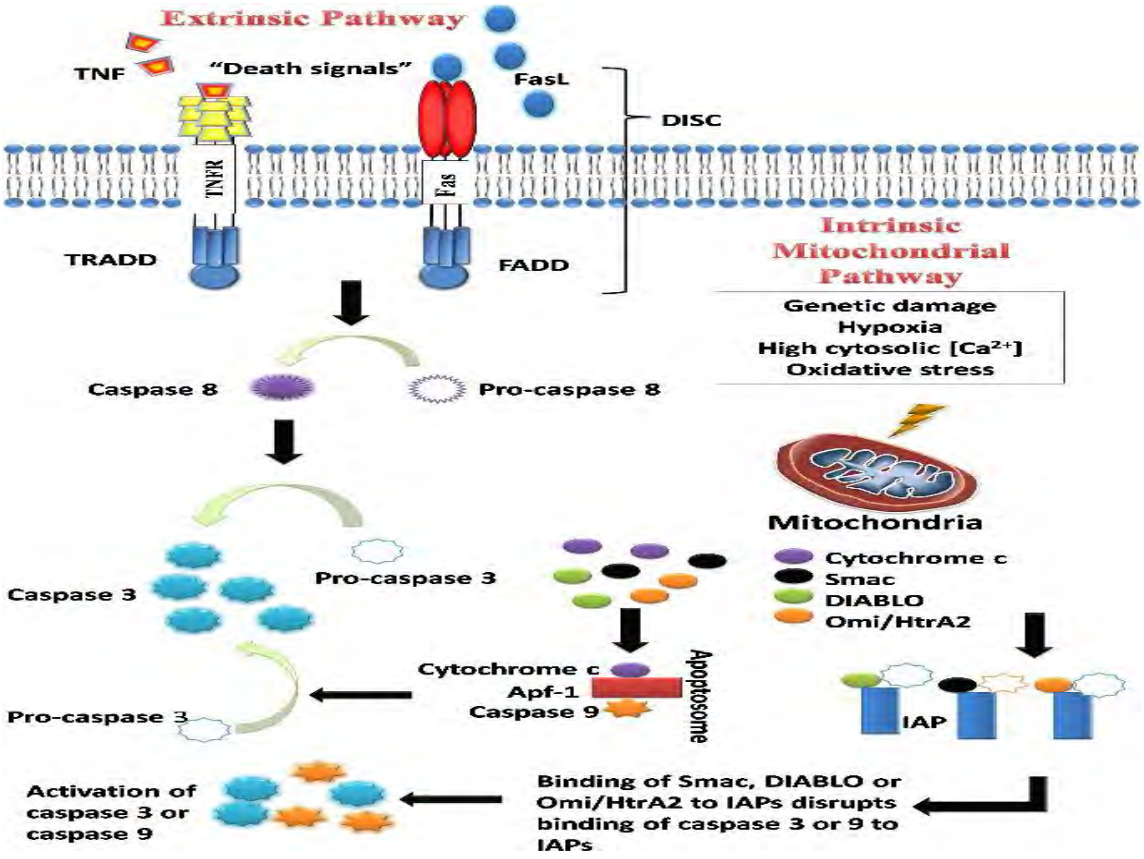

Fig.1.3: Apoptosis signaling pathways trigger cell death: a) the mitochondrial (the intrinsic) pathway and b) the death receptor (the extrinsic) pathway (adapted from Wong *et al.*, 2011).

Generally, there are main five types of cancer, which includes:

- **Carcinoma.** Carcinoma is the most common type of cancer. This type of cancer affects organs and glands, such as the lungs, breasts, pancreas and skin.
- **Sarcoma.** This cancer affects soft or connective tissues, such as muscle, fat, bone, cartilage or blood vessels.
- **Melanoma.** These cancers affect the cells which pigment your skin.
- **Lymphoma.** This cancer affects your lymphocytes or white blood cells.
- **Leukemia.** This type of cancer affects blood.

1.3.3. Cancer treatment

During previous decades a few options were available for cancer treatment among patients which mainly included surgery, radiation therapy, and chemotherapy either singly or in combination. However, in recent years several advanced and innovative approaches have been developed that has improved cancer therapy dramatically, with their benefits and challenges. These advanced cancer therapies include stem cell therapy, targeted therapy, ablation therapy, nanoparticles, natural antioxidants, radionics, chemodynamic therapy, sonodynamic therapy, and ferroptosis-based therapy (Debela *et al.*, 2021). Hence, the cancer treatment methods are divided into two different catagories as conventional (traditional) and advanced or novel or modern methods.

1.3.3.1. Conventional cancer therapies

The highly recommended conventional cancer treatment method includes surgical resection of tumors followed by radiotherapy or chemotherapy (Arruebo *et al.*, 2011). Surgery is the first option for direct removal of solid tumors located in one area. But the method is only effective at early stage of disease progression, whereas, radiotherapy can act on genetic material of tumor cells. Radiation therapy targets an affected area with high-energy waves that are often the location of a tumor or the place where a tumor has been removed during surgery in order to destroy any remaining cancer cells. However, higher doses of radiation can damage healthy cells, organs, and tissues (Debela *et al.*, 2021). Whereas chemotherapy, with the use of very toxic drugs, helps slow down or stop tumor growth which has reduced morbidity and mortality, but the therapeutic agents used to treat the diseases are powerful chemicals that can damage the healthy cells, especially rapidly dividing and growing cells (Moses *et al.*, 2003).

1.3.3.2. Advanced cancer therapies

As many hurdles exist in cancer treatment with drug resistance, drug delivery system and harmful side effects being the most common problems and hence, several new therapeutic approaches and drugs have been developed. The following are the advanced and innovative cancer therapy types: -

1.3.3.2.1. Stem cells therapy

The insufficient and nonspecific targets of traditional therapeutic approaches in cancer treatment often led to therapy resistance and cancer recurrence. Meanwhile, stem cell therapeutic strategy involves stem cells from different sources in the procedures, has provided a hopeful option to fight against cancer. However, various stem cell-based strategies under investigation are in preclinical trials, and they show both great promises and challenges

for cancer treatment (Gomes *et al*., 2017). The stem cell therapy is also considered to be safe and effective.

Type of Stem Cells for Cancer Treatment

The type of stem cell therapy depends on the source of stem cells that exhibit different capacities of proliferation, migration, and differentiation, which in turn determine their application in anti-tumor therapy (Chu *et al*., 2020).

(i) *Pluripotent Stem Cells (PSCs)*

Pluripotent stem cells (PSCs) are a type of stem cell that has the ability to differentiate into any cell type in the body. These cells have the potential to regenerate damaged tissues and organs, making them valuable in medical research and regenerative medicine. There are two types of PSCs: embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs). ESCs are derived from the inner cell mass of a developing embryo, whereas iPSCs are generated by reprogramming mature cells, such as skin cells, to an embryonic-like state. Currently, both ESCs and iPSCs are used for the induction of tumor-specific effector T- and NK cells, which are another type of immune cell that can attack cancer cells (Hermanson *et al*., 2018). It holds great promise for the development of effective cancer therapies that harness the power of the immune system to fight cancers (Ouyang *et al*., 2019).

(ii) *Adult Stem Cells (ASCs)*

Adult stem cells are the basis of essentially all successful stem-cell based therapies. The ASCs are useful for tissue regeneration and tissue replacement after severe injuries, and in fact, they are the tool of choice for regenerative medicine (Shin *et al.*, 2013). ASCs can give rise to many specialized cell types of the tissue and organ. In this group, hematopoietic stem cells (HSCs), mesenchymal stem cells (MSCs), and neural stem cells (NSCs) are often utilized in cancer treatment. HSCs located in bone marrow can form all types of mature blood cells. Till now, the infusion of HSCs derived from cord blood is the only stem cells approved by the FDA to treat multiple myeloma, leukemia, and some kinds of blood system disorders (Copelan *et al*., 2006).

(iii) *Cancer Stem Cells (CSCs)*

Cancer stem cells (CSCs) are generated in normal stem cells or precursor/progenitor cells by the epigenetic mutations process. Their role in tumor treatment includes control in cancer growth, metastasis and recurrence therefore; targeting CSCs could provide a promise to treat various types of solid tumors (Chang *et al*., 2016).

1.3.3.2.2. Targeted drug therapy

Targeted drug therapy is a type of cancer treatment that involves the use of drugs or other substances that specifically target cancer cells while sparing healthy cells. This approach is based on the fact that cancer cells have unique molecular features that distinguish them from

normal cells, and targeting these features can help to selectively kill cancer cells. In the targeted drug therapy uses drugs that target the specific genes and proteins associated with survival and proliferation of cancer cells. Targeted therapy may affect the tissue environment where cancer cells multiply or it may target the cells required to develop cancer, such as blood vessel cells (Padma *et al.*, 2015). The targeted drug therapy can treat different types of cancer in combination with other cancer treatments, such as chemotherapy. Targeted therapies are a rapidly growing field of cancer research and many new targets and drugs are still under clinical trials. Although various types of targeted therapies are available, the most common types are monoclonal antibodies and small-molecule drugs (Gerber *et al.*, 2008).

(i) *Monoclonal antibodies*

Monoclonal antibodies are laboratory-produced proteins that can be designed to interact with specific targets on the surface of cancer cells or might also be the area around the cancer cells. When a monoclonal antibody binds to its target, it can interfere with the signaling pathways that promote cancer cell growth and survival, or it can trigger an immune response that leads to cancer cell death. They also help chemotherapy and radiation therapy reach cancer cells better. The monoclonal antibodies which help to turn the immune system against cancer are considered a type of immunotherapy. Rituximad is an example, which binds to a protein called CD20 on B cells, a type of white blood cell and some types of cancer cells, causing the immune system to kill them (Zahavi *et al.,* 2020).

(ii) *Small-molecule inhibitors*

Small molecule inhibitors are drugs that can block specific signaling pathways that are critical for cancer cell growth and survival. These drugs are usually taken orally and can target various cellular processes, including DNA repair, cell cycle regulation, and angiogenesis (the formation of new blood vessels that supply nutrients to tumors). Some small molecule inhibitors of cancer targets are: gefitinib, an inhibitor of epidermal growth factor receptor (EGFR) kinase; erlotinib, an inhibitor of EGFR in non-small cell lung cancer (NSCLC); the lapatinib, an inhibitor of EGFR/ERBB2 for ERBB2-positive breast cancer; and the sorafenib, an inhibitor of vascular epidermal growth factor receptor (VEGFR) kinase in renal cancer (Hoelder *et al.*, 2012).

(iii) *Gene therapy*

Gene therapy is an experimental approach that involves the introduction of genetic material into cancer cells to alter their function. This can include the insertion of genes that encode tumor suppressors or the deletion of genes that promote cancer cell growth and survival (Touchefeu *et al*., 2010)

1.3.3.3. DNA metabolism enzymes as target for cancer therapeutics

DNA metabolism enzymes play a critical role in the replication and repair of DNA, and therefore, are attractive targets for cancer therapeutics. In cancer cells, these enzymes can be overexpressed or mutated, leading to aberrant DNA metabolism and genomic instability.

Targeting these enzymes with small molecule inhibitors can interfere with the ability of cancer cells to replicate and repair their DNA, leading to cell death or sensitization to chemotherapy or radiation therapy.

Some of the key DNA metabolism enzymes that are being targeted for cancer therapy include:

1. **DHFR (dihydrofolate reductase)**: The enzyme plays a key role in the folate metabolism pathway, which is critical for DNA synthesis and repair. DHFR catalyzes the conversion of dihydrofolate to tetrahydrofolate, which is required for the synthesis of purines and thymidylate, the building blocks of DNA (Lan *et al.*, 2018). DHFR is an attractive target for cancer therapeutics because cancer cells require rapid DNA synthesis and repair to sustain their growth. Several drugs have been developed that target DHFR as a cancer therapeutic (Vander *et al.*, 2011). Methotrexate (MTX) is one of the most well-known DHFR inhibitors and is used as a chemotherapy drug for various types of cancers, including acute lymphoblastic leukemia, osteosarcoma, and choriocarcinoma (Neradil *et al.*, 2015). MTX is a structural analog of folate and competes with folate for binding to DHFR. By inhibiting DHFR, MTX interferes with the folate pathway, leading to a decrease in purine and thymidylate synthesis and ultimately causing cell death (Qin *et al.*, 2021). Another DHFR inhibitor is pemetrexed, which is approved for the treatment of non-small cell lung cancer and mesothelioma. Pemetrexed is a multitargeted antifolate that inhibits several enzymes in the folate pathway, including DHFR, thymidylate synthase, and glycinamide ribonucleotide formyltransferase (Adjei *et al.*, 2004). DHFR inhibitors can also be combined with other chemotherapeutic agents to enhance their efficacy. For example, MTX is often used in combination with 5-fluorouracil (5-FU), which is a thymidylate synthase inhibitor, for the treatment of colorectal cancer (Longley *et al.*, 2003).

2. **DNA polymerases**: These enzymes are responsible for replicating the DNA during cell division. Inhibiting them can disrupt DNA replication and cell proliferation. For example, nucleoside analogs such as gemcitabine and cytarabine are commonly used chemotherapeutic drugs that inhibit DNA polymerase (Galmarini *et al.*, 2002).

3. **Topoisomerases**: These enzymes are responsible for unwinding and winding the DNA during replication and repair. Inhibiting topoisomerases can lead to DNA damage and cell death. Several topoisomerase inhibitors such as etoposide, doxorubicin, and irinotecan are used as chemotherapeutic agents (Xu *et al.*, 2015).

4. **DNA methyltransferases**: These enzymes are responsible for adding methyl groups to DNA, which can affect gene expression. Inhibiting DNA methyltransferases can re-activate tumor suppressor genes and induce cancer cell death. For example, the drug azacitidine is a DNA methyltransferase inhibitor that is used to treat myelodysplastic syndrome (Huang *et al.*, 2019).

5. **PARP enzymes**: These enzymes play a critical role in repairing DNA damage. Inhibiting PARP can lead to the accumulation of DNA damage, which can selectively kill cancer cells that have defective DNA repair pathways. PARP inhibitors such as olaparib and rucaparib are approved for the treatment of ovarian and breast cancers with BRCA mutations (Zheng *et al.*, 2020).

Targeting DNA metabolism enzymes is a promising approach for cancer therapy, and many drugs targeting these enzymes are currently in development or clinical trials. However, these drugs can also affect normal cells, leading to toxicity and side effects. Therefore, identifying specific targets and optimizing drug delivery are important challenges in developing effective cancer therapeutics.

1.3.4. Targeting Oxidative Stress as Anticancer Therapy

For normal physiological functions of growth and differentiation, cellular ROS plays a critical role in cell signaling cascades as secondary messengers (Sena *et al.*, 2012; Covarrubias *et al.*, 2008). However, their excessive production can damage nucleic acids, proteins, lipids, membranes and organelles, which can promote tumorigenesis, metastasis, and angiogenesis (Schieber *et al.*, 2014; Mitra *et al.*, 2019). Studies have shown that cancer cells have increased ROS level compared to normal cells due to high metabolic rate and mitochondrial dysfunction (Trachootham *et al*., 2009; NavaneethaKrishnan *et al*., 2018). Thus, additional surge in ROS level is likely to cause cancer cells to reach their oxidative stress threshold sooner than normal cells, which leads cell death (Trachootham *et al*., 2009; NavaneethaKrishnan *et al*., 2018). The redox imbalance feature of cancer cells may be exploited for targeted therapy (Krishnan *et al.*, 2019). Almost a decade ago, ROS inducers were proposed as anticancer strategy, in order to overcome the specific threshold of ROS level beyond which cancer cells undergo ROS-mediated cell death (Gorrini *et al*., 2013; Trachootham *et al*., 2009). Therefore, it is not surprising that several natural dietary bioactive compounds causing increased ROS levels have shown to selectively target cancer cells (Mileo *et al.*, 2016). For instance, dietary phytochemicals, such as polyphenols, flavonoids, and stilbenes exhibit the capacity to inhibit cancer cell proliferation, and to induce apoptosis and autophagy (Vallinas *et al*., 2013). Although most of the dietary bioactive compounds possess antioxidant capacity at low doses, their high doses can induce proxidant activity that induces cancer cell death (NavaneethaKrishnan *et al*., 2019).

These compounds also influence mitochondrial functions by altering mitochondrial enzymes, oxidative phosphorylation, and mitochondrial pathways (Gibellini *et al.*, 2015). An earlier report showed that flavanone naringenin, a naturally occurring bioactive compound of citrus fruit, has anti-proliferative effects in different cancer cell lines (Frydoonfar *et al*., 2002). The compound showed toxic effect at higher concentration (300 mM) with induction of apoptosis in various human cancer cells. Such compound is considered as ideal compound which act as active cancer specific compounds in drug screening and drug discovery mechanisms as many antitumor agents are non-selective and kill normal cells (Kanno *et al*., 2005; Park *et al*., 2008; Matsuo *et al*., 2005).

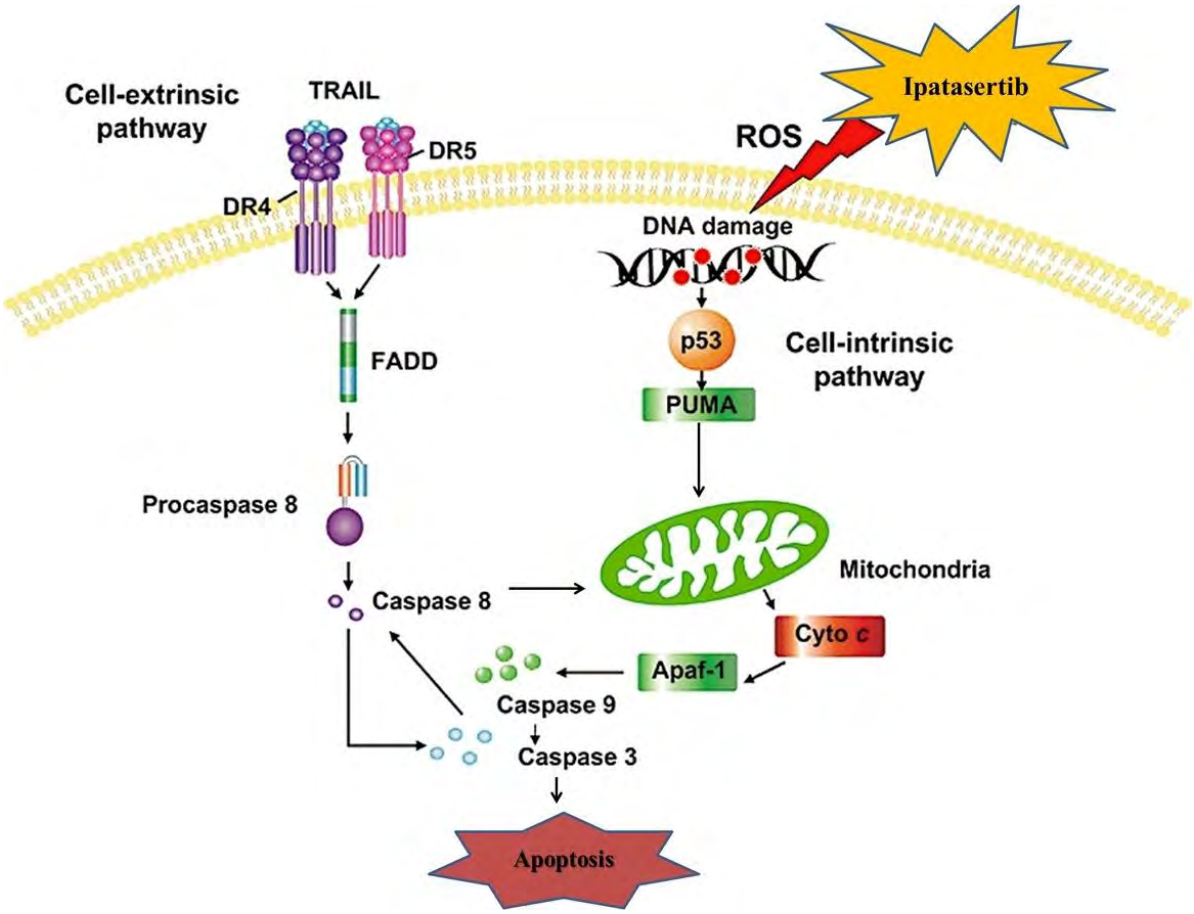

Fig.1.4: Mechanism of Reactive oxygen species (ROS) mediated DNA damage and apoptotic cell death through various signal transduction pathways (adapted from Lie *et al.*, 2019).

1.3.5. Importance of medicinal plant in drug discovery

With the early nineteenth century the use of medicinal plants involved the isolation of active components. The isolation of compounds resulted in their use in western prescription drugs with much safer and more effective dosing, which can be considered as a major advancement compared with the prescribed herbal materials. At the beginning of twentieth century, significant progress in the field of chemistry led to development of semisynthetic and synthetic drugs in required amounts (Süntar *et al.*, 2020).

Medicinal plants have been the major source of phytochemicals with therapeutic potential. Moreover, natural compounds as drugs derived from plants have reduced side effects due to their regular intake as components of vegetable food (Zhang *et al.*, 2020). Further, the well-recorded and widely practiced knowledge of herbal medicine can substantially reduce the time to identify development candidates. The use of ethnomedicinal plants to cure pharmacological diseases, such as skin disorders, inflammatory, infectious, parasitic and viral diseases can be taken into account for identification of anticancer drugs because they reflect disease states bearing relevance to cancer or a cancer symptom (Cordell *et al.,* 1991). The major categories of plant derived compounds with medicinal properties are

the terpenoids (such as taxol and various steroids), the glycosides (such as digitalis and various flavonoids) and the alkaloids (such as reserpine and various opiates) (Thatoi *et al.,* 2011).

Scientific studies on ethnomedicinal plants have resulted in discovery of several therapeutic drugs. In excess of 20% of the ethical turnover, the pharmaceutical companies today are generated medicinal products from plants. Some of these are the original natural product, others the synthetic equivalent or synthetic derivatives designed to improve efficacy or decrease associated side effects. The most important of these, representing historically accepted for medical practice are listed in **Table 1.1.**

Table 1.1: Significant plant-derived pharmaceutical products

Source plant	Drug	Indications
Atropa belladonna	Atropine	Anti-cholinergic, pupil dilation
Digitalis lanata	Digoxin	Cardiotonic
Mucuna deeringiana	(L)-Dopa	Anti-parkinsonism
Dioscorea deltoidea	Diosgenin	Anti-fertility
Papaver somniferum	Morphine,Codeine	Analgesic and antitussive
Colchicum autumnale	Colchicines	Anti-tumour, anti-gout
Cinchona ledgeriana	Quinine	Anti-malarial
Catharanthus roseus	Vincristine, Vinblastine	Anti-tumour
Galanthus woronowiis	Galanthamine	Alzheimer
Podophyllum peltatum	Podophyllotoxin	Anti-cancer, anti-viral
Camptotheca acuminate	Camptothecin	Anti-cancer
Artemisia annua	Artemisinin	Anti-malarial
Taxus brevifolia	Taxol	Anti-cancer
Tricosanthes kirilowii	Tricosanthin	Anti-cancer, anti-viral
Coleus forskolii	Forskolin	Glaucoma, impotence
Cannabis sativa	Cannabadiol	Appetite stimulation, multiple sclerosis, spasticity
Silybum marianum	Silymarin (Madaus)	Pain management, hepatic disorders, amanita poisoning
Salix spp.	Aspirin	Analgesic, anti-pyretic, cardiovascular

1.3.6. Approaches to drug discovery from natural products

Natural products (NPs) have been used to combat human diseases for thousands of years and they play important role in drug discovery and development (Fulda *et al*, 2009). History of medicine from natural products (NPs) has been acknowledged for dates back before the dawn of modern medicine. The NPs as drugs is one of the most enduring approaches due to their multiple biologically specific targets, reduced side-effects and low cost (Kamble *et al.*, 2019). In addition, NPs offer special features in comparison with conventional synthetic molecules, which confer both advantages and challenges for the drug discovery process. Eventually, they provide more structural diversity than standard combinatorial chemistry and thus they offer

major opportunity for finding novel remedial leads that are potent against a wide range of assay targets. The development costs of NPs as medicines are also likely to be much lower than that of biotechnological products or compounds produced from combinatorial chemistry (Alan *et al.,* 2000). Additionally, their use in traditional medicine may provide insights regarding efficacy and safety. By and large, NPs have assumed a key part in drug development, particularly for malignant growth, such as cancer and infectious diseases (Atanasov *et al*., 2015 and Harvey *et al*., 2015), yet additionally in other therapeutic areas, including cardiovascular diseases (for instance, statins) and multiple sclerosis (for instance, fingolimod).

The biologically active NPs with drug like properties are small molecules that are capable of being absorbed and metabolized by the body. They also have greater molecular rigidity compared with synthetic compound libraries (Feher *et al*., 2003; Clardy *et al*., 2004). These differences can be advantageous; for example, the higher rigidity of NPs can be important in drug discovery tackling protein–protein interactions (Atanasov *et al.*, 2021). NP screens include a library of extracts from natural sources (**Fig. 1.5**), which may not be compatible with traditional target-based assays (Henrich *et al*., 2013). At the initial crude extraction process, there are unlimited challenges exist which include the presence NPs that are already known, NPs that do not have drug-like properties or insufficient amounts of NPs for characterization. These challenges can be addressed through the development of methods for dereplication, extraction and pre-fractionation of extracts. Finally, at the last stage, when bioactive compounds are identified by phenotypic assays, significant time and efforts are typically needed to identify the affected molecular targets. The classical NP- based drug research begins with biological screening of 'crude' extracts to identify a bioactive 'hit' extract, which is generally involves the testing of extracts of source organisms, like plant, marine or microbes, in appropriate in vitro assays (cytotoxicity/cytostatic or enzyme/target based), followed by bioassay-guided fractionation of the active extract and isolation and purification of active constituent(s). Although bioactivity guided isolation is a laborious process with various limitations, various strategies have been developed to deal with the problems. Those constituent showing significant *in vivo* activity in appropriate animal models is considered as lead molecule which may be selected as candidate for preclinical development **(**Cragg *et al.,* 2005). Earlier reports have confirmed that most of the anticancer agents were discovered mainly through their inhibitory potential on the metabolic pathways crucial to cell division; however, their exact mechanism of action was often a subject of retrospective investigation. The recent growth in molecular biology and the advances in genomics and proteomics have generated several kinds of potential new drug targets (Narang *et al.,* 2009).

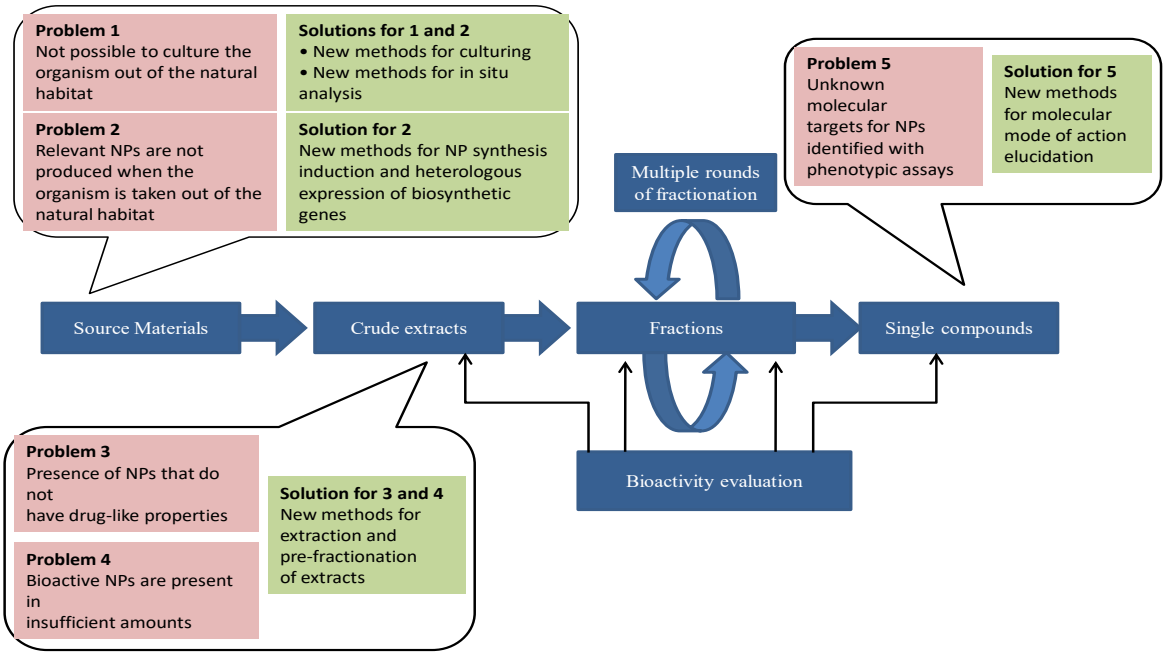

Fig.1.5: Outline of bioactivity-guided procedures in natural product drug discovery. Various steps of the process are shown in purple boxes, with associated key limitations shown in red boxes and advances that are helping to address these limitations in modern natural product (NP)-based drug discovery shown in green boxes (adapted and modified from Atanasov *et . al.*, 2021).

1.3.7. Natural products as anticancer agents

For many years, NPs and their structural analogues have made a huge contribution to pharmacotherapy, especially for cancer and infectious diseases (Lahlou *et al*., 2007; Atanasov *et al*., 2015; Harvey *et al*., 2015). Over the past 40 years, NPs have been investigated for their anticancer potential and served as a source of new anticancer agents due to existence of extensive chemical diversity. It is estimated that between 1981 and 2019, about 25% of all newly approved anti-cancer drugs were related to NPs (Newman *et al*., 2020; Huang *et al*., 2018). Some of the widely-used anticancer therapeutics originated from natural sources include irinotecan, vincristine, vinblastine, taxol, etoposide and paclitaxel from plants, actinomycin D and mitomycin C from bacteria, and marine-derived bleomycin (Orlikova *et al.*, 2014). Some of these compounds have historic achievements in cancer therapy and they are also expected to play a pivotal role in the future therapy. Among them the most successful drugs include Taxol, vinblastine, and camptothecin; and they are structurally unique agents and have novel mechanism of action. Camptothecin, first isolated and identified from wood and bark of *Camptotheca acuminata*, has the ability to kill cancer cells uniquely via specifically trapping topoisomerase I, an enzyme critically involved in both DNA replication and transcription processes, and form topoisomerase-DNA complexes. The topoisomerase-

DNA complex formation inhibits the ongoing process of DNA replication or transcription leading to genomic stress and ultimately to cell death (Huang *et al*., 2021). Food and Drug Administration (FDA) approved topotecan and irinotecan, the first-generation analogues of camptothecin as drugs, which are currently being used to treat various types of cancers, such as ovarian, breast and colon cancers, small-cell lung carcinoma, and several second-generation analogues of camptothecinare in clinical trials (Oberlies *et al*., 2004).

Taxol, first isolated and identified from bark of *Taxus brevifolia*, was found to inhibit cancer cell growth via the stabilization of microtubules. The compound was found to bind microtubules and cause dysfunction in microtubules dynamics, resulting in mitotic catastrophe of cancer cells (Huang *et al*., 2021). The compound represents typical journey of NPs to reach bedside. Initially the amount of compound yield was very low with finite resource later the problem was resolved by a commercially feasible semi-synthetic procedure. Further the complex structure of the compound also played as hurdle which was resolved with the collective assistance of mass spectrometry, X-ray crystallography and NMR spectroscopy. The last obstacle was dealt with the poor solubility which was solved by a special formulation made of castor oil. Finally, FDA approved the compound for clinical trials in the year 1992, after more than twenty years of initial report of its isolation and structure (Wall *et al*., 1995; Oberlies *et al*., 2004).

The discovery of two important chemotherapeutic agents, vinca alkaloids i.e. vinblastine and vincristine, were isolated from *Catharanthus roseus* (L.) G. Don. Earlier, the plant extract was famous for the treatment of diabetes in folklore medicine. These alkaloids are structurally almost same except for the presence of an additional carbonyl group in vincristine and they are in clinical use for the treatment of variety of cancers (Noble *et al*., 1990). The mechanism of action of both the drugs, vinblastine (Velban®, Velsar®) and vincristine (Oncovin®) is almost similar. They bind to the microtubular proteins of the mitotic spindle to prevent the tubulin dimers from polymerizing to form microtubules and thus prevent formation of the mitotic spindle (Thirumaran *et al*., 2007). In this manner, they induce a terminal mitotic arrest that ultimately leads to cell death (Jordan *et al*., 2002). However, these drugs not only affect the division of cancer cells but also of normal cells resulting of many side effects and hence making it necessary for the very specific administration of the drug (Morris *et al*., 2008).

There are several newer compounds, including steroids and terpenoids that have been isolated from plants and are currently under study or clinical trial for their potential therapeutic applications. Some of the important compounds like betulinic acid which is a triterpenoid that is found in the bark of white birch trees and other plants have shown anti-tumor activity (Mullauer *et al.*, 2010). Similarly, artemisinin is sesquiterpene lactone that is isolated from *Artemisia annua*, commonly known as sweet wormwood. Artemisinin and its derivatives have potent anti-malarial activity and are currently used as first-line treatments for malaria. They have also shown promise as potential cancer therapeutics due to their anti-tumor activity (Meng *et al.*, 2021). Ganoderic acid is a triterpenoid that is isolated from *Ganoderma lucidum*, commonly known as the reishi mushroom which has shown potent anti-tumor activity (Akihisa *et al.*, 2007). Ursolic acid is also a triterpenoid that is found in various

plants, including apple peels, rosemary, and basil. The compound also has shown anti-tumor activity against various cancers (Mandal *et al.*, 2021). Withanolides are a group of steroidal lactones that are found in various plants, including *Withania somnifera*, commonly known as ashwagandha. The compound has shown promising effect against cancers (Mirjalili *et al.*, 2009). All these compounds have shown anti-tumor activity in preclinical studies and are being investigated them as potential cancer therapeutics. Clinical trials are currently underway to evaluate the safety and efficacy of the above compounds in various types of cancer.

1.3.8. Darjeeling Himalayan medicinal plants

1.3.8.1. Overview of Darjeeling medicinal plants

According to a report only 10% of the world's biodiversity has been tested for biological activity, still many more useful natural lead compounds are remains to be identified (Dias *et al.*, 2012). A large number of NPs, especially plant-derived drugs, continue to be discovered on the basis of traditional or empirical local medical practices. India is one of the mega biodiversity centers of the world with northeastern region being the biodiversity hotspot (Saha *et al,* 2011). The floristic wealth of this region is nearly 43% of the country's total flora and good number of known and and unknown medicinal plants are found in the forest of Darjeeling district of West Bengal and the state of Sikkim, India (Mukherjee *et al,* 2014). The region is rich in diversity and density of plants due to the variation in the topography of the land, soil type and climatic conditions. The diversity of plants is related with a wide range of herbal products as this region supports approximately 18,440 species of plants. The tribal people of this region possess a rich knowledge on the use of medicinal plants for the treatment of various kinds of diseases, like wounds, diarrhea, dysentery, diabetes, jaundice and skin infection etc (Hussain *et al,* 2006). But the reports on plants as specific antitumoral agents are rarely found mainly because cancer is a disease involving a complex set of signs and symptoms (Monagelli *et al,* 2000). The ethnopharmacological usages, such as skin disorders, inflammatory, infectious, parasitic and viral diseases, can be taken into account for identification of anticancer drugs because they reflect disease states bearing relevance to cancer or a cancer symptom (Cordell *et al.,*1991; Popoca *et al.,*1998).

1.3.8.2. Selection of Darjeeling Himalayan medicinal plants for the present study

Astilbe rivularis *Tupistra nutans* *Zanthoxylum oxyphyllum*

Bergenia ciliata *Artemesia vulgaris* *Eupatorium glandulosum*

Fig.1.6: Photographs of some selected Himalayan medicinal plants used in the present study.

Many systems of herbal medicine are in practice in Darjeeling Himalayas including Bhutias system, Nepali system and Lepcha system (Bantawa *et al*., 2009). The local people treat their ailments using their own medicinal knowledge, mostly prepared from locally available herbs, animal parts, salts and other materials, locally termed as Pahade Dabai (Chettri *et al*., 2007). A report revealed that a total of 281 species of plants belonging to 108 different families are used in the Darjeeling Himalayan folk-medicine (Chettri *et al*., 2005; Jain *et al*., 1991). However, their therapeutic potentials are not explored scientifically.

Accordingly, in the present study, the main focus was the exploration of some indigenous species of medicinal plants from Darjeeling Himalayan region of West-Bengal as potent candidate of natural compound(s) against cancer. This was done through a series of bioassay-guided plant extraction and isolation protocols which are considered as a principle approach for the isolation of new lead compounds. For the study total six plant species were selected for the cytotoxic activity screening (**Fig. 1.6**). All these plants were selected on the basis of their traditionally known therapeutic potential, and frequent use by local ethnic people. These plants have been used for various ethnopharmacological properties such as antidiabetic, anti-inflammatory, anti-infectious, anti-diarrheal or anticancer activity. Additionally, the plants were also selected for the study on the basis of their availability in sufficient amount but availability of little scientific information.

Table 1.2: List of selected Himalayan medicinal plants with their traditional uses

Plant species	Family	Local name	Traditional uses
Astilbe rivularis	Saxifragaceae	Budho Okhati	Bleeding during child birth, inflammation, body Ache,diarrhea, and dysentery (**Timalsena *et al.*, 2019**).
Tupistra nutans	Asparagaceae	Nakima	Body pain and weakness; high blood pressure, diabetes and stomach-ache **(Thuy *et al.*, 2022).**
Zanthoxylum oxyphyllum	Rutaceae	Timur	Stimulant, stomachic, sudorific; used in colic; in fever, dyspepsia, also for asthama, bronchitis, rheumatism and toothache (**Khare *et al.*, 2007**)
***Bergenia ciliata* .Leaf**	Saxifragaceae	Pakhanbed	Cough, cold, fever, pulmonary infections, heart diseases, ophthalmic, hemorrhoids and stomach disorders (**Ahmad *et al.*, 2018**)
***Bergenia ciliata* .Rhizome**	Saxifragaceae	Pakhanbed	Diarrhea, vomiting, fever, cough, diabetes, cancer, pulmonary disorders and wound healing (**Zafar *et al.*, 2019**).
Artemesia vulgaris	Asteraceae	Titepati	Cuts and wound, diabetes, epilepsy, psychoneurosis, depression, irritability, insomnia, anxiety and stress (**Pandey *et al.*, 2017).**
Eupatorium cannabium Linn	Asteraceae	Banmara	Cuts and wound, treatment of liver diseases and diarrhea **(Judzentiene *et al.*, 2016).**

1.3.8.3. *Astilbe rivularis,* a Himalayan herb

1.3.8.3.1. Geographical distribution

Astilbe rivularis is well known with the different common names, such as River astilbe, Gosy, Pothee, Budho Okhati, Thulo Aushadhee, Budho Aushadhee, Bedaanggo and Gaane Gurjo (Timalsena *et al.*, 2019 and Kunwar *et al.*, 2010). It is native to South-East Asia (SEA) and Northern America along the mountain ravines and woodland (Manandhar *et al.*, 2002 and Brickell *et al.*, 2004). The plant grows well in most part of north-eastern part of Himalayas, Darjeeling, Kashmir, Assam in India and its neighboring countries like Nepal, Bhutan, South Tibet, Thailand, North Indo-China, West China (Manandhar *et al.*, 2002 and Hussain *et al*., 2007) (**Fig.1.7**). The wide varieties of *Astilbe* are well adapted to shade and water-logged conditions and some of them can also tolerate clay soils well, at altitudes of 2000-3600 m (Manandhar *et al.*, 2002).

1.3.8.3.2. Taxonomic classification

Kingdom: Plantae

Clade: Tracheophytes

Clade: Angiosperms

Clade: Eudicots

Order: Saxifragales

Family: Saxifragaceae

Genus: Astilbe Buch.-Ham. ex D.Don

species: *rivularis*

Botanical name: *Astilberivularis* Buch.-Ham. Ex D.Don

Fig.1.7: The growth habitat of *A. rivularis* worldwide.

1.3.8.3.3. Morphology

A. rivularis is a perennial herb that grows to 1-1.5 m tall. The leaves are compound and the lower leaflets usually further divided. The leaflets are 3-8 cm long, ovate, long-pointed, base sometimes heart-shaped, stalked to stalkless, rough, especially on the veins. The leaf-stalks and rachis are covered with long hairs, tufted at the base of leaflet-stalks. Stipules are about 1 cm long, adnate to the leaf-stalk. The flowering pattern consists of long terminal branch pyramidal clusters of tiny greenish yellow flowers. Peduncle is glandular villous. Flowers are bracteate, bracts lance shaped, about 2 mm long. Sepals are 5, 1.5 mm long, ovate, basally adnate to the ovary, persistent. Petals are absent. Stamens are 5, opposite the sepals, filaments 2.5 mm long. Carpels are connivent, oval, about 1.5 mm, each prolonged into a short style, less than 1 mm long. Capsules are about 4 mm long, splitting longitudinally into 2 valves. Mainly the germination is by vegetative method, but the plant also germinates via seeds. The plant flowering season is July to October.

1.3.8.3.4. Phytochemistry

Table 1.3: List of chemical compounds isolated and characterized from *A. rivularis*

Compound	Plant part	Isolation and Identification Method	Reference
Bergenin	Rhizome	^{1}H NMR, ^{13}C NMR and mass spectroscopy	Kaundinnyayana et al., 2013 and Rajbhandari *et al*., 2011
Arbutin	Rhizome	^{1}H NMR, ^{13}C NMR and mass spectroscopy	Rajbhandari *et al*., 2011
dimer of bergenin	Rhizome	^{1}H NMR, ^{13}C NMR and mass spectroscopy	Rajbhandari *et al*., 2011
11-*O*-galloylbergenin	Rhizome	spectroscopic methods	Kengo *et al*., 2018
(+) catechin	Rhizome	physical and spectral data	Kengo *et al*., 2018
(−) catechin	Rhizome	physical and spectral data	Kengo *et al*., 2018
(−) afzelechin	Rhizome	physical and spectral data	Kengo *et al*., 2018
(−) epiafzelechin	Rhizome	physical and spectral data	Kengo *et al*., 2018
3b-trans-p-Coumaroyloxy-olean-12-en-27-oic acid	Rhizome	MS and NMR spectroscopic	Kang *et al*., 2017
6bhydroxy-3-oxoolean-12-en-27-oic acid	Rhizome	MS and NMR spectroscopic	Kang *et al*., 2017

All higher plants produce secondary metabolites for defense against pathogens. They can also serve as source of drug leads for the treatment of several diseases and their different pharmacological activity is the scientific basis for their use. The methanolic extract of rhizome of *A. rivularis* contains several major classes of phytochemicals, such as alkaloids, tannins, terpenoids, flavonoids, coumarins, phenols, glycosides and saponins (Adhikari *et al*., 2012). The principal chemical compounds earlier reported from the plant include aesculetin, astilbic acid, astilbin, aticoside, dimethylaesculetin, daucosterol, eucryphin, palmitine, peltoboykinoleic acid, scopoletin, sitosterol and stilbene (Kunwar *et al.*, 2010); whereas other bioactive compounds such as bergenin, arbutin and bergenin derivatives were isolated from the rhizome. According to the report the compounds arbutin and dimer of bergenin were first time reported in this plant (Rajbhandari *et al*., 2011). Moreover, β-amyrin and β-sitosterol were also isolated from aerial parts (Timalsena *et al.*, 2019). The potent antioxidant constituents, such as 11-ogalloylbergenin, (+)catechin, (−)catechin, (−)afzelechin, (−)epiafzelechin and 2(*β*-D-glucopyranosyloxy)- 4-hydroxylbenzenacetonitrile were isolated from the rhizomes (Kengo *et al.*, 2018). A new pentacyclic triterpene, 3β-trans-p-coumaroyloxy-olean- 12-en-27-oic acid and previously known 6β-hydroxy-3-oxo-olean-12-en-27-oic acid, the pentacyclic triterpenoids substituted with a carboxylic acid at the C-27 position were isolated from the plant first time (Joo *et al.*, 2015).

1.3.8.3.5. Biological properties

A. rivularis has been played important role in traditional medicine system and Ayurveda system because it has immense potential to treat various ailments. Earlier reports suggested that the plant extracts from its different parts like leaves, rhizomes, roots, seeds and individual isolated compounds, such as triterpenoids, bergenin, arbutin etc., have shown anti-inflammatory, antioxidant, antidiabetic, anti-bacterial, anti-proliferative, anti-peptic ulcerogenic properties.

A. rivularis has been considered as high valued medicinal plant hence there is increase in the demand at local and international level pharmaceutical industry. The varied ethnopharmacological uses of *A. rivularis* have led to the initiation of various pharmacological investigations. Based on the previous research the plant extracts and its some isolated compounds had shown a wide range of biological activities, such as antibacterial, antifungal, antiviral, anticancer, anti-inflammatory, antidiabetic, and antioxidant activities.

Antimicrobial activity

To combat the threat of antibacterial resistance there is need to develop new antibacterial drugs. In this regards the natural products can solve this challenge and further replace those expensive drugs and minimize the side effects. The plant extract and its constituents played a significant role as antimicrobial and antiviral activity. In one of the study the methanolic extract of *A. rivularis* was evaluated for antibacterial activity against *E. coli* using the agar disc diffusion methods with Ciprofloxacin as positive control. The extracts exhibited zones of inhibition ranging from 11-13 mm which was comparable with zone of inhibition exhibited by Ciprofloxacin of 23-25 mm. The minimum inhibitory concentration (MIC) of methanolic extract of *A. rivularis* was found to be 11 µg ml^{-1} (Adhikary *et al.*, 2012). Another study reported that the plant rhizome extract of *A. rivularis* also showed strong antibacterial activity against *E. coli* (Aligiannis *et al.*, 2001). In addition, the methanolic rhizome extract showed potent anti- herpes viral activity with IC50 values of < 6.25 µg ml^{-1} without cytotoxity to the vero cells with CC 50 value of 67µg ml^{-1} (Rajbhandari *et al*., 2009).

Anti-peptic ulcer activity

An in vivo study was carried out to assess the anti-peptic ulcer activity of the *Astilbe* root extract in rat. The powdered root of *A. rivularis* was administered as drug to the rats before inducing the ulcer where esomeprazole was taken as positive control. Eventually both the drugs showed significant effects against both gastric and duodenal ulcers induced by ethanol and cysteamine, respectively, in dose dependent manner. As the ulcer index was calculated the plant extract showed nearly 50% reduction in ulcer formation at the dose of 2 g/kg in

ethanol as well as in cysteamine treated rats. When compared with control, the anti-peptic ulcer of root powder effect less than that of omeprazole (Mitra *et al.*, 2008).

Antioxidant activity

The antioxidant activity of methanol bark extract of *A. rivularis* was evaluated by Subedi *et al.* (2014) using DPPH and Ferric ion reducing assays. The extract showed potent free radical scavenging activity with the EC_{50} values of 4.05 µg ml^{-1} and also showed increasing ferric ion reducing ability in dose dependent manner with 90% reduction at 100 µg ml^{-1} of extract. The total phenolic and flavonoid contents of the extract were 183.11 mg GAE/g dry extract weight and 857.26 mg QE/g dry extract weight, respectively. According to Kaundinnyayana *et al.* (2013), the rhizome extract also showed potent DPPH radical scavenging activity with IC_{50} 7.88 µg ml^{-1}, whereas the ascorbic acid had IC_{50} value of 5.03 µg ml^{-1}.

According to a report the extract was subjected to isolation of important antioxidant compounds which included bergenin, 11-*O*-galloylbergenin, (+) catechin, (−) catechin, (−) afzelechin, (−) epiafzelechin and 2-(*β*-D-glucopyranosyloxy)-4-hydroxyl-benzenacetonitrile. Using DPPH scavenging assay, it was revealed that all the seven compounds showed high antioxidant property as compared to the standard or positive control Trolox. Among these compound, 11-*O*-galloylbergenin had the highest scavenging potential with EC_{50} 9.6 µM followed by (+) catechin, (−) catechin, (−) afzelechin, (−) epiafzelechin and trolox with their EC_{50} as 13.5, 13.6, 21.8, 20.9 and 48.8 µM, respectively (Kengo *et al*., 2018). The season wise analysis of antioxidant activity of leaves revealed maximum activity during the rainy season i.e. June-July which could be due to the high content of antioxidant compounds like phenol, flavonoids, ascorbic acid and carotenoids during the season (Mitra *et al*., 2007).

Anti-inflammatory activity

Inflammation is a vital part of the immune system's response to injury and infection. It is the process to heal and repair damaged tissue through the signaling immune system as well as protects the body from foreign invaders, such as viruses and bacteria (Linlin *et al*., 2018). However, the imbalance in inflammatory process may leads the development and progression of severe diseases, such as cardiovascular disease or stroke, and autoimmune disorders, like rheumatoid arthritis and lupus (Sugimoto *et al.,* 2016).

A. rivularis has been long known for its anti-inflammatory activity. A report revealed the anti-inflammatory effect showed by dried rhizome in the carrageenan induced Albino rat paw. It was able to inhibit inflammation by 56.20% at a dose of 200 mg/kg body weight. This anti-inflammatory activity was comparable with standard dose (20 mg/kg body weight) of non-steroidal anti-inflammatory drug, the Ibuprofen (Mandal. *et al.,* 2009). The observed anti-inflammatory activity may be due to the precence of flavanoids and some bioactive compounds, like bergenin and astilbic as mojor constituents in *A. rivularis*. As previous studies revealed, these compounds contribute in the inhibition of cyclooxygenase-2 (COX-2)

activity halting the release of prostaglandins which play a key role in the generation of the inflammatory response (Ricciotti *et al.*, 2011).

In another study, the two compounds were investigated for the anti-inflammatory activity which were isolated from *A. rivularis*, and characterized as 3β-trans-p-coumaroyloxy-olean-12-en-27-oic acid and 6β-hydroxy-3-oxoolean-12-en-27-oic acid. They are pentacyclic triterpenoids bearing a carboxylic acid group at the C-27 which is a unique characteristics constituent of the genus Astilbe. Here they were investigated on TGFBIp-mediated vascular inflammatory responses (Kang *et al*., 2017). As it was found that 100 ng ml^{-1} LPS stimulated the release of TGFBIp in HUVECs. Hence, to investigate the effect of each compound on the LPS-mediated release of TGFBIp, HUVECs were stimulated with 100 ng ml^{-1} LPS for 1 h, followed by treatment with increasing concentrations of each compound for 6 h. Each compound inhibited the release of TGFBIp in HUVECs, with an optimal effective concentration >5 mM. The high plasma concentrations of TGFBIp in patients with sepsis are known to be related to the severity of sepsis. Therefore, the prevention of CLP induced TGFBIp release by both compounds suggests the potential of compounds which can be used for the treatment of vascular inflammatory diseases. Further the effects of compound 1 and 2 on barrier integrity in HUVECs were determined by using permeability assay. The result suggested that both the compounds (1 and 2) were able to inhibit TGFBI p-mediated hyperpermeability in endothelial cells, with the optimal effect occurring at a concentration above 5 mM. Therefore, both can be considered as potential therapeutic agents against vascular inflammatory diseases. CLP-induced sepsis mouse model was also carried out to investigate whether 1 and 2 can protect mice from CLP-induced sepsis lethality. Both the compound 1 and 2 were administered as 24.1 mg/mouse and 18.8 mg/mouse respectively twice at different time interval of 12 and 50 h after CLP, which resulted in an increase in the survival rate from 40 to 50%, according to the Kaplan-Meier survival analysis. It was also found that the treatment with both the compounds 1 and 2 resulted in the downregulation of TGFBIp- induced levels of VCAM-1, ICAM-1, and E-selectin, suggesting that 1 and 2 inhibit the adhesion and migration of leukocytes to the inflamed endothelium. Hence, the compounds 1 and 2 were inhibiting the expression of CAMs in vascular endothelial cells which can be considered a promising therapeutic approach for treating vascular inflammatory diseases.

Anti-diabetic activity

The pentacyclic triterpenoids substituted with a carboxylic acid at the C-27 position isolated from *A. rivularis* was assigned as new pentacyclic triterpenoids 1 and 2 by spectroscopic data interpretation (Joo *et al*., 2015). Both the compounds 1 and 2, were evaluated for the activity of glucose uptake and glucose transporter 4 (GLUT4) translocation in C2C12 myotubes. Both the compounds showed significantly increase basal and insulin-stimulated glucose uptake and GLUT4 translocation to plasma membrane. They stimulated the phosphorylation of insulin receptor substrate-1 (IRS-1), protein kinase B (Akt), and extracellular signal-regulated kinase 1/2 (Erk1/2). The study suggested that compounds 1 and 2 activated both the IRS-1/Akt and Erk1/2 pathways and subsequently stimulated GLUT4 translocation, leading to enhanced

glucose uptake. Thus, these observations suggest that C-27-carboxylated-pentacyclic triterpenoids may serve as scaffolds for development as agents for the management of blood glucose levels in disease states such as diabetes.

Ethnomedicinal Properties

As according to Evans *et al*., 2002 all the parts of *A. rivularis* contributes as medicine in their traditional medicine system. Root paste is mixed with honey and taken to control post-partum diarrhoea and dysentery. The stem-bark are used in disease conditions like stomach ache, ascites, headache, cough, rheumatism, back pain, wound healing, weakness, avian plague, yellow fever, and malaria. A few pieces of fresh rhizomes are used either for infusion or poultice or as powder for the treatment of toothache (Maity *et al.*, 2004). The dried rhizomes parts have been traditionally used for the treatment of inflammatory diseases, headache, peptic ulcer, infertility, hemorrhages and prolapse of uterus from the ancient period in India (Manandhar *et al.*, 2002, Mandal *et al.,* 2009). The fruit pulp has been used as laxative whereas the leaves parts are used as diuretic, antipyretic, analgesic and used for the treatment of pleurisy and burns. The seed is used for treatment of pneumonia. The juice of the plant is applied traditionally to sprains and muscular swelling. The rhizome part of plant has also been widely used astonic for uterine and menstrual disorders in Nepal and India (Watanabe *et al*. 2013). The observations from these studies suggest the ethno medicinal use of *A. rivularis* which could be commercially exploited by the pharmaceutical industry.

Threats towards its extinction

The Ayurveda and traditional medicine system has described many important ethno pharmacological properties of the plant *A. rivularis*. The plant being used as alternative remedies with their long experiences and practices. However, the indigenous knowledge related to this medicinal plant are highly threatened due to the continuous exploitation of the individual plant species from wild, legally, or illegally, substantial loss of their habitats and various human related activities like deforestation, habitat destruction, unsustainable harvesting of forest products etc (Negi *et al.*, 2010). Another major reason for the declining in population of the species is related with the biotic factor where the attack of pathogenic organism especially in the leaves of this plant leads to the occurrence of chlorosis on leaf lamina (Singh *et al.*, 2011). So, these all activities resulted in declining the population of this high valued medicinal plant species. Due to this the plant has been categorized as rare plant species and hence there is need to immediate conservation in order to sustain its existence in nature.