INTEGRATED EVALUATION OF PHYTOCHEMICAL COMPOSITION, ANTIMICROBIAL, ANTIOXIDANT POTENTIAL, AND MOLECULAR DOCKING STUDIES OF *RHODODENDRON*

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BIOTECHNOLOGY

By

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To

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May 2023

CERTIFICATE

I hereby declare that the work presented in this report entitled "**Integrated evaluation of phytochemical composition, antimicrobial, antioxidant potential, and molecular docking studies of *Rhododendron*** " in partial fulfillment of the requirements for the award of the degree of Master of Technology in Biotechnology submitted in the department of Biotechnology and bioinformatics, Jaypee University of Information Technology Waknaghat is an authentic record of my own work carried out over a period from August 2022 to june 2023 under the supervision of **Dr. Udayabanu, M and Dr. Raj Kumar.**

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LIST OF FIGURES

CHAPTER 2

CHAPTER 4

CHAPTER 5

LIST OF TABLES

TABLE OF CONTENT

ABSTRACT

A particularly dangerous and aggressive kind of brain cancer with few effective treatments is glioblastoma, recently, increased attention has been paid to natural chemicals as potential sources of cutting-edge anti-cancer medications. Rhododendron, a plant known for its wide range of phytochemicals, has shown potential as a therapeutic agent for a number of diseases. The goal of this study was to employ molecular docking studies to examine the phytochemical components of Rhododendron and evaluate any potential interactions with proteins associated with glioblastoma cancer. A phytochemical analysis of rhododendron extracts revealed the presence of several bioactive compounds, including alkaloids, flavonoids, terpenoids, and phenolics. By concentrating on several cellular pathways linked to the development of cancer, these drugs' anti-cancer properties have been demonstrated. To examine potential interactions between Rhododendron phytochemicals and glioblastoma cancer proteins, molecular docking studies were conducted. The glioblastoma-related proteins' three-dimensional structures were obtained from publicly available sources, and state-of-the-art software was used to do molecular docking simulations. The docking tests indicated that certain phytochemicals from Rhododendron appeared to have a strong affinity for the functional regions of the glioblastoma cancer proteins. According to the study's findings, novel glioblastoma treatments may be developed using rhododendron phytochemicals as viable candidates. To establish the anti-cancer efficacy and safety of these medications, additional in vitro and in vivo research is required. Additionally important details regarding their therapeutic potential would come through preclinical research and understanding the underlying mechanisms of action.

CHAPTER 1

INTRODUCTION

Cancer is a genetic mutation where a collection of cellular organelles ignores the dynamic guidelines for the division of cells and multiplies uncontrollably. Because cancerous cells are somewhat self-sufficient, they don't react to the signals which trigger regular cell cycle, which causes the unchecked development and proliferation of altered cells. If malignant cells continue to proliferate, it can be lethal. In reality, 90% of cancer deaths are caused by over spreading of tumors to the tissues, a process known as metastasis [1].

During the process of mitosis, regular cells exhibit a reliance on external growth factors for their development and rely on interdependent interactions. Cell proliferation stops when the flow of all these growth signals declines or stops. Conversely, tumor cells exhibit uncontrolled growth independent of any stimulation or signaling. Normal cells also possess the ability to avoid contact with one another. They cease cell division when a specific threshold is reached or when they have a sufficient number of neighboring cells. Cancer cells, on the other hand, lack this interaction inhibition capability, which leads to the creation of an undesirable cell cluster. A normal cell multiplies just 50 times before its existence is well-programmed to end by apoptotic and then being substituted by a new cell. Given that repetitive replication shortens telomeres, this is compatible with the limited efficiency of DNA replication. Contrarily, cancer cells have high levels of the enzyme telomerase, which continuously repairs the telomere ends that are broken or worn out, enabling unrestricted cell growth [2].

The Ericaceae family includes *Rhododendron arboreum*, an angiosperm with striking brilliant red blooms. Rhodo in Greek stands for Rose, and dendron means tree , hence the name "*rhododendron*." It is indigenous to Nepal, India, China, and Bhutan. It is incredibly common in Northern and North-Eastern India's high elevations. R. *arboreum* is the official tree in Uttarakhand as well as the official flower of the nation of Nepal, respectively. This evergreen tree has importance both economically and horticulturally. Additionally, it is commonly utilized by North Indian tribal tribes for cooking and conventional medical treatments[3].

In Himachal Pradesh's mountainous regions, jams, jellies, and native beer are made from R. *arboreum* flowers. Fresh flowers from R. arboreum are used to treat diarrhea and dysentery, while dried flowers are used to treat dysentery that is accompanied by bleeding. According to reports, R. *arboreum* works well as an astringent, choleretic, diuretic, and treatment for persistent diarrhea and IBS. This plant's young, toxic leaves are applied to the forehead to relieve headaches [4]. A variety of pharmacological actions, including antioxidant, anti-diabetic, hepatoprotective, anti-nociceptive, antidiarrheal, and anti-inflammatory have been linked to various *Rhododendron* components. Numerous writers have also claimed that it is a source of certain phytoconstituents with medicinal significance. Despite the fact that R. *arboreum* is frequently utilized in traditional treatment procedures, there aren't enough scientific studies that support its antimutagenic and anticancer qualities [5]

The process of molecular docking, another name for docking research, is used in the fields of computational chemistry and for the development of drugs. They predict how a substance called ligand will adhere to an intended receptor's active pocket at a certain time, location, and in a particular way. Docking studies support leads maximizing virtual testing, and comprehension of structure-activity correlations by examining interactions among molecules. By identifying novel therapeutic possibilities and comprehending how interactions between protein and ligand function, they significantly speed up the drug's manufacturing procedure[6].

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CHAPTER 2

REVIEW OF LITERATURE

2.1 TUMOR BIOLOGY

When cell division proceeds without the involvement of growth factors, it leads to a cascade of events, ultimately resulting in the formation of tumors. In the initial stages, uncontrolled cell growth gives rise to a significant accumulation of cells known as hyperplasia. Dysplasia, which is distinguished by atypical cell growth, is the next in line. These abnormal cells then proceed to lose its intended purpose as they spread within a constrained area of the tissues. The word for this phase is anaplasia. The tumor is now regarded as benign because it is not invasive. At a later stage, tumor cells attain the capacity to metastasize [7]. Cells start to spread through the circulation and into surrounding tissues in addition to faraway ones. It is quite challenging to treat a malignant condition at this time. Not all tumors progress to this extent, though, if caught early. While tumor cells may grow even in the absence of growth hormones, they still need food and oxygen to survive. All healthy tissues include a large number of capillaries, which enable oxygen and nutrients to penetrate any cells. Contrary to this, as a tumor grows, angiogenesis—the process of creating new blood vessels—is carried out to make sure that nutrients get to the tumor's core's cells that have access to regular blood arteries [8].

2.2 VARIOUS TYPES OF TUMORS

2.2.1 On the basis of cell type that was first changed

Tumors are called for the type of cell that gave rise to them. These include:

- Carcinomas, which are caused by abnormal epithelial cells. They have the greatest ratio of any form of cancer.
- Sarcomas are cancerous growths in the muscle, fat, connective tissue and bone.
- Leukemia, caused by malignant white blood cells (WBCs).

- Lymphoma is a kind of tumor which impacts the lymph system, or bone marrow-derived cells.
- Specific WBCs that create antibodies, known as myelomas, are malignant [9].

2.2.2 Grade classification

The difference between cells and their natural environment is this aberration. According to the severity of the irregularities, the grade ranges from 1 to 4. Moderate tumors have specialized cells, which bear a resemblance to healthy cells. Compared to the nearby tissues, cells that have undergone aberrant differentiation are incredibly abnormal [4]. These tumors are extremely dangerous:

- Grade 1 cancers are well-differentiated with few anomalies,
- Grade 2 tumors are much more advanced and atypical.
- Grade 3 cells are significantly aberrant and incorrectly differentiated as a result of defective chromosomes; they also produce hazardous substances that influence neighboring cells and have the potential to enter the bloodstream.
- Grade 4 cells are immature, undifferentiated, and juvenile [9].

2.3 CAUSE OF CANCER

Cancer develops and spreads due to a variety of causes within the cell, (mutation, immunological states, and hormone) along with external environmental influences (smoking, chemicals, infectious organisms, and radiation). These elements work together to produce abnormal cell behavior and unrestrained development. The ensuing aberrant body's cell mass travels periodically to other areas of the body, enlarges, and damages nearby healthy tissues (metastasis). (Fig. 2.1) [9].

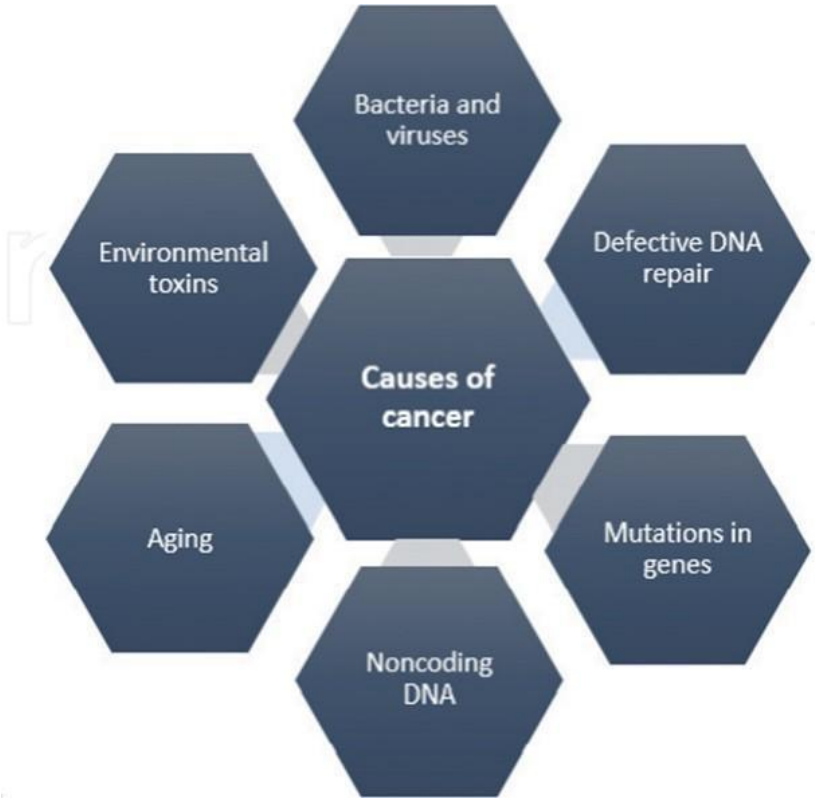


Fig 2.1 - Cancer Causing Factors

The generally recognised theory of cancer genesis proposes that oncogenes and tumor suppressor genes are significantly mutated during cancer development.. Another hypothesis suggests that a mutation in a central gene responsible for regulating cell division may lead to the replication of abnormal chromosomes, potentially resulting in the duplication or loss of substantial portions of chromatin. Despite there being no real need, this change in the genetic makeup of the cells causes an inordinate amount of a certain protein to be generated. Cancer can result from any chromosomal mutation that alters a protein that is essential for the cell cycle, either quantitatively or qualitatively [9].

The incorrect insertion or hypomethylation of the methyl groups from genes which regulate the cellular cycle, repair of DNA, and apoptosis has also been linked to various malignancies, according to research. It's crucial to keep in mind that cancers may require months or years to amass sufficient DNA alterations for the ensuing tumor masses to also be recognized. The progression of the tumor may therefore be influenced by a variety of routes. Finding the real cancer's cause becomes more difficult as a result of this [10].

2.4 TUMOR SUPPRESSOR GENE (p53) MUTATION

The p53 gene's normal operation is essential for human carcinogenesis since it is integral to the biological processes of transcription of genes, synthesis of DNA, apoptosis, and DNA repair.. Primary cancers are caused by changes and mutations in the p53 gene. Multiunit protein machines carry out biochemical steps associated with the proper operation of the p53 gene. Certain oncoproteins disrupt the functions of these mechanisms by binding to p53 and disrupting its interactions with various intracellular proteins [10].

2.5 CANCER TREATMENT

Chemotherapy, radiation, or surgery, either alone or in combination, have often been the mainstays of medical cancer treatment for many years. However, things are evolving quickly. Today, novel methods like immunotherapies and specialized medicines are becoming accessible, and there are many more being developed in research. The new therapies frequently work better and have fewer negative effects [9,10].

2.6 GLIOBLASTOMA

Glioblastoma, the most prevalent and aggressive form of primary brain cancer, originates from supportive glial cells in the brain. It is characterized as a grade IV tumor due to its highly malignant nature. Glioblastoma primarily affects adults and accounts for a significant proportion of brain cancer cases. While these tumors can emerge in different regions of the brain, they often develop in the cerebral hemispheres, which constitute the brain's largest portion. The precise causes of glioblastoma remain incompletely

understood; however, certain risk factors have been identified. Factors that contribute to glioblastoma include being exposed to ionizing radiation, having certain genetic disorders like neurofibromatosis type 1 and Turcot syndrome, and having a family history of glioblastoma. The symptoms of glioblastoma can vary based on the size and location of the brain tumor. Common signs incorporate headaches, seizures, cognitive decline, changes in personality, difficulties with speech or motor skills, and vision or hearing impairments. These symptoms tend to worsen progressively over time. Typically, the treatment approach for glioblastoma involves a combination of surgical intervention, radiation therapy, and chemotherapy. However, complete surgical removal of the tumor is often not feasible due to the infiltrative nature of glioblastoma cells and their ability to spread within the brain. Additionally, glioblastoma is highly resistant to treatment, and recurrence is frequent. Despite advancements in therapy, glioblastoma remains a challenging disease with a grim prognosis. The average survival time following diagnosis typically ranges from 12 to 15 months, and the five-year survival rate is low. Nonetheless, individual outcomes can vary, and certain patients may respond more positively to treatment or have the opportunity to participate in clinical trials investigating novel therapies. A customary approach to treating glioblastoma involves a combination of surgery, radiation therapy, and chemotherapy. However, complete surgical removal of the tumor is often not achievable due to the infiltrative nature of glioblastoma cells and their propensity to spread throughout the brain. Glioblastoma is highly resistant to treatment, leading to frequent tumor recurrence. Despite advancements in therapeutic approaches, glioblastoma remains a difficult disease to manage and has a bleak prognosis. On average, patients survive for about 12 to 15 months after diagnosis, with a low five-year survival rate. Nevertheless, individual outcomes can differ, and some patients may show better responses to treatment or have the opportunity to participate in clinical trials investigating innovative therapies [10, 11, 13, 18, 22, 23].

Glioblastoma tends to be more commonly detected in older individuals, although it can manifest at any age. The median age of diagnosis is around 64 years, and the likelihood of developing glioblastoma rises with increasing age. The prognosis for glioblastoma is generally poor, with relatively low survival rates. On average, patients survive for around 12 to 15 months after diagnosis. The survival rate after five years is below 5%, and the survival rate after ten years is even more minimal [11,13].

Glioblastoma exhibits a high level of resistance to treatment. Despite the aggressive approaches used, such as surgery, radiation therapy, and chemotherapy (e.g., temozolomide), tumor recurrence is a common occurrence. The response to treatment can vary among individuals, and certain factors such as age, performance status, and molecular characteristics of the tumor may influence outcomes [13].

Glioblastoma can be categorized into different molecular subtypes based on genetic and molecular markers. The most prevalent subtypes are the proneural and mesenchymal subtypes, each characterized by distinct genetic alterations and clinical features [14].

2.6.1 Glioblastoma Pathway

Glioblastoma is influenced by multiple pathways that impact its development, progression, and response to treatment [15].

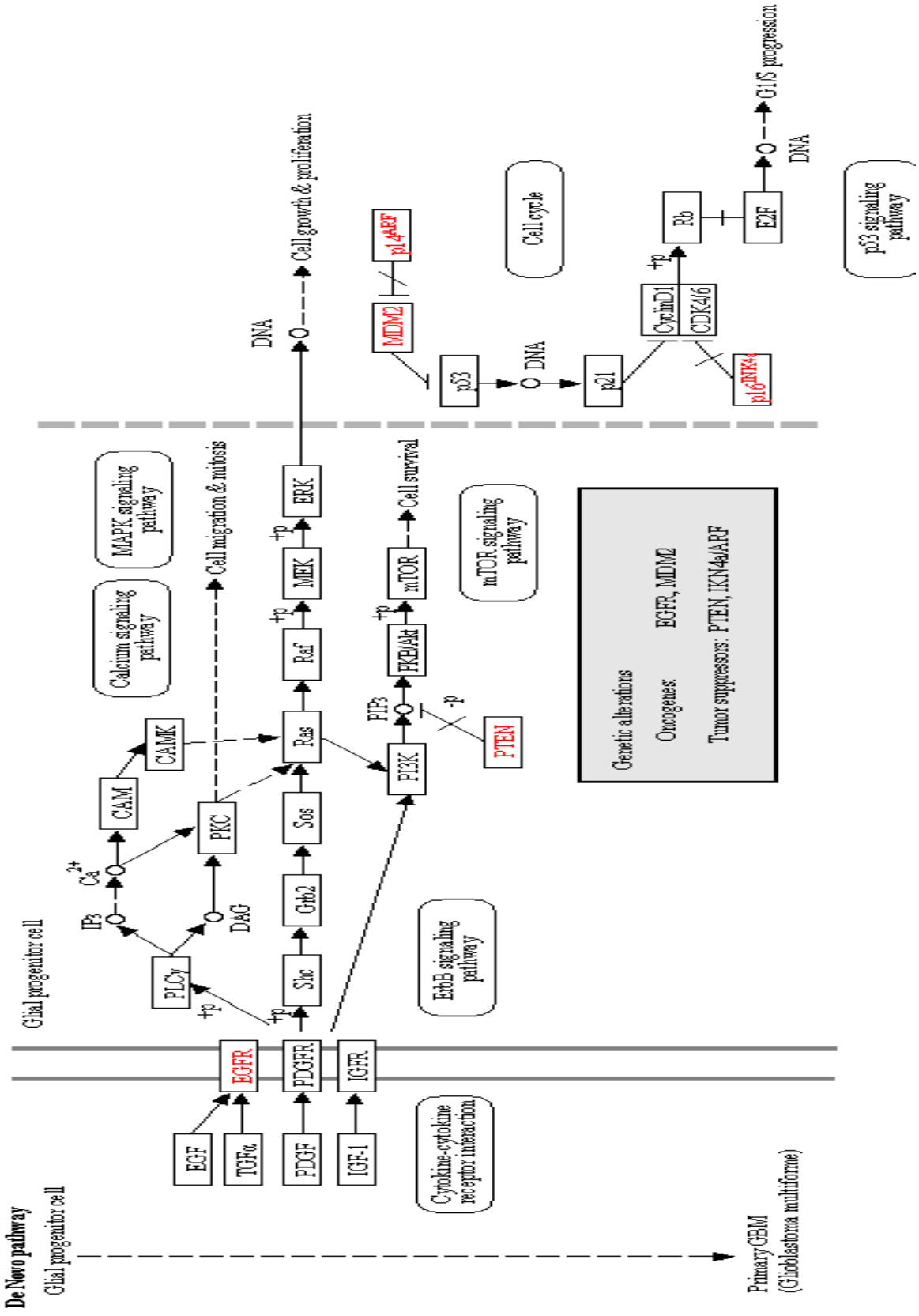


Fig 2.2 - Glioblastoma Pathway

Here are some key pathways associated with glioblastoma:

Epidermal Growth Factor Receptor (EGFR) Pathway: The EGFR pathway, which plays a role in regulating cell growth and division, is associated with glioblastoma. Glioblastoma commonly shows mutations or excessive expression of EGFR, resulting in increased cell proliferation, survival, and angiogenesis. These factors contribute to the growth of the tumor [16].

Phosphatidylinositol-3-Kinase (PI3K)/Akt/mTOR Pathway: The PI3K/Akt/mTOR pathway, involved in cell growth, survival, and metabolism, is implicated in glioblastoma. Genetic abnormalities often disrupt this pathway in glioblastoma, leading to enhanced tumor cell survival, invasion, and resistance to therapy [16].

Ras/Raf/MEK/ERK Pathway: The Ras/Raf/MEK/ERK pathway regulates differentiation, cell proliferation, and survival. Mutations or activation of this pathway, including BRAF gene mutations, have been identified in glioblastoma, promoting cell growth and survival, contributing to tumor progression [17].

TP53 (p53) Pathway: TP53 is a tumor suppressor gene that safeguards against cancer development. TP53 mutations are relatively common in glioblastoma, disrupting p53's normal function and impairing cell cycle control and DNA repair mechanisms [17].

RB Pathway: The RB pathway regulates cell cycle progression and prevents uncontrolled cell growth. Mutations in the RB1 gene can disrupt this pathway, contributing to glioblastoma development and progression [18].

Notch Pathway: The Notch pathway influences cell fate determination and differentiation. Dysregulated Notch signaling is implicated in glioblastoma, promoting tumor growth and the ability of cancer stem-like cells to regenerate themselves [19].

In summary, these pathways, including EGFR, PI3K/Akt/mTOR, Ras/Raf/MEK/ERK, TP53, RB, and Notch pathways, play significant roles in glioblastoma, impacting various aspects of tumor biology such as survival, cell growth, invasion, and self-renewal [20].

2.6.2 MAPK signaling pathways

Activating transcription factors via transmitting impulses from cell surface receptors, the mitogen-activated protein kinase cascade is a signaling system that controls gene expression. This route controls gene expression, the process of cell cycle advancement and apoptosis, depending on the particular stimuli and cell type.. Key enzymes within this pathway, BRAF and MEK, are target kinases that have essential roles in cell cycle regulation[21]. Consequently, targeting this pathway for therapeutic intervention is a suitable approach. With understanding in pathway advances, the complexity of the Ras/Raf/MEK/ERK cascade increases with the discovery of new kinase and transcription factor members. Protein phosphorylation plays a significant role in activating or deactivating these components. The diversity of signals transmitted through this pathway is expanded by the formation of heterodimers among various family members, resulting in the transmission of distinct signals. Additionally, several pathways that transmit signals engage in interactions with the MEK path, affecting its functioning in both positive and negative ways and changing the degree to which downstream targets are phosphorylated[22].

2.6.3 MEK (MAPK/ERK kinase)

When Raf is activated, its catalytic domain at the C-terminal interacts with MEK, leading to phosphorylation of the serine residue in its catalytic VIII subregion.MEK has molecular mass of 44, and 45 kDa, respectively, and is made up of the MEK1 and MEK2 subtypes. In order to activate ERK, it is a special kinase that phosphorylates both Thr and Tyr regulating sites. The specific mechanism underlying MEK's ability to phosphorylate both Tyr and The remains unclear. Nevertheless, this characteristic is of significant physiological importance due to the central role of the ERK signaling pathway in the cell's signal transduction network. Any variations in how it activates can have a significant impact on cellular functions. The signal transduction precision is substantially improved and mistakes in ERK activation are prevented by the detection and activation process that permits MEK's dual specificity[23].

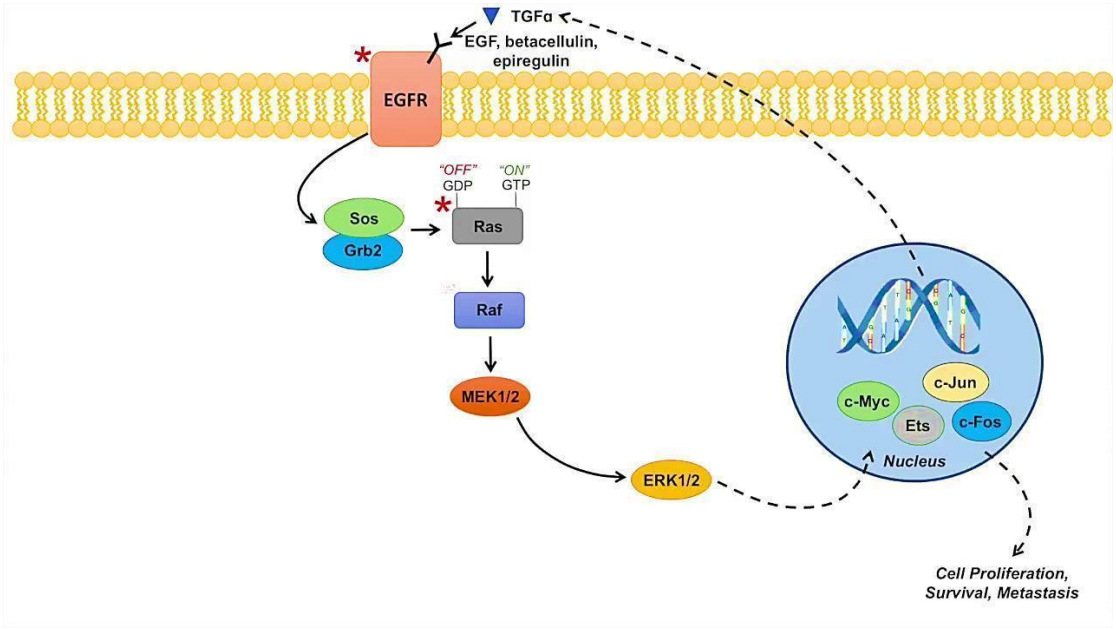


Fig 2.3 - Ras Raf MEK ERK Signaling Pathway overview

Since RAF signaling relies on the activation of MEK1 and MEK2, both of these proteins are considered promising targets for drug intervention, especially in RAF-addicted tumor cells. Strong allosteric inhibitors that don't compete with ATP can attach to MEK1 and MEK2 because they have a characteristic hydrophobic pocket close to their ATP-binding site.

However, it's worth noting that MEK1 and MEK2 mutations are rare in cancer, which means that MEK inhibitors (MEKis) do not specifically target tumor cells over normal tissue. Consequently, there is a potential risk of toxicity in normal tissues when using MEK inhibitors [23].

2.6.4 MEK1 protein function

A key function within the MAP kinase communication pathway is played by the double specificity protein kinase. Activation of cell-surface receptors by extracellular ligands like growth factors, cytokines, and hormones triggers the activation of RAS, which subsequently initiates the activation of RAF1. Several dual-specificity protein kinases MAP2K1/MEK1 and MAP2K2/MEK2 are then activated by RAF1. The kinases MEK1 and MEK2 have a particular function within the MAPK/ERK cascade. They activate MAPK3/ERK1 and MAPK1/ERK2 proteins by phosphorylating a threonine and a tyrosine residue, respectively, which causes the signal to spread across the MAPK/ERK cascade. [28].

Depending on the biological setting, the MAPK/ERK cascade performs a variety of tasks. It controls cell proliferation, adhesion, survival, and differentiation.. This cascade has an impact through regulating the transcription process, metabolism, and cytoskeletal changes. One of its targets is the nuclear receptor peroxisome proliferator-triggered receptor gamma (PPARG), which promotes differentiation and apoptosis. PPARG has been seen to be exported from the nucleus with the aid of MEK1/MAP2K1. The control of endosomal dynamics also involves the MAPK/ERK cascade, which controls activities including lysosome processing as well as endosome rotation via the perinuclear reusing compartment (PNRC). Additionally, it contributes to the Golgi apparatus's fragmentation during mitosis [23,24].

2.6.5 MEK Inhibitors

Activation of ERK1/2 triggers multiple cellular and nuclear events while simultaneously suppressing Raf activity through a feedback cycle, thereby controlling the activity of the MAPK pathway. Inhibition of MEK1/2 deactivates both ERK1/2 and the feedback inhibition of Raf. Drugs that specifically inhibit MEK proteins can stop cell development and cause cell death in the occurrence of certain mutations [25].

MEK inhibitors exhibit selectivity due to their high affinity for a specific region near the ATP binding pocket of the protein kinase. This selectivity is maintained even when the ATP binding site is highly conserved, as they avoid interacting with other protein kinases. MEK inhibitors induce conformational changes that limit the movement of the activation loop, which is responsible for triggering the kinase enzyme. As a result, MEK's kinase activity is inhibited, delaying the Raf-mediated phosphorylation of MEK and making the enzyme's catalytically inactive. The signaling route is effectively interrupted by this. [25].

As a result, MEK1/2 inhibitors selectively deactivate ERK1/2 without impacting other signaling pathways. Because of their interaction via a non-ATP binding site, they frequently don't have to compete with the abundant ATP found in cells. Furthermore, there is ongoing development of a novel ATP-competitive inhibitor that effectively targets mutants susceptible to ATP-noncompetitive inhibitors [25].

As compared with the use of a BRAF inhibitor by itself, the benefits of combined MEK inhibitor treatment include improved progression-free survival and less toxicity [25].

Trametinib and cobimetinib, two MEK inhibitors, have received FDA and European Medicines Agency (EMA) approval [26].

(I) Trametinib

The FDA initially authorized a MEK inhibitor for the treatment of melanoma in the month of May 2013 under the name trametinib (GSK1120212). Its chemical structure is depicted in Figure. The inhibitor functions as an allosteric inhibitor that does not compete with ATP, exhibiting potent action against pure MEK1 and MEK2 kinases with half maximum inhibitory concentrations (IC50) of 0.7 nM and 0.9 nM, respectively.

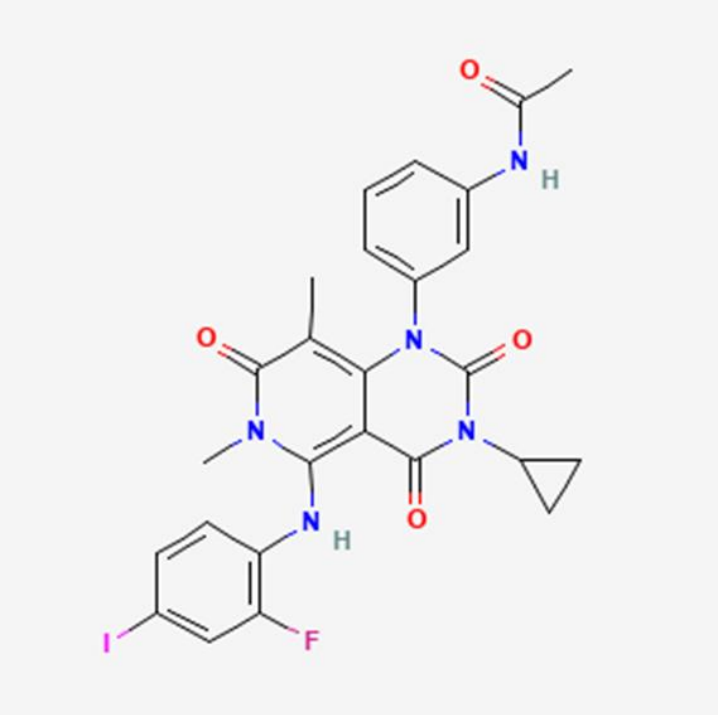


Fig 2.4 - 2D structure of Trametinib

(II) Cobimetinib

The next MEK inhibitor to receive approval is cobimetinib (GDC-0973, XL518), a product of Exelixis and Genentech (Roche). In the month of November 2015, The drug's use in combination with vemurafenib for the treatment of unresectable and metastatic melanoma that carries the BRAF V600E as well as V600K mutation was approved by the FDA. The FDA designated the medicine used to treat malignant melanoma that carries the BRAF V600e mutant an orphan drug in 2014.

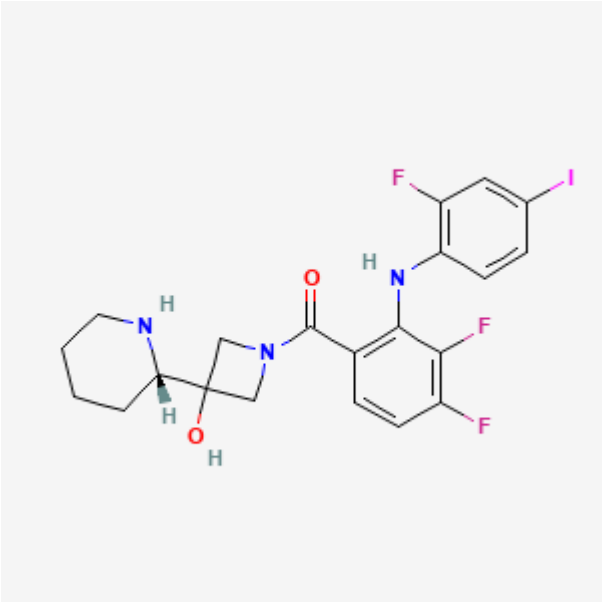


Fig 2.5 - 2D Structure of the Cobimetinib

2.6.6 MEK Inhibitors under Clinical Developments

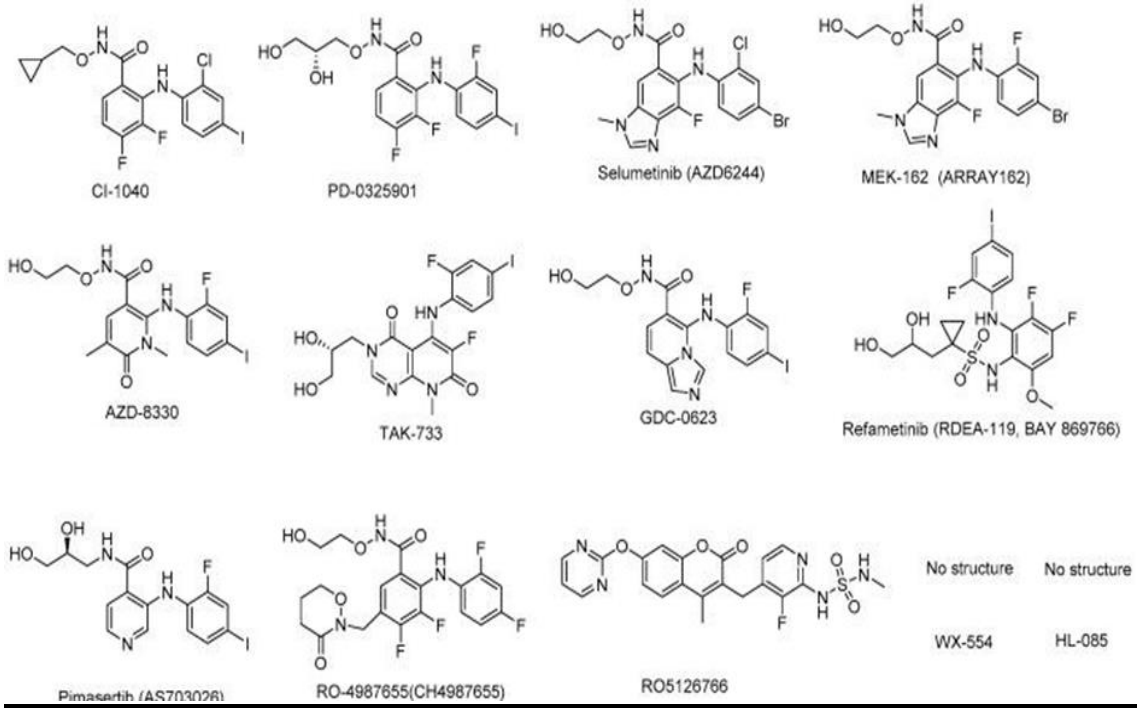


Fig 2.6 - MEK inhibitors in clinical study [30]

There are several MEK inhibitors under preclinical developments as well, and their structures are shown in Figure.

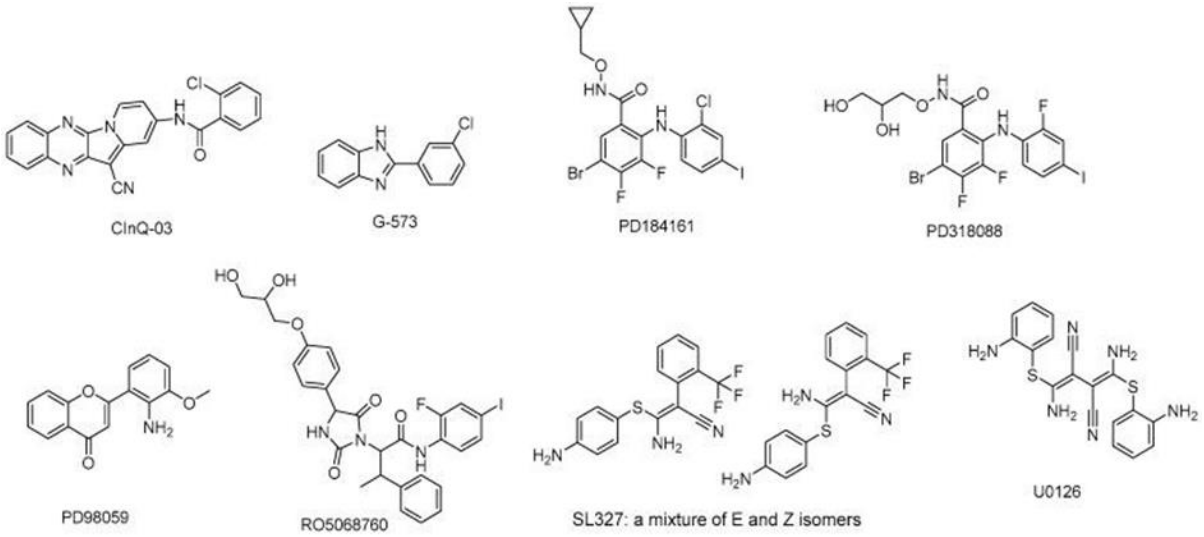


Fig 2.7 - MEK inhibitors in preclinical study

2.6.7 Uses of MEK inhibitors

The MAPK pathway is the target of extremely selective cancer-fighting drugs called MEK1 and MEK2 inhibitors. In BRAF- and NRAS-mutant cancer cell lines, selective inhibitors of MEK can reduce symptoms and temporarily halt the course of the disease. Being more efficient and less harmful than monotherapy using a BRAF inhibitor by itself,The gold standard of care for those with BRAF-mutated melanoma is currently a combination of MEK inhibitor therapy and a BRAF inhibitor..

Additionally, by blocking BRAF activity, the MEK1/2 agent has been given the go-ahead to treat advanced lung cancer that is not small cells having BRAF mutations.

For a variety of tumors, including BRAF-mutated melanoma and KRAS/BRAF-mutated colorectal cancer, where MAPK/ERK is overactive, more inhibitors of MEK are now undergoing preclinical and clinical studies [27].

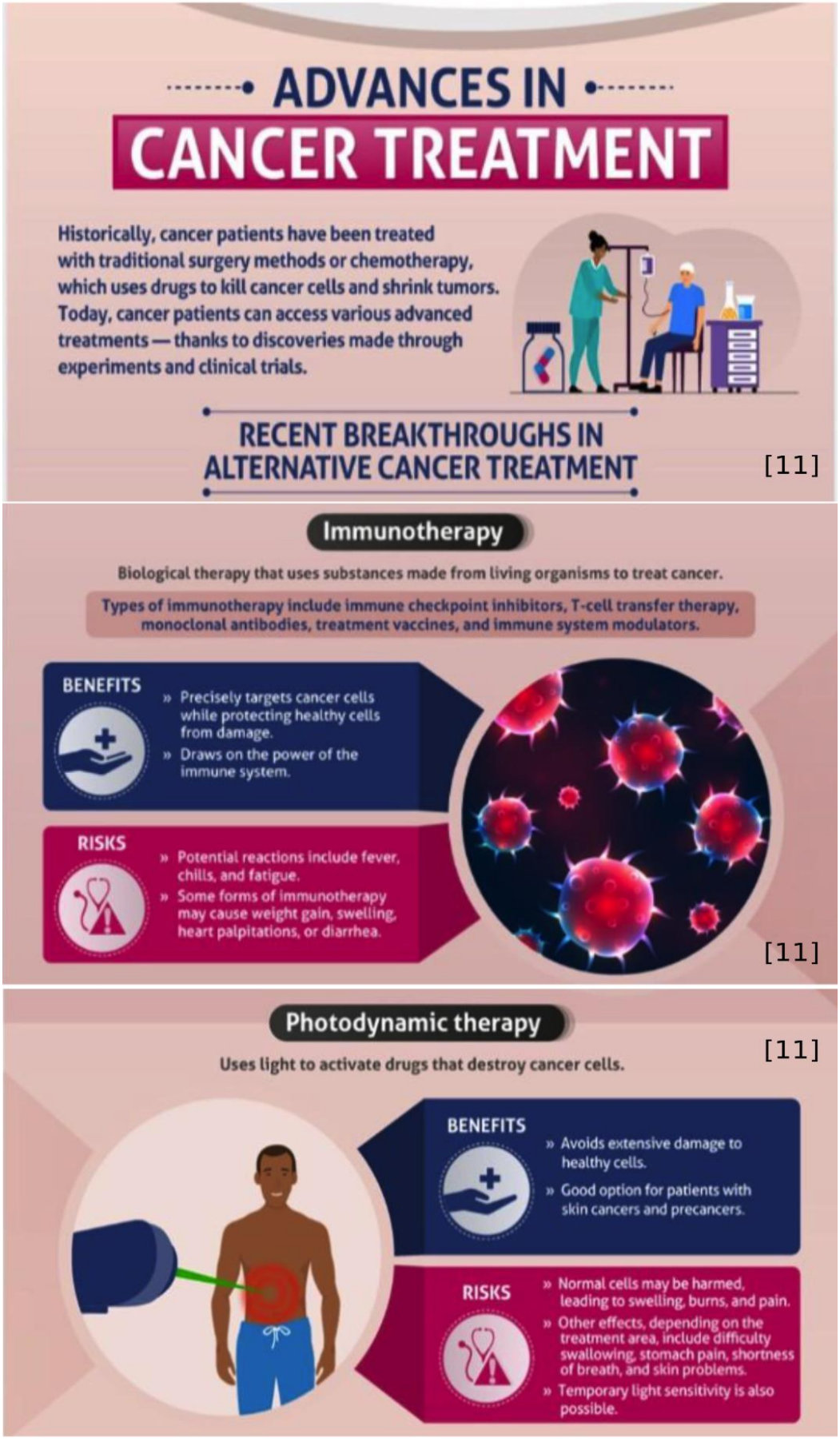


Fig 2.8 – Cancer Treatment Alternatives (a)

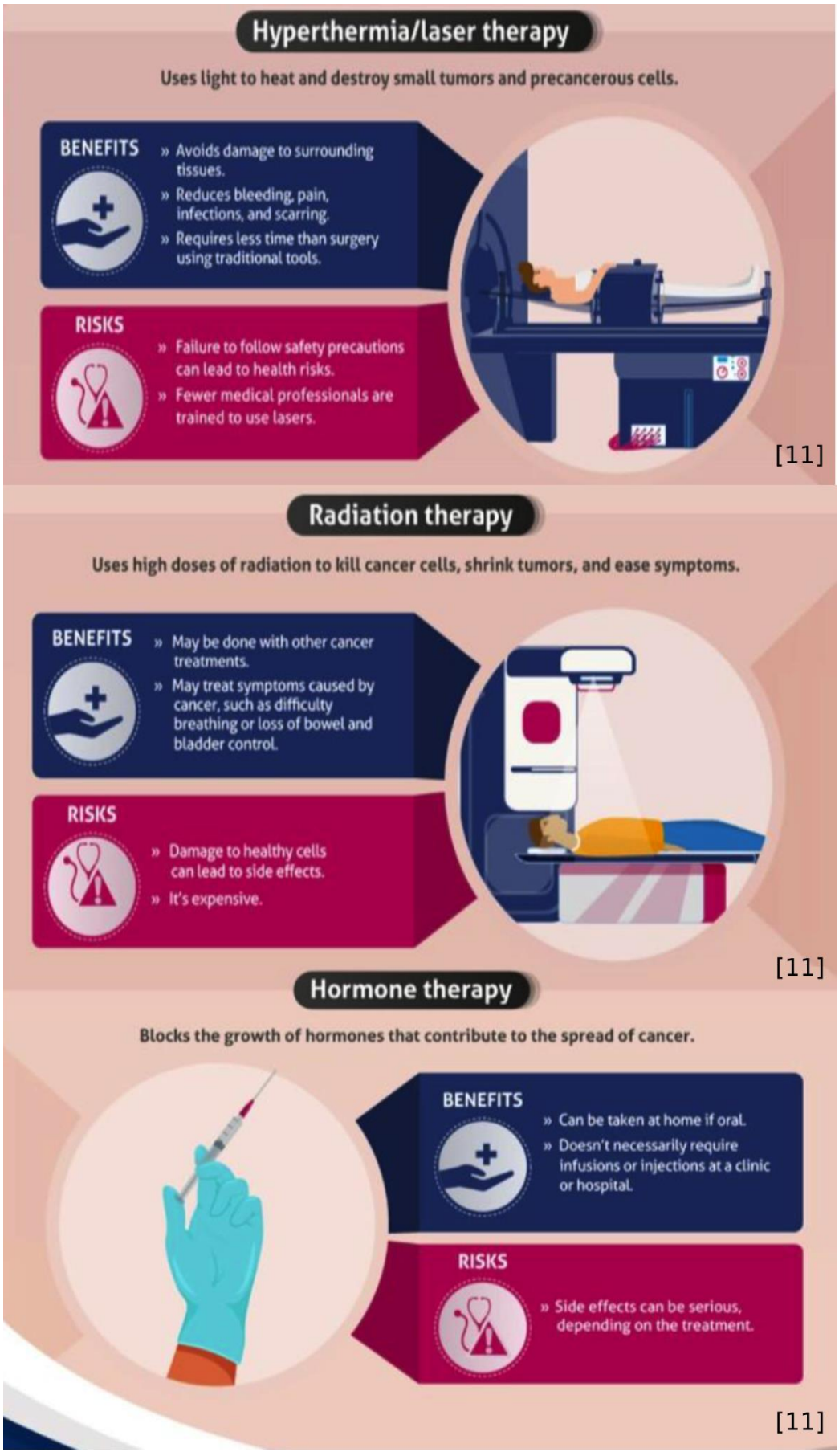


Fig 2.9 - Cancer Treatment Alternatives (b)

2.7 *RHODODENDRON*

Fig 2.10 –Flower of *Rhododendron*

According to taxonomy, the plant is [28]

Plant's Kingdom: Plantae

Phylum: Magnoliophyta

Class: Angiospermae

Order: Ericales

Plant's Family: Ericaceae

Genus: *Rhododendron*

Species:*arboreum*

We have access to a wide range of plants in nature that have many various uses, such as ornamentation, medicine, growing things, with fruiting. The practice of growing plants for therapeutic as well as commercial purposes is a long-standing one today, and researchers are looking at neglected species that have historically been used for various purposes [3]. India consists of a large biodiversity and rich medicinal herb traditions (Ayurveda), that give a good approach to the study of many plants used in primary care. The rhododendron is an example of a plant that is assuming a distinctive role in the overall cultural as well as economic life of individuals. Greek is the language that gave us the terms "rhodo" (which translates to "rose") and "dendron" (which means "tree"). It is a member of the Ericaceae family and Carl Linnaeus first described it in 1837 [10,11,14,28]. The *rhododendron* was first discovered in the Himalayan valley, Kashmir , Assam, and Manipur in India, as well as in some parts of Bhutan. Visitors are drawn in by the fully developed flowers' aesthetic splendor during the blooming season. These factors have led to the flower being designated as Nepal's national flower and Himachal Pradesh's state flower (India) [28].

India has long been regarded as a veritable gold mine of species of aromatic and medicinal plants. WHO estimates (2000), 80% of Indians employ plant-based remedies to treat a range of illnesses, and 65% of the worldwide people utilize medical herbs for therapy [28]. *Rhododendron*, a plant that grows organically, has a multitude of health benefits, including, such as the capacity to cleanse the body and decrease inflammation, as well as the potential to avoid, cure heart, dysentery, and diarrheal illnesses [27]. Strong antioxidant qualities may be found in the leaves. Utilize the leaf to alleviate migraines. The wood from this shrub may be used to create posts, khukri handles, saddles, goodie bags, and gunstocks. With very few exceptions, The food and pharmaceutical sectors' researchers and processors have not yet made use of the flower's restricted availability.. To spread knowledge about the *rhododendron*, this article discusses its classification, location, area of production, ingredients, methods of distribution, health benefits, applications, and prospects[27].

2.8 DISTRIBUTION AND CLASSIFICATION

Biologists have developed several classes based on physical details such as flowers, leaves, hair, and other characteristics because of the diversity of species within a particular phylum. The major genus of the Ericaceae family, Rhododendron, contains more than 1200 species that are distributed over many different geographic areas, include the northern part of Asia, Europe and Asia, the western part of Europe, and North America.. China is the only place where more than 70% of the 500 *Rhododendron* species may be found. George Forrest earned the moniker "King of *Rhododendron*" for his discovery and identification of *R. protistum* var. *giganteum*, one of the oldest and tallest *Rhododendron* trees, in 1919. Table.1 includes the most frequent subspecies of *Rhododendron arboreum*, the most widely distributed *Rhododendron* species [27].

Due to its cultural applications, economic benefits, and medical properties, the *Rhododendron*, one of the most well-known horticultural plants, has gained popularity in gardens and as street trees. The cultivation of Rhododendron is extensive on a global scale, encompassing Southeastern Asia from the Northwestern Himalaya to regions such as Western and Central China, Upper Burma, Arunachal Pradesh, Bhutan, Nepal, the northern state of Si Eastern Tibet, and Sikkim. These locations are home to 90% or more of the wild *Rhododendron* population on earth [26].

Table 2.1 - Subspecies of *Rhododendron arboreum*

Subspecies	Characteristics and distribution
Rhododendron arboreum spp. Arboreum	Red flower, found in Western Himalayas
Rhododendron *arboreum* spp. Cinnamomeum	White, pink or red flower, found in Central Himachal
Rhododendron *arboreum* spp. *Delavayii*	Red flower, found in Eastern Himalayas
Rhododendron *arboreum* spp. Nilagiricum	Red flowers, found in Nilgiri
Rhododendron *arboreum* spp. Zeylancium	Orange red flowers, found in Sri Lanka

2.8.1 *Rhododendron* Distribution

A total of 1200 Rhododendron species are thought to exist in the globe, with China having the most with 571 species, 409 of which are native. About 80 species, and 10 subspecies, as well as 14 variations may be found in India. Historical data indicates that Sikkim harbors approximately 72% of the total Indian species found in the Himalayan region, accounting for 98% of the species diversity in that area [27].

2.9 COMPOSITION OF *Rhododendron arboreum*

Rhododendrons contain the minerals Manganese, iron, copper, sodium, chromium, Co, lead, Mb, Ni, Pb, and As. Minerals are needed for the maintenance of specific physico-chemical phenomena that are required for life. A number of metabolic processes need the important cofactors Mn, copper, Se, Zn, Fe, and Mb, which are found in the molecular makeup of several enzymes. Sodium is necessary for the maintenance of osmotic equilibrium between cells and interstitial fluid [27].

Due to their potent antioxidants, antimutagenic, and anticancer activities, researchers have recently concentrated in particular on phytochemicals originating from plants. minimal toxicity to mammals, and therapeutic qualities that help sustain human health. Researchers believe that eating foods high in antioxidants, such as those that contain phenolic compounds and a variety of other phytochemicals with antioxidant properties, can reduce oxidative stress and its effects, such as DNA mutations and the emergence of cancer. The adverse effects and long-term toxicity of synthetic antioxidants are detrimental to health. As a result, scientists are becoming increasingly interested in locating and purifying the natural antioxidants present in plants. Although R. *arboreum* is frequently employed in conventional treatment procedures, there are few scientific studies that support its antimutagenic and anticancer qualities [27,28].

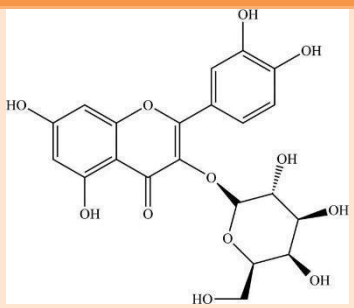
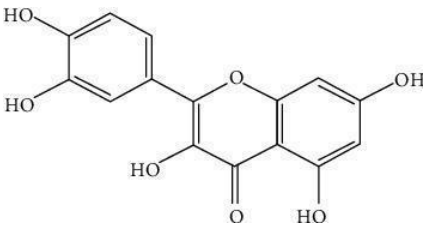
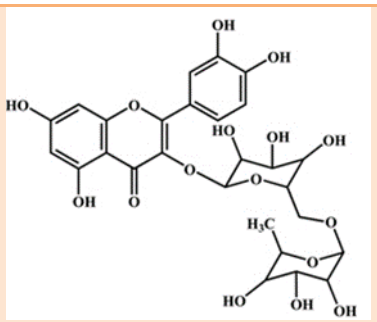
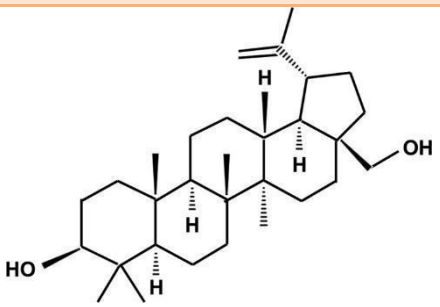
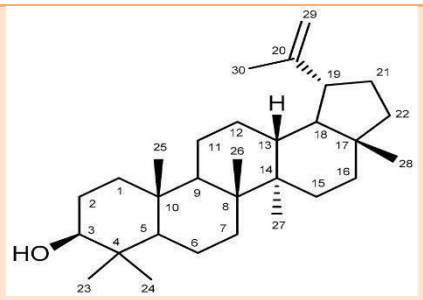
Table 2 emphasizes the broad variety of non-nutritive plant compounds with protective as well as disease-preventive effects, the phytochemical capacity of different Rhododendron plant sections. These secondary metabolites, including alkaloids, flavonoids, glycosides, saponins, tannins, steroids, and phlobatannins, contribute to the

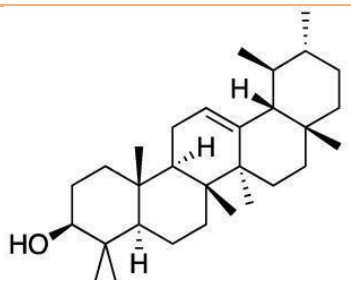
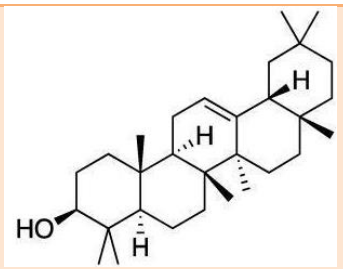
overall phytochemical profile of the plant. Secondary metabolites are crucial for the plant's growth and vitality, and they also hold significance for human health [28].

Table 2.2 - Phytochemicals present in *Rhododendron arboreum* plant

S. No.	Part of plant	Compound
1.	Bark	Ursolic acid acetate Betulinic acid Leuco-pelargonidin
2.	Leaves	Glucoside Ericolin Ursolic acid Quercetin Hyperoxide Flavone glycosides Flavonoids
3.	Flowers	Quercetin-3-rhamnoside Phenolic compounds: Rutin Coumaric acid

Table 2.3 : Structures of a few phytochemicals

Compound Name	Structure
Hyperoside	
Quercetin	
Rutin **(Flavonol glycoside)**	
Betulin **(Triterpenoid)**	
Lupeol **(Triterpenoid)**	

α- amyrin (Pentacyclic Triterpenoid) **β- amyrin**	
β- amyrin (Pentacyclic Triterpenoid)	

2.10 MEDICAL PROPERTIES OF R.arboreum PLANT

TABLE 2.4 - Medicinal uses of diff. parts of Plants

Plant part	Bioactive compounds	Medicinal uses	Remarks
Flower	Contains profile of phenolic compounds quercetin (C15H10O2), rutin (C, coumaric acid (CHgO), saponins, xanthoprotein, steroids tannins, etc., sugar, amino acid vitamins, organic acid etc.	Anti-diabetic, beneficial against diabetic nephropathy (DN), anti-diarrheal activity, antimicrobial activity anti- inflammatory, dyspepsia, anti-nociceptive Hepato-protective, fungal infection, free radical scavenging activity, anti-allergy.	Since the flower contains anti-diabetic potential it (2 can be used as nutraceutical or functional food for diabetes and its complications Phenolic compounds present in it to enhance the medicinal properties and therefore has been used widely for healing many diseases.

Leaves	Leaves contain glucoside, ericolin, ursolic acid a-amyrin, epifriedelinol Campanulin, quercetin, hyperoside They also contain flavore glycoside, dimethyl ester of terephthalic acid and certain flavonoids phenols, epicatechin, catechins, anthocyanidins	Antioxidant activity, anti-inflammatory gout and rheumatism (dried leaves), alleviate headache and fever, diuretics, fungal infection, to relief toothache, sciatica syphilis, treatment of cold cough, asthma, bronchitis, post-delivery complications, indigestion, lung infection, hepatic disorders, analgesic activity, anti-allergy	Flavonoids are present in the leaves and vitamin C is [2] found to possess antioxidant activity and the leaves are also helpful in the treatment of gout and rheumatism and are used traditionally for healing many diseases like cough, cold, fever, indigestion, headache, etc.
Root	Alkaloids, terpenoids, tannins, reducing sugars, steroids, saponins, anthraquinones.	Anticancer anti-nociceptive, anti-inflammatory, prevent cardiovascular diseases.	The presence of secondary metabolites signifies that [2] the root of *rhododendron* can be used as a the apeutic agent.
Bark	Alkaloids steroids terpenoids tannins, saponins and reducing sugars the bark is also found to be a rich source of taraxerol, betulinic acid, ursolic acid (C4O)	Excellent cold reliever, antimicrobial agent anti-inflammatory, anti-oxidative anti-inflammatory anti-oxidative anti-carcinogenic, anti-mutagenic anti-atherosclerotic, anti-hyperlipidemic, and antimicrobial effects	Alkaloids help to control the development system of [2 the living organism and play a metabolic role by providing the protective properties Tannins have the reducing power which prevents liver injury by inhibiting the lipid peroxides Ursolic acid has a few biological as well as medicinal properties
Stem	Alkaloids steroids, terpenoids anthraquinones tannins, glycoside saponins and reducing sugar.	Hemorrhage, hay fever, bronchial asthma, anticancer prevent cardiovascular diseases.	Bioactive substances can promote good health, [4] and they are used for healing many diseases.

2.11 BIOLOGICAL AND PHARMACOLOGICAL CHARACTERISTICS

1. Adaptogenic activity

The existence of secondary compounds with antioxidant activities has recently attracted a lot of attention to the therapeutic usefulness of plants.These substances help plants defend themselves from a variety of enemies, including insects, mammals, and microbes. According to Som et al. (2019), *Rhododendron* sp. has a variety of compounds that have been isolated, including flavonoids, diterpenes, alkaloids, triterpenes, phenolics, steroids, tannin, quercetin, saponin, gallic acid, tannin, and glycosides, that have potent anti-stress and strong antioxidant properties, suggesting that they may be the source of adaptogenic activity[29].

2. Antidiarrheal activity

Rhododendron *arboreum* flowers had strong antidiarrheal activity when ethyl acetate was added to the mixture. In the castor oil-induced diarrhea, the fraction considerably reduced the number of diarrheal stools (Verma et al., 2010)[29].

3. Anti-inflammatory and Analgesic properties

In a study conducted by Gautam et al. (2018), it was observed that the bark of a *Rhododendron* tree exhibited anti-inflammatory properties in reducing rat paw swelling. Additionally, another study revealed that the ethyl extract derived from the flower of the plant displayed both anti-inflammatory and pain-relieving effects. The presence of several phytochemicals can be credited with these beneficial benefits., including flavonoids, tannins, and saponins, which may act individually or synergistically. It is believed that the high concentration of flavonoids in the ethyl extract is responsible for its significant anti-inflammatory activity[29].

4. Antioxidant and antimutagenic activity

With urbanization and environmental pollution on the rise, plant-derived antioxidants and anti-mutagens are becoming increasingly important for safeguarding human health. The hexane, the solvent chloroform and ethyl acetate (ETA) fractions of R. *arboreum* extracts of leaves were shown to be efficient in lowering nitric oxide radical generation and suppressing lipid peroxidation in a research. The extract's beneficial properties were likely due to the combined effects of various phytochemicals present, which were identified through GC-MS profiling. Furthermore, the antioxidant activity of the extract may be attributed to the presence of vitamin E[29].

In a study conducted by Acharya et al. (2011), Comparing the methanol extract of the plant's leaves to conventional quercetin, it was found that it had a little antioxidant effect. On the other hand, Bhandari and Rajbhandari (2014) found that the ethanolic extract of the flowers demonstrated high antioxidant activity and activation of nitric oxide synthase. Sonar et al. (2012) focused on extracting quercetin from the flower petals and assessing the overall flavonoid content, phenolic content, and antioxidant activity of various parts of the *Rhododendron arboreum* plant[28].

5. Cancer Prevention

Bhandary and Kuwabata (2008) found that the ethanolic extract of leaves had a significant anti-tumor effect against Agrobacterium tumefaciens-induced tumors in potato discs. This effect was observed to be dose-dependent, indicating that the higher the dose, the greater the activity against the tumor. The investigators also identified two compounds, rutin and quercetin, found in the extract, which could potentially contribute to the anti-tumor properties[28].

6. Anti-diabetic activity

Parcha et al. (2017) investigated the anti-diabetic activity of *Rhododendron arboreum* flowers and isolated the active compounds present in them. They found that the ethyl acetate soluble fraction of the flower was more potent in inhibiting the activity of a-glucosidase, which is a key enzyme involved in carbohydrate digestion, than the water-soluble fraction[28].

7. Cardioprotective activity

According to Murty et al. (2010), certain secondary metabolites found in Rhododendron *arboreum* may be able to neutralize dangerous and toxic compounds. Rats receiving isoproterenol treatment were given an ethanolic extract from the whole plant.. They observed significant reductions in the activity of enzymes such as alanine transaminase (ALT), aspartate transaminase (AST), and lactate dehydrogenase (LDH), as well as levels of Mass drug administration (MDA) in both serum and heart tissue. Moreover, the extract increased the activity of antioxidant enzymes including Superoxide dismutases (SOD), catalase, glutathione peroxidase (GPx), and glutathione (GSH). Comparatively, the ethanolic extract of the flower was found to be more effective than the aqueous extract in reducing the release of lactate dehydrogenase and creatine kinase in albino rats. Notably, the n-butanol fraction of the ethanolic extract exhibited the highest level of cardio-protective activity among all the tested extracts[30].

8. Hypolipidemic effect

Verma et al. (2011) conducted a study in which they orally administered a combination of Hippophae rhamnoides fruit juice and *Rhododendron arboreum* flower juice in a 1:4 ratio to test its effectiveness in reducing lipid levels. The researchers found that the administration of this combination significantly decreased total cholesterol, triglycerides, low-density lipoprotein, and the atherogenic index.

9. Hepatoprotective and Immuno-modulatory activity

Painuli et al. (2015) discovered that the ethyl acetate fraction of *Rhododendron* arboreum flower extract possessed hepatoprotective properties in preventive and curative models against carbon tetrachloride (CCl4) induced liver damage. The researchers observed that the ethyl acetate fraction demonstrated a dose-dependent prevention of elevated hepatic malondialdehyde formation and depletion of reduced glutathione content in rats intoxicated with CCl4. Similarly, Acharya et al. (2011) reported significant hepatoprotective effects of the ethanolic leaf extract of R. arboreum against liver damage induced by carbon tetrachloride in rats. The extract reduced the levels of serum enzymes such as Alkaline phosphatase (ALP), serum glutamic oxaloacetic transaminase (SGOT), and serum glutamic pyruvic transaminase (SGPT), as well as triglycerides,Bringing back to normal levels were the elimination of ascorbic acid, total bilirubin, and cholesterol. Furthermore, Bhandary and Kuwabata (2008) discovered that the alcoholic extract of R. *arboreum* leaves exhibited safe and effective immunosuppressive properties[30].

2.12 TOXICOLOGY AND ANTIMICROBIAL ACTIVITY

According to Ali et al. (2008), the leaves of *R. arboreum* displayed high cytotoxicity, while the stem and roots exhibited moderate effects, and the bark showed the least significance. The extracts contained glycosides, alkaloids, and flavonoids, which were considered potential contributors to the observed activity. In terms of antimicrobial potency, According to Ali et al. (2008) as well as Prakash et al. (2008), the petals, leaf, stem, and root of the plant had a substantial impact on Bacillus subtilis, Salmonella typhi, as well as S. aureus. The leaf extract was found to be more effective than the flower extract in inhibiting the growth of microorganisms, according to Prakash and others(2008). Methanol as well as aqueous leaf extracts were both effective, according to Chauhan et al. demonstrated a zone of inhibition against *S. aureus*, *Klebsiella pneumoniae*, *Streptococcus pyogenes*, and *E. coli.* Additionally, the ethanolic floral extract shown notable effectiveness against Salmonella typhi, E. coli, B. subtilis, and S. aureus. as indicated by Sharma et al. (2013). Bhandary and Kuwabata (2008) reported that ethanol and methanol extracts, as well as isolated quercetin, displayed effectiveness against *E. coli* and *S. aureus*. The ethanolic floral extract revealed antifungal properties over Aspergillus flavus, the

yeast Candida albicans, as well as Aspergillus parasiticus in addition to playing an important part against Escherichia coli, S. epidermidis, as well as S. aureus. Saranya and Ravi (2016) found that the water extract was efficient against Aspergillus flavus, Aspergillus parasiticus, and Candida albicans. Effective antifungal activity was seen over Fusarium solani, a species of Aspergillus niger, M. canis, Cantharellus flavus, yeast albicans, plus Candida glabrata in methanol plus ethyl acetate extracts. According to Ali et al. (2008), the hydrophilic character of betulin and 3-acetoxy urs-11, 12-epoxy-13 may be the cause of their high activity. Furthermore, as reported by Acharya and colleagues (2011) and Painuli et al. (2015), the ethanolic leaf extract significantly decreased serum enzyme stages, bilirubin, and cholesterol in mice treated with carbon tetrachloride, while the ethanolic flower extract demonstrated liver-protective potential towards carbon tetrachloride (CCl4)-induced harm to the liver across both preventive and curative models [30].

CHAPTER 3

OBJECTIVES

1. Collection, authentication, pretreatment of the collected plant samples from two different regions of Himachal Pradesh.

2. Preparation of Methanolic Leaf Extract (MEL) Extract of Rhododendron *arboreum* and Rhododendron maximum using Soxhlet method.

3. Concentrating on the MEL, using rota-evaporator. Lyophilization of the MEL.

4. To analyze the chemical composition of the Rhododendron leaf extracts and identify bioactive compounds such as flavonoids, terpenoids, and alkaloids.

5. Employing a variety of assays, quantify the antioxidant capacity of Rhododendron leaf extracts and assess their efficacy as natural antioxidants.

6. Evaluate the potential of Rhododendron leaf extracts as natural antibiotics by looking at their antibacterial activities against bacteria and fungus.

7. By comparing the chemical makeup of these chemicals with their biological functions, it will be possible to pinpoint the putative bioactive substances that are behind the antioxidant, antibacterial, and cytotoxic properties of Rhododendron leaf extracts.

8. To contribute to the scientific understanding of the potential health benefits of Rhododendron and its potential applications in fields such as medicine, agriculture, and biotechnology.

9. To perform Molecular Docking Studies using the plant's phytochemicals against Cancerous protein.

CHAPTER 4

METHODS AND MATERIALS

4.1 USED CHEMICALS

Sodium hydroxide, ferric chloride, lead acetate, molisch's reagent, dilute HCL, concentrated sulphuric acid, wagner's reagent, mueller hinton Agar/Broth, DMSO, Gallic acid, Folin-Ciocalteu reagent, 1,1-diphenyl-2-picrylhydrazyl (DPPH), ascorbic acid, quercetin, aluminum chloride, sodium acetate, and sodium carbonate. Solvents like ethanol and methanol were also used during the experiments.

4.2 PLANT MATERIAL

The leaves of *Rhododendron arboreum* and *Rhododendron maximum* were obtained from two different regions of Himachal Pradesh during the months of August and September. The leaves were then identified and verified at the herbarium of Dr. YS Parmar University of Horticulture and Forestry, Nauni.

Location 1: Advance Study, Summer Hills

Fig 4.1 – Rhododendron *maximum*

(Source Internet)

Location 2: Tara Devi, Shimla

Fig 4.2 – Rhododendron *arboreum*

(Source Internet)

4.3 PREPARATION OF SAMPLES

The pretreatment of the leaves for both the samples was done according to the procedure provided by [V. Gautam et al]. The materials which are used for this pretreatment includes: Tween-80, bavistin and double distilled water. The procedure for the same are as follows:

I. Separated clean leaves to a clean tray.
II. Washing of leaves with tween-80 solution.
III. Transferred the washed leaves to the bavistin solution.
IV. Final washing with double distilled water.
V. The leaves were then dried in an incubator below 40 degrees Celsius overnight, and then crushed to thin powder in a grinder.

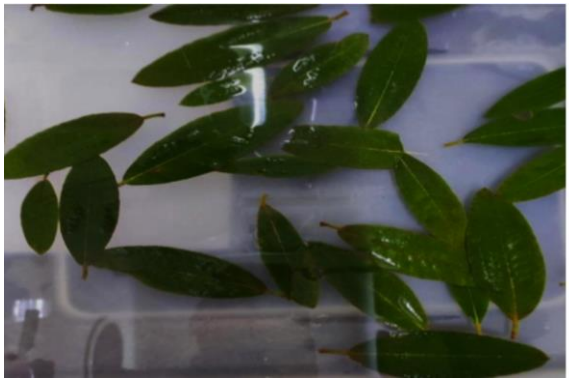


Fig 4.3 - Leaves inTween-80 solution (Anti-bacterial detergent)

Fig 4.4 - Leaves in bavistin solution (Anti-fungal solution)

4.4 PLANT EXTRACTION

Rhododendron *arboreum* and Rhododendron maximum plant samples were then extracted using soxhlet method. The Soxhlet method is a procedure utilized to extract organic compounds from solid materials. It involves the use of a specialized apparatus called a Soxhlet extractor to repeatedly extract the sample with a solvent. The solvent is boiled in the flask, and the resulting vapor condenses in the condenser, eventually dripping down into the thimble holding the sample. This allows the solvent to extract the target compound from the solid material. Afterward, the solution is heated to evaporate the

solvent, leaving behind the extracted compound. Finally, the condensed solvent vapor is collected and returned to the flask, completing the extraction process.The extraction takes around around 3 to 4 days, or even 1 to 3 weeks depending on the type of sample being extracted.

The procedure for the extraction process are as follows:

1. Pre-treatment of the leaves were done.

2. The dried leaves were then weighted 20 gms each, and rolled inside a muslin cloth and sealed using a stapler.

3. The soxhlet assembly was assembled as per the instructions.

4. The folded muslin cloth was put inside the thimble.

5. With the help of a funnel, 400 ml of pure Methanol was then poured from the top of the condenser inside the thimble.

6. The water supply was kept on.

7. The Soxhlet apparatus were kept over a heating mantle with a set temperature of 50 degrees.

8. The plant extraction proceeded. This extraction was done until there was a change in color inside the thimble (from dark green to colorless).

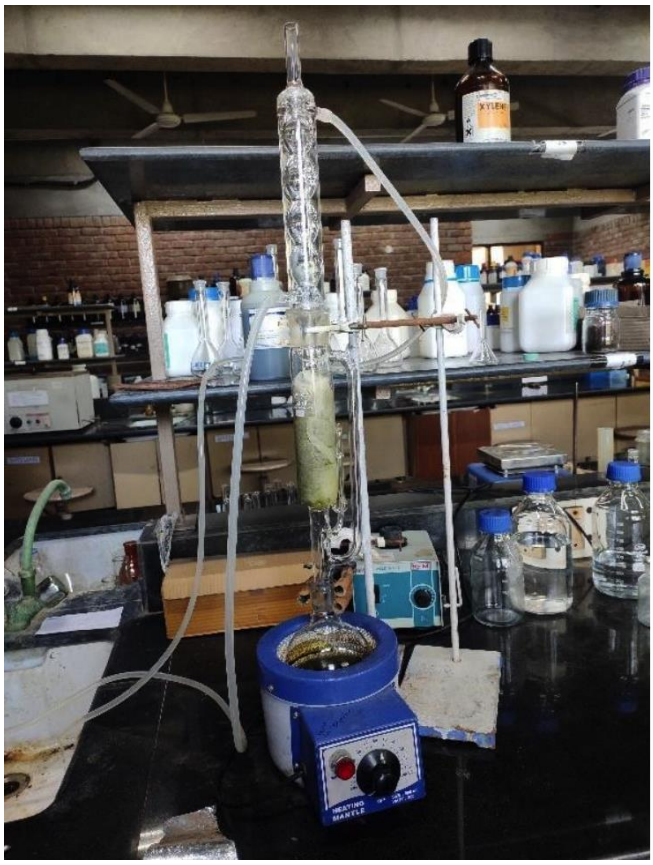


Fig 4.5 - Soxhlet extractor used for obtaining plant extract in methanol

4.5 DETERMINATION OF PERCENTAGE YIELD (%)

To calculate the extract's percentage yield, the weight of the dried extract (a) and the weight of the initial sample material (b) were measured, and the following formula was utilized:

$$\textbf{Percentage yield (\%)} = a/b \times 100$$

This calculation was performed for each extract to determine its extraction yield.

4.6 CONCENTRATION PROCESS

After the extraction was over, the extracts for both the plant samples were concentrated using a rota-evaporator. A rotacap or rotary evaporator is a laboratory tool that is utilized to evaporate solvents from a sample. It comprises a heat bath that controls the temperature of the sample, a rotating flask that holds the sample, and a condenser that cools and condenses the evaporated solvent. By adding the sample and solvent into the rotating flask and heating it up, the solvent is evaporated and condensed back into a liquid formed through the condenser. This separates the solvent from the sample, enabling the concentration and purification of the sample. This apparatus is widely used in chemistry, biochemistry, and cannabis industries for various applications.

I. The rotational speed of the flask causes the ingredients to form a significant layer of thin film on the inner wall of the evaporation flask.

Fig 4.6 - Rota evaporator used for the concentration of the extracts

4.7 LYOPHILIZATION PROCESS

Lyophilization, also known as freeze-drying, is a preservation process that involves removing moisture from perishable materials. Freezing, basic drying, plus secondary drying are the three steps in this procedure. The material is rapidly frozen during the first stage to maintain its structure and prevent the formation of ice crystals. During primary drying, the pressure is reduced, and the temperature is slowly increased to vaporize the ice crystals. In the second drying stage, residual moisture is eliminated by further decreasing the pressure and increasing the temperature. This process is commonly used in various industries such as pharmaceutical, biotechnology, and food to stabilize and preserve products, including vaccines, enzymes, proteins, and fruits. Here, we needed to lyophilize the extract for 19 hours; to get the extract in powdered form.

Fig 4.7 - Lyophilizer used for the drying of the extracts

4.8 POLYPHENOL QUALITATIVE ESTIMATION

Different techniques and processes were used to analyze the plant samples both qualitatively and quantitatively. The plant samples were measured and weighed according to the test's requirements.

4.8.1 Tests for Carbohydrate

Molisch's Reagent Use:

i. Fill a test tube with 3 ml containing the test extract.

ii. To the test tube, add a few drops of the alpha-naphthol solution.

iii. Mix the contents of the test tube thoroughly.

iv. Add a few drops of concentrated sulfuric acid to the mixture, while being careful to avoid splashes or spills.

v. Observe the interface of the two liquids for the formation of a violet ring.

vi. Note the presence or absence of the violet ring and interpret the results accordingly.

4.8.2 Test for Alkaloid

Using Mayer's Reagent

i. TIn a test tube, place 2 ml of the solution extract.

ii. Add 2 ml of 2% hydrochloric acid to the test tube.

iii. Mix the contents of the test tube thoroughly.

iv. Drops of Mayer's reagent should be added to the solution.

v. Observe the solution for the occurrence of reddish-brown precipitate.

vi. The formation of a reddish-brown precipitate indicates the liquid extract contains alkaloids.

vii. Interpret the results accordingly.

4.8.3 Tests for Flavonoids

i. Fill a test tube with 2 ml containing the test extract.

ii. To the test tube, add a couple of drops of the NaOH solution.

iii. Mix the contents of the test tube thoroughly.

iv. Observe the appearance of an intense yellow color in the solution.

v. Add a few drops of diluted HCl to the solution.

vi. Mix the contents of the test tube thoroughly again.

vii. Observe the solution for the disappearance of the yellow color and the development of a colorless solution.

viii. Note the presence or absence of a colorless solution as an indicator of the presence of flavonoids.

4.8.4 Tests for Saponins

i. Fill a test tube with 1 ml containing the test extract.

ii. Drop of 1% solution of lead acetate should be added to the test tube.

iii. Mix the contents of the test tube thoroughly.

iv. Observe the mixture for the appearance of an intense white precipitate.

v. If an intense white precipitate forms, it indicates the presence of saponins in the test extract.

vi. Note the presence or absence of the precipitate and interpret the results accordingly.

4.8.5 Test for Sterols

i. Fill a test tube with 2 ml containing the test extract.

ii. Fill the test tube with 2 mL of pure sulfuric acid.

iii. Mix the contents of the test tube thoroughly.

iv. Observe the mixture for the formation of a red precipitate.

v. Note the presence or absence of the red precipitate and interpret the results accordingly.

vi. The existence of sterols within the test extract is shown by the formation of a crimson precipitate.

4.8.6 Tests for Tannins

i. Take 1 ml of the test extract in a test tube.

ii. Add 1 ml of 3% ferric chloride solution to the test tube.

iii. Mix the contents of the test tube thoroughly by swirling gently.

iv. Observe the color of the mixture.

v. Look for the development of a brownish green color.

vi. If a brownish green color appears, it indicates the presence of tannins in the extract.

vii. If there is no color change, it suggests that tannins are absent in the extract.

4.9 QUANTITATIVE POLYPHENOL ESTIMATION

4.9.1 Total Carbohydrate Content Calculation

The anthrone technique was used with spectrophotometry to estimate the overall carbohydrate content in a plant extract. In this method, glucose was employed as the standard, and the total carbohydrate content was expressed as milligrams of glucose equivalent per gram of sample (mg GE/g sample). This was calculated by comparing the absorbance of the sample to that of the glucose standard. Using this method, the total carbohydrate content of the sample was quantified and expressed as glucose equivalents, which allows for direct comparison to glucose-based standards.

4.9.2 Total Protein Content Determination

The Lowry method was employed to determine the total protein content of the sample. In this method, 1 mL of sample was combined with 4 mL liquid alkaline copper reagent as well as allowed to stand for 20 minutes. The mixture was then incubated in the dark at 37°C approximately 15 minutes with 0.5 ml containing Folin's phenol reagent. Standard solutions containing BSA at different concentrations were treated in a similar manner. The resulting blue color was measured at 750 nm using a spectrophotometer. The total protein content of the sample was expressed as milligrams of protein per gram of tissue. This method is widely used for protein quantification due to its sensitivity, specificity, and compatibility with various sample types, making it a reliable and commonly employed assay.

4.9.3 Complete Phenolic Content (TPC) Determination

The total phenolic amount (TPC) of the extract was determined using the Folin-Ciocalteu technique, a spectrophotometric assay. TPC was calculated in milligrammes of the equivalent of gallic acid (GAE) each gramme of material. Gallic acid was utilized as a standard in the experiment to establish a reference for comparing the phenolic content of the extract. This method is widely utilized in assessing phenolic content due to its straightforwardness, sensitivity, and accuracy. It is commonly applied to various biological samples, including plant extracts and food products, to determine the phenolic composition.

4.9.4 Complete Flavonoid Content (TFC) Determination

The aluminum chloride technique, an increasingly common spectrophotometric test, was employed to assess the extract's total flavonoid concentration (TFC). Quercetin, a well-known flavonoid compound, served as a standard for comparing the flavonoid content of the extract. TFC was calculated as milligrammes per the equivalent of quercetin (QE) per gramme of material. This method is commonly employed to quantify flavonoids in plant extracts and food products due to its simplicity, sensitivity, and reliability in generating reproducible results.

4.10 ANTIOXIDANT ACTIVITY DETERMINATION

Assay for radical scavenging by ABTS

The ABTS radical ions decolorization test was used to assess the antioxidant activity in plant materials. The experiment involves producing an ABTS+ cation radical via combining 7 mM ABTS using 2.45 mM potassium persulfate, this was then left to incubate in the dark for 12-16 hours at room temperature. After diluting the resultant ABTS+ solution with methanol, an absorption value of 0.700 on 734 nm was obtained. Plant extracts were added to the diluted ABTS·+ solution, and the absorbance was measured after a 30-minute incubation period. A solvent blank was used as a control in each assay. The percentage suppression at absorbance at 734 nm was estimated using the formula: ABTS+ scavenging effectiveness (%) = ((AB - AA) / AB) 100, where AB represents ABTS radical + methanol absorbance and AA represents ABTS radical + samples extract/standard absorbance. Trolox was used as a control drug in this test. To guarantee the precision and dependability of the results, the measurements were performed at least three times.

4.11 ANTIMICROBIAL SUSCEPTIBILITY TESTING (AST)

The agar well diffusion technique was used to test the antimicrobial properties of an aqueous and solvents extracts. A bacterial culture with a concentration of 106 colony-forming units (cfu)/ml for an individual bacterial strain; a sterile swab dipped in the bacterial solution was used to disseminate the microorganisms over nutrient agar plates. The plant extract, which had a concentration of 25 mg/ml, was then poured into wells having a dimension of 8 mm that had been made in sterile medium consisting of agar. The plates were subsequently left to incubate at 37°C for 24 hours after the extracts had been allowed to diffuse for two hours at room temperature. The widths of the areas of inhibition were determined in millimeters, and both negative and positive controls using distilled water and Amphotericin B had been included. The findings were presented as the mean standard deviation.

4.12 IN-SILICO STUDIES

4.12.1 Preparation of protein target:

Prior to running docking simulations, protein preparation is a vital stage in molecular docking investigations. This entails optimizing and fine-tuning the protein structure. For the purpose of preparing proteins for molecular docking, Discovery Studio software offers a variety of tools and functions. A summary of the procedure is provided below:

The initial stage in protein retrieval is to acquire the desired protein structure. The structure can be downloaded from open repositories like the Protein Data Bank (PDB) or used in conjunction with experimental data to do this. Protein structures may be imported and retrieved in a variety of formats, including PDB files, using Discovery Studio.

Protein Cleaning: Prior to starting the docking procedure, it's crucial to purify the structure of proteins by getting rid of all non-protein molecules, like water molecules, ligands, as well as cofactors, that can obstruct the docking procedure. The newly discovered capabilities of clean proteins Using methods that estimate their placements on the basis of the 3D structure that comprises the protein, Studio is used to produce proteins that complete the structure of a protein by adding missing hydrogen atoms along with additional essential elements. For proper representation of the protein's interactions during docking, this is crucial. It also takes into account the pH settings of the docking experiment when assigning the proper protonation states and tautomeric forms to ionizable residues in the protein. As a result, the charge distribution of the protein is accurately represented throughout the docking process. The protein structure is optimized for energy reduction and any steric conflicts or poor connections are eliminated using the UCSF Chimaera programme. This process aids in the protein's relaxation and conformational refinement, improving its suitability for docking investigations.

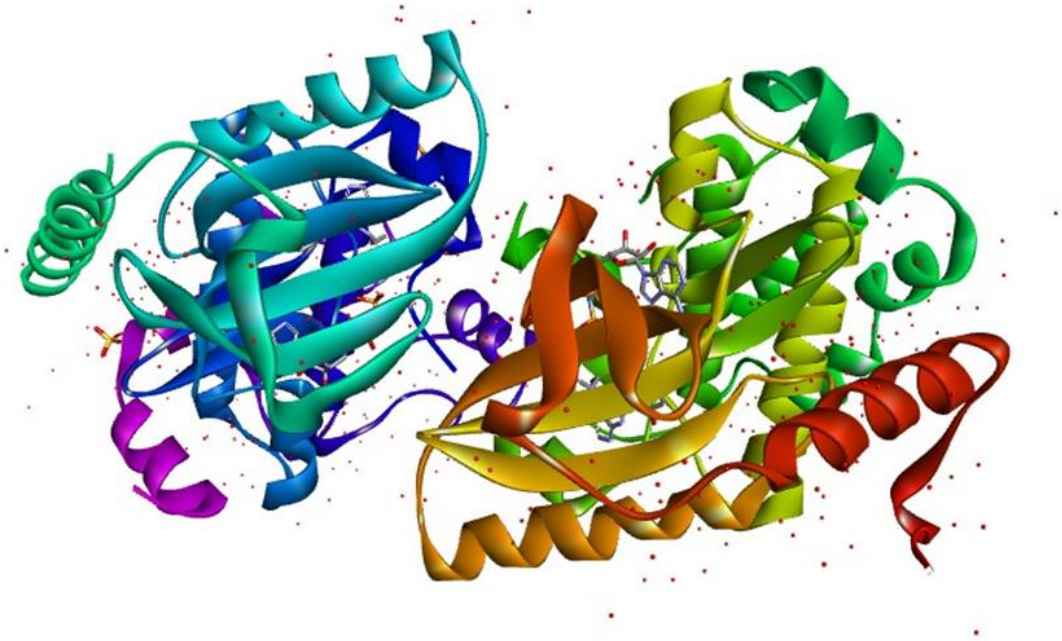


Fig 4.8 - Overall view of protein 7B7R, represented as a ribbon diagram

4.12.2 Preparation of ligands:

The PubChem Compound Database included Structure Data File (SDF) documents containing the structures of various ligands. Utilizing Discovery Studio Biovia (DSB), these chemical patterns in the form of SDF were afterwards translated to PDB format. Making sure the ligand is clear of any impurities or mistakes is crucial before running any molecular docking simulations. The ligand structure may be cleaned using the tools provided by Discovery Studio's prepare ligand functionality, which carries out tasks including eliminating water molecules, rearranging bonds, and looking for any missing atoms and bonds.

Ligand optimization entails structuring the ligand to enhance geometry and get rid of any steric strain or conflicts. Energy minimization plus molecular dynamics simulations are two of the many optimization techniques that Discovery Studio provides. These techniques can aid in ligand relaxation and are more realistic conformational. Use of the discovery studio's functionalities is made for this rapid reduction (dreiding).

4.12.3 Pre Docking Studies

Pre-docking studies are essential to molecular docking investigations and are frequently carried out to improve the speed and precision of the docking procedure. The re-docking procedure is used to evaluate the precision of the discovery studio's LibDock programme.

Protein and ligands that have been prepared earlier are used in the docking process. This hotspot's parameters are 100, 0.25 docking tolerance, quick search as the preferred docking technique, true conformation, and fast conformation method. The RMSD value is assessed, and it is decided that further docking studies may be conducted if the docking approach produces conformations of less than 2.

4.12.4 Docking based virtual screening

Large databases of small compounds are tested against a target protein in virtual screening to find possible binders. The virtual screening technique uses the same parameter that is utilized in pre-docking investigations. The protein (PDB ID: 7B7R) is screened using compounds from table 2.3, and the results are analyzed.

CHAPTER 5

RESULTS

5.1 PERCENTAGE YIELD OF THE EXTRACTS

The results indicated that *Rhododendron maximum* leaves had the highest yield at 20.2%, while *Rhododendron arboreum* had a lower yield of only 6.0%. These findings suggest that the yield of the extract can vary significantly depending on the *Rhododendron* species. Further studies may be necessary to identify the factors contributing to the difference in yield.

5.2 THE EXTRACTED SAMPLES

i. For Tara Devi - Initial temperature – 45 degrees

One Cycle Complete - 21 minutes

ii. For Advanced Study - Initial temperature – 50 degrees

One Cycle complete – 1 hour 10 minutes

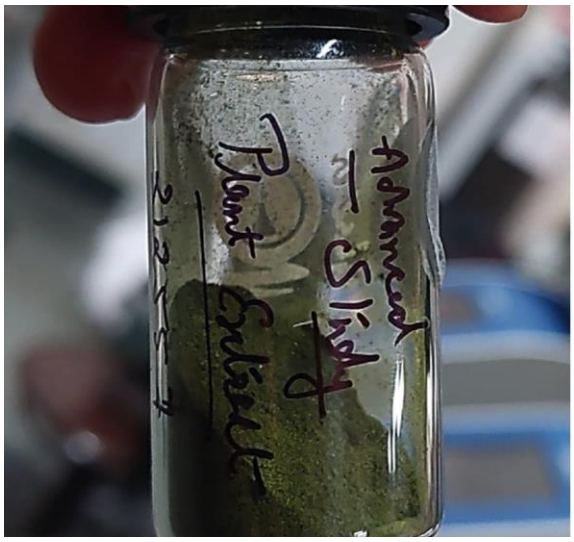


Fig 5.1 - Sample 1 (R. maximum)

Fig 5.2 - Sample 2 (R. arboreum)

5.3 PHYTOCHEMICAL SCREENING

5.3.1 Qualitative Estimation

The analysis of two distinct plant species indicated that their respective extracts contained different phytochemical compounds. The results for *Rhododendron maximum* and *Rhododendron arboreum* vary, showing the presence of a particular phytochemical in one while absence in the other species. The qualitative testing for *Rhododendron maximum* is depicted using **Table 5.1**. While, the qualitative testing for the other species which is *Rhododendron arboreum* is shown using **Table 5.2**.

Table 5.1 - Rhododendron *maximum* qualitative analysis

S.No	Compounds	Test	Interference	Results
1.	Carbohydrates	Molisch's reagent	Appearance of Violet ring at the interface	Positive
2.	Alkaloids	wagner's reagent	Occurrence of reddish-brown ppt	Negative
3.	Flavonoids	Few drops of NaOH -> intense yellow color appearance add few drops of dilute HCL	Solution turns calories	Negative
4.	Saponins	1% Lead Acetate	White ppt	Positive
5.	Sterols	2 ml of Conc. H2SO4 -> 2 ml of extract	Formation of red ppt	Negative
6.	Tannis	3 % Ferric Chloride Soln	Brownish green color	Positive

Table 5.2 - Rhododendron *arboreum* qualitative analysis

S.No	Compounds	Test	Interference	Results
1.	Carbohydrates	Molisch's reagent	Appearance of Violet ring at the interface	Positive
2.	Alkaloids	wagner's reagent	Occurrence of reddish-brown ppt	Negative
3.	Flavonoids	Few drops of NaOH -> intense yellow color appearance add few drops of dilute HCL	Solution turns calories	Negative
4.	Saponins	1% Lead Acetate	White ppt	Positive
5.	Sterols	2 ml of Conc. H2SO4 -> 2 ml of extract	Formation of red ppt	Positive
6.	Tannis	3 % Ferric Chloride Soln	Brownish green color	Positive

5.3.2 Quantitative Estimation

Total Carbohydrate content

Table 3 presents the total carbohydrate contents of two crude extracts. The calibration curve equation for the glucose standard is stated below which showed its higher absorbance at 630 nm.

$y = 1.782x + 0.8313$, with an

R^2 value of 0.9445

Based on the results, it can be inferred that the leaf extract of Rhododendron *arboreum* had a higher amount (0.84 mgQE/g) of total carbs content compared to Rhododendron

maximum.

Table 5.3 - Total Carbohydrate Content

Rhododendron Species	Sample	Total Carbohydrate Content (mgGE/gram)
Rhododendron *maximum*	MEL	0.069
Rhododendron *arboreum*	MEL	0.084

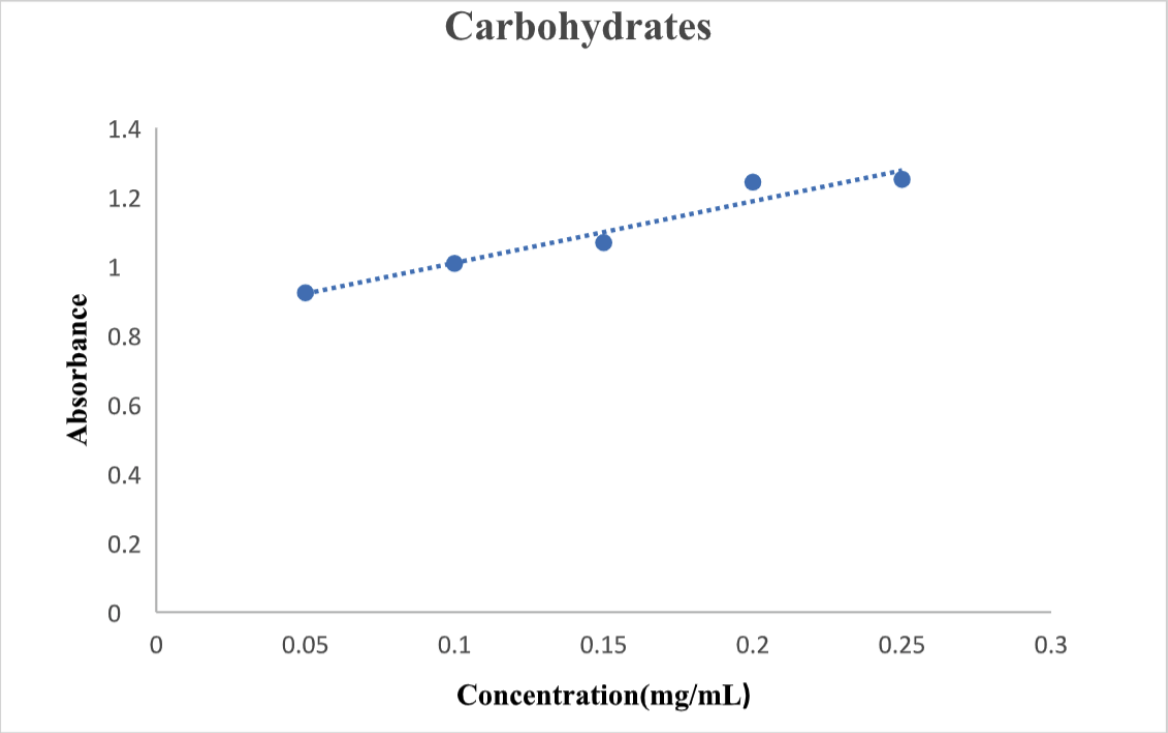


Fig 5.3 - Calibration curve using Glucose as a standard

Total Protein Content

According to **Table 4**, the total protein content was measured in two crude extracts of Rhododendron *arboreum* and Rhododendron maximum. The calibration curve was established using a BSA standard, which showed higher absorbance at 750 nm, and the equation obtained was:

y = 0.1215x - 0.1344, with an

R^2 value of 0.9743

The findings indicated that the overall protein content was higher inside the leaf extract of *Rhododendron maximum* (0.7 mg BSA/g) compared to the extract from *Rhododendron arboreum*.

Table 5.4 - Table Protein Content

Rhododendron Species	Sample	Total Carbohydrate Content (mgGE/gram)
Rhododendron *maximum*	MEL	0.70
Rhododendron *arboreum*	MEL	0.60

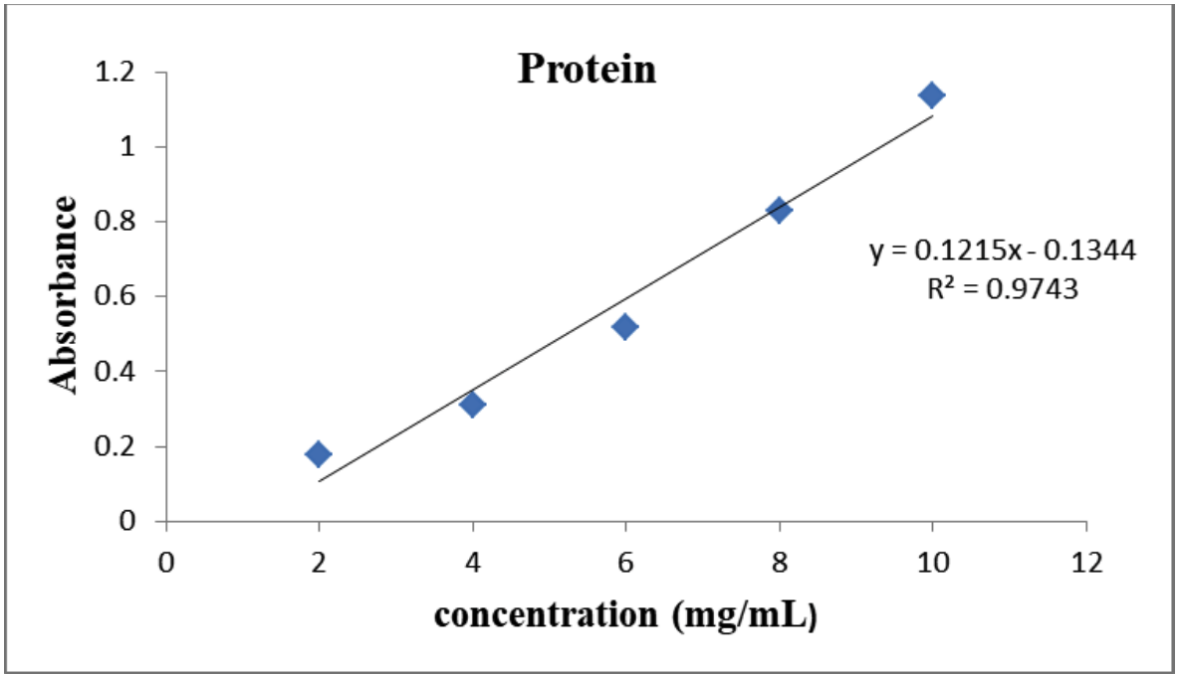


Fig 5.4 - Calibration curve using BSA standard

Total Flavonoid Content

Table 5 presents the total flavonoid content of two crude extracts, which displayed the higher absorbance at 415 nm and the quercetin standard calibration curve equation is

y = 0.35x + 1.242, with an

R^2 value of 0.9526

In comparison to Rhododendron maximum, the leaf extract of Rhododendron *arboreum* had a higher concentration of total flavonoid content, specifically 90ugQE/g.

Table 5.5 - Total Flavonoid Content

Rhododendron Species	Sample	Total Carbohydrate Content (mgGE/gram)
Rhododendron *maximum*	MEL	84
Rhododendron *arboreum*	MEL	90

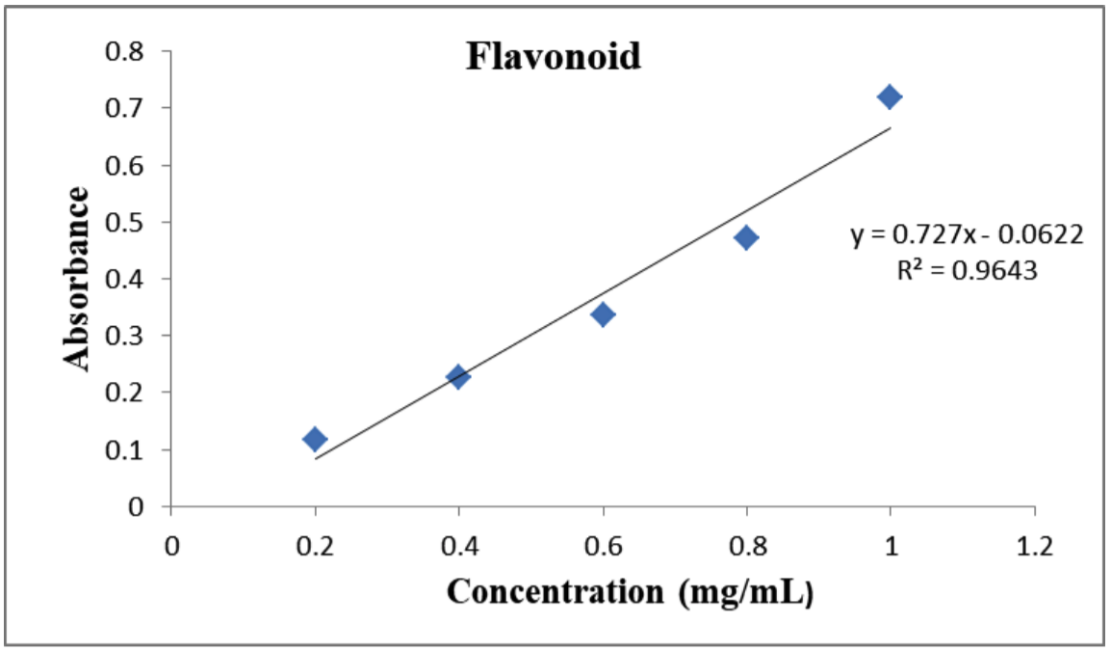


Fig 5.5 - Calibration curve for flavonoid using quercetin standard

Total Phenolic Content

Table 6 presents the findings of the total phenolic content analysis conducted on 70% methanolic extracts of various Rhododendron species. The calibration curve was created using gallic acid, and it displayed the highest absorbance at λ765 nm. The obtained calibration equation is:

y = 0.727x - 0.0622, with an

R^2 = 0.9643

Using the Folin-Ciocalteu technique, the two crude extracts' total phenolic content was determined, and the findings were represented by means of the gallic acid equivalents.

Table 5.6 - TPC of Samples

Rhododendron Species	Sample	Total Carbohydrate Content (mgGE/gram)
Rhododendron *maximum*	MEL	0.143
Rhododendron *arboreum*	MEL	0.147

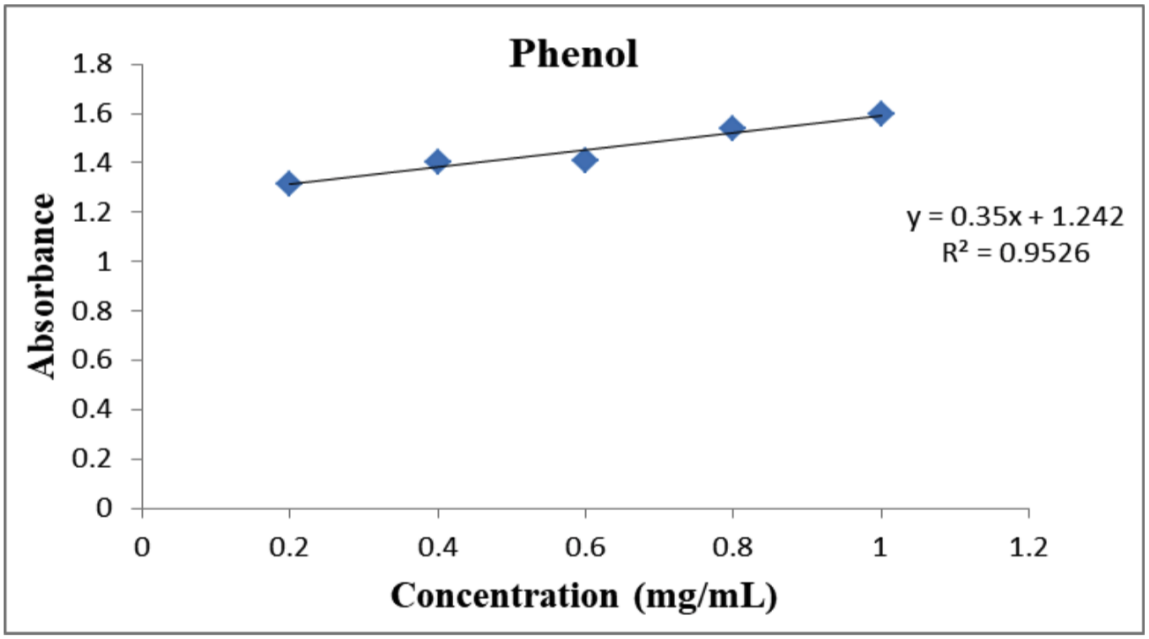


Fig 5.6 - Calibration curve shown by Gallic acid

5.4 Antimicrobial Activity

The antibacterial properties of methanolic, and water extracts were investigated, and it was found that only the methanol extract demonstrated antibacterial activity. The water extract did not exhibit any antibacterial effects, as no inhibition zone was observed. Table 5 presents the outcomes of the antibacterial assay, which was performed by gauging the inhibition zone's diameter of antibiotic-resistant isolates of three bacterial strains, namely *Bacillus subtilis*, *S typhi*, along with *S aureus*. The results were then compared to the control disks.

Table 5.7 - Antimicrobial analysis

sample	Staphylococcus aureus MTCC3160(mm)	Bacillus subtilis MTCC121(mm)	Salmonella *typhi* MTCC98(mm)
Positive control (Ampicillin)	41 mm	23 mm	22 mm
Negative control	Not detectable	Not detectable	Not detectable
R.arboreum	30 mm	33 mm	37 mm

Fig 5.7 - Inhibitory zone exhibited on Staphylococcus aureus

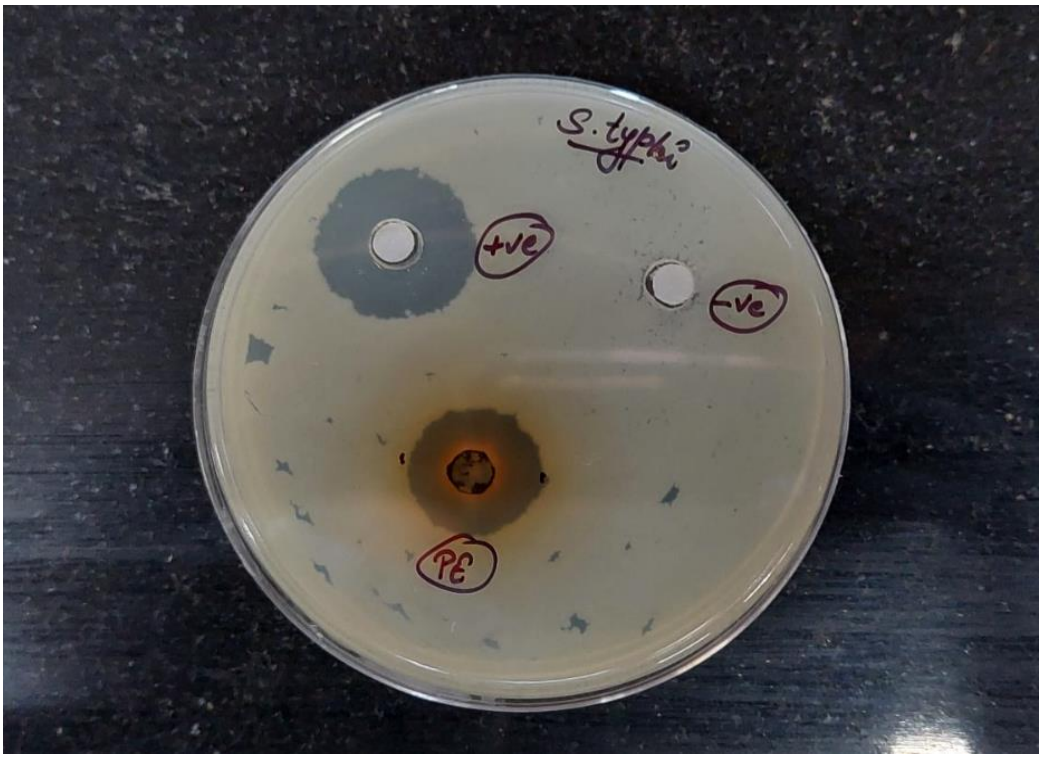


Fig 5.8 - Inhibitory zone exhibited on Salmonella typhi

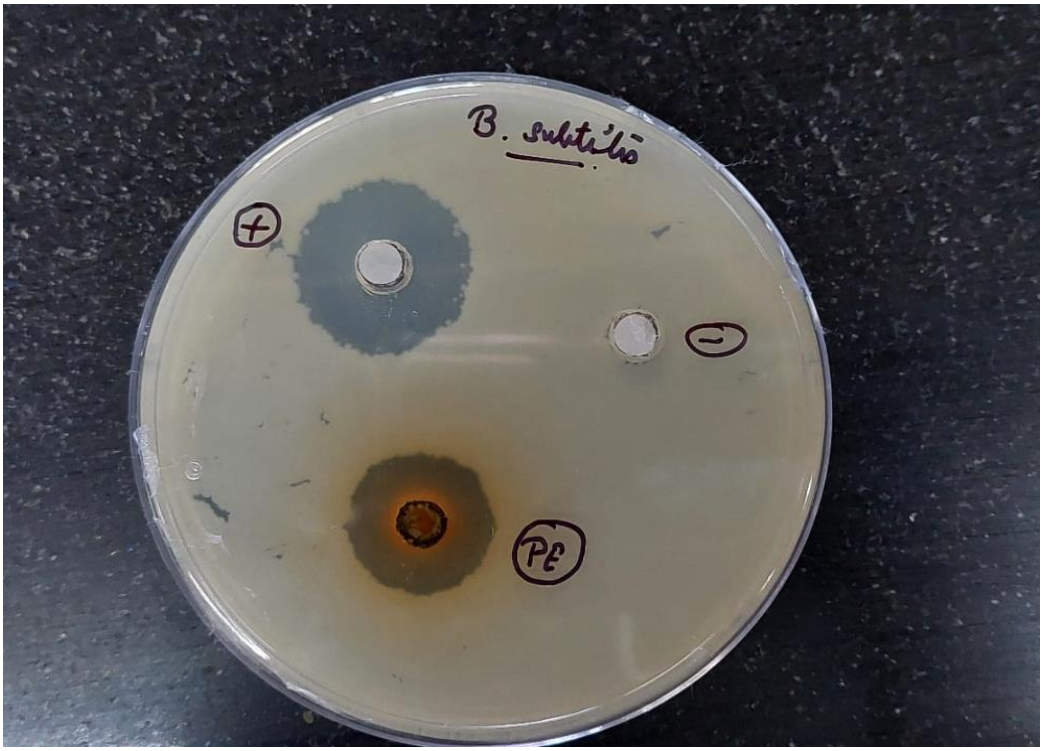


Fig 5.9 - Inhibitory zone exhibited on Bacillus subtilis

5.5 Antioxidant Activity

Table 5.8 and 5.9 displays the efficacy of the extract in eliminating ABTS radical.

Table 5.8 Antioxidant ABTS scavenging activity

R.maximum	
Concentration	% Inhibition
20	75
40	76.42
60	79.71
80	80.14
100	80.57
IC50 - 321.80 ug	

Table 5.9 Antioxidant ABTS scavenging activity

R.arboreum	
Concentration	% Inhibition
20	77.42
40	77.57
60	78.71
80	81.28
100	83
IC50 - 338.29 ug	

5.6 High-throoughput virtual screening

Virtual screening was performed using the three-dimensional structure of 7B7R to identify potential phytochemicals that could interact with the target protein in plants. The docking studies involved comparing the binding affinity of reference inhibitors with the target protein, measured by a parameter called the libdock score. A libdock score of 103.526 was set as the cutoff for candidate drug screening.

To further narrow down the potential candidates, the molecular interactions exhibited by the reference inhibitors with the active site of the protein were analyzed. The reference inhibitors were found to form hydrogen bonds with specific amino acid residues such as PHE209, SER212, and VAL211. Therefore, it was decided to retain phytochemicals that could form at least one hydrogen bond with these residues.

Following these criteria, two phytochemicals were identified as potential candidates, as they exhibited favorable binding properties and satisfied the requirements of forming hydrogen bonds with the aforementioned residues.

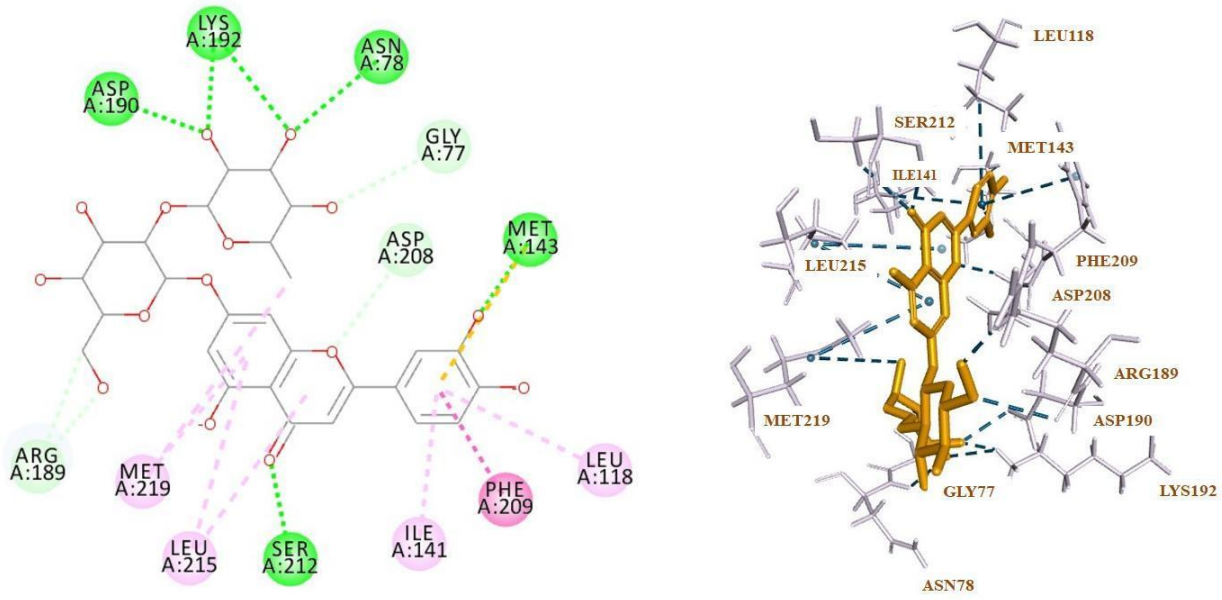


Fig 5.10: (a) 2D Interaction of ligand Flavonol glycoside with the receptor. (b) 3D Interaction of ligand Flavonol glycoside with the receptor (7B7R).

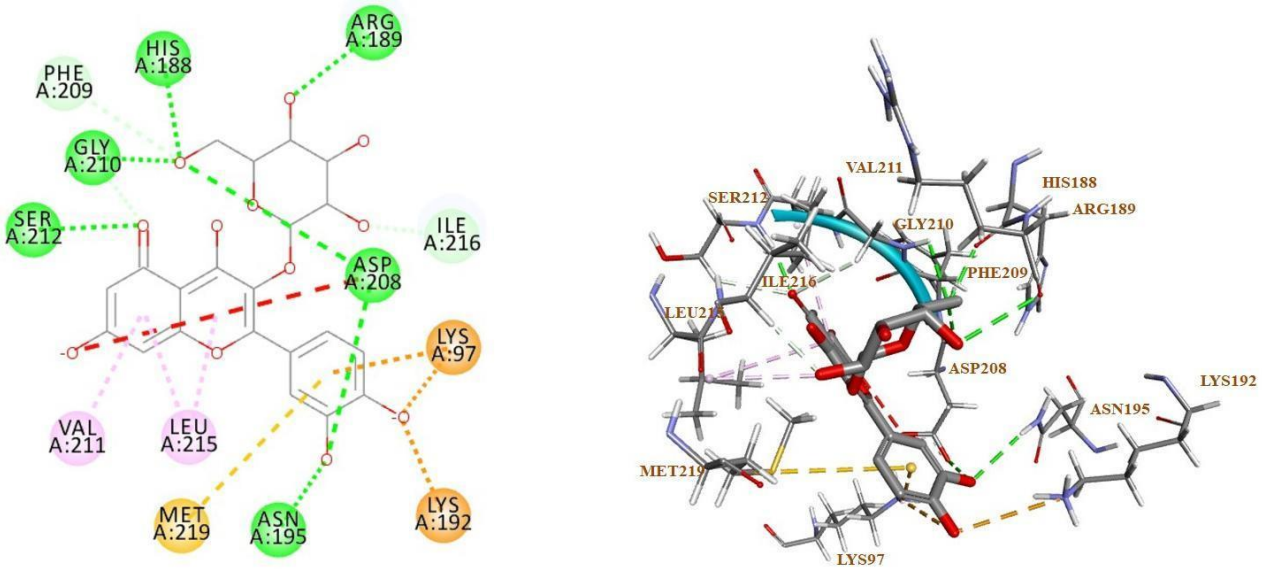


Fig 5.11: (a) 2D Interaction of ligand Hyperoside with the receptor. (b) 3D Interaction of ligand hyperoside with the receptor (7B7R).

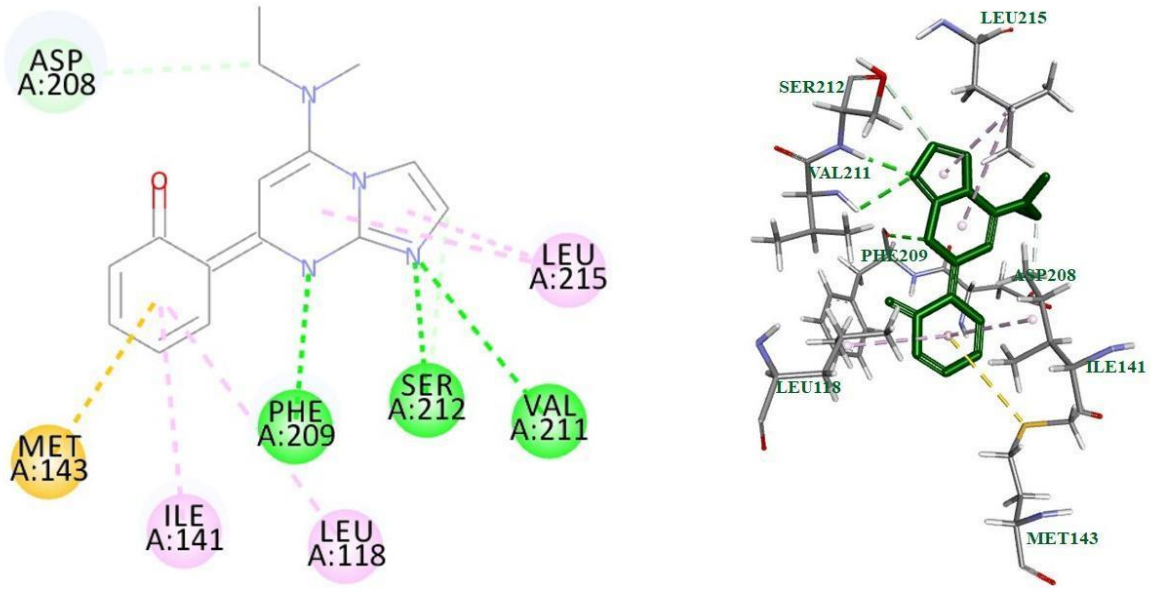


Fig 5.12: (a) 2D Interaction of reference structure. (b) 3D Interaction of reference structure.

Table 5.10 Hits and Reference compounds with their LibDock Score and Hydrogen interactions

S. No.	Compound Name	LibDock Score	H-bonds
1.	**Flavone glycosides**	**159.998**	**ASN78, MET143, ASP190, LYS192**
2.	**Hyperoside**	**112.862**	**HIS188, ARG189, ASN195, ASP208, GLY210, SER212,**
3.	**7B7R**	**98.0858**	**PHE209, VAL211, SER212**

CONCLUSION

In this study, the phytochemical makeup of Rhododendron was examined in order to determine whether it may be used as a treatment for glioblastoma cancer. After thorough phytochemical examination, it was found that Rhododendron contains a variety of bioactive substances, including as polyphenols, flavonoids, and alkaloids, which have previously been demonstrated to have anticancer activities. Docking tests were also carried out to see how well the bioactive compounds interacted with the glioblastoma tumor proteins. Intriguing relationships between Rhododendron chemicals and the target proteins were discovered by the data, suggesting that these compounds may be able to alter how these proteins function and maybe stop the development of cancer cells.

The results of this study add to the mounting body of research that indicates rhododendron and its phytochemical components may be a promising source for the creation of innovative therapeutic drugs for the treatment of glioblastoma. The discovered bioactive chemicals, which show promise as possible research targets, may be further investigated using in vitro and in vivo investigations to determine their effectiveness and safety features But it's vital to keep in mind that this research was restricted to in silico docking tests. Therefore, in-depth experimental investigations, including cell-based assays and animal models, should be included in future research to show the efficacy of Rhododendron chemicals against glioma.

This study primarily emphasizes the potential of Rhododendron as a source of bioactive compounds for the treatment of glioblastoma. Further research and validation of these findings may aid in the identification of novel, effective therapeutic strategies to combat this deadly form of brain cancer.

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